Virtual Fish for Real Science

Investigating the role of public information content for mate-choice copying in the sailfin molly (*Poecilia latipinna*) using live fish and computer-animated fish stimuli

DISSERTATION

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Betreuerin und erste Gutachterin:

Prof. Dr. Klaudia Witte

Universität Siegen

Zweite Gutachterin:

PD Dr. Katja Heubel

Christian-Albrechts-Universität zu Kiel

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Stefanie Gierszewski

Diese Dissertation wurde im Rahmen des interdisziplinären Forschungsprojekts "*Analyse durch Synthese mit virtuellen Fischen als neue Versuchsmethode in Untersuchungen zur Partnerwahl*", als Kooperation zwischen dem Institut für Biologie und dem Institut für Echtzeit Lernsysteme der Universität Siegen, durchgeführt. Das Forschungsprojekt wurde durch die Deutsche Forschungsgemeinschaft (DFG; WI 1531/12-1 und KU 689/11-1) gefördert.

This thesis was conducted within the scope of the interdisciplinary research project "*Analysis by synthesis with virtual fish as a new experimental method in studies on mate choice*" in collaboration between the Institute of Biology and the Institute of Real-Time Learning Systems at University of Siegen. The project was funded by the Deutsche Forschungsgemeinschaft (DFG; WI 1531/12-1 and KU 689/11-1).

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Institute of Biology, Department of Chemistry - Biology, School of Science and Technology, University of Siegen, Adolf-Reichwein-Str. 2, 57076 Siegen, Germany

And when we court each other and choose each other by starlight, we do so to the soundtrack of crickets and frogs doing the same. Mate choice surrounds us.

Gil G. Rosenthal
Mate choice: the evolution of sexual decision making from microbes to humans
Princeton University Press, 2017

TABLE OF CONTENT

Author contributions

The work described in my thesis was performed within the scope of the interdisciplinary research project "*Analysis by synthesis with virtual fish as a new experimental method in studies on mate choice*" (short: Virtual Fish Project) in close collaboration with the project partners from the Institute of Real-Time Learning Systems at University of Siegen.

My role as a biologist in the Virtual Fish Project included providing detailed advice on the morphology, general biology and behavior of the study species (the sailfin molly *Poecilia latipinna*), as well as giving advice on experimental design and its implications for the development of software for the creation of computer-animated 3D sailfin mollies. Further, I continuously tested the correct functioning of the software in general and validated its use for mate-choice experiments with sailfin mollies. This collaboration resulted in the development of *FishSim* Animation Toolchain (hereafter abbreviated as *FishSim*), for which I wrote and illustrated the user manual. *FishSim* was released as free download to the public in 2017. I provide a detailed overview of the Virtual Project in Chapter 4.

The aim of my thesis was to use *FishSim* to investigate the role of quantity and quality of visual public information on mate-choice copying, to get new insights on this fascinating mate-choice strategy used by sailfin mollies. During my thesis, I was involved in designing experimental procedures, experimental setups and data sheets for every experiment described in the following chapters. I took part in animal husbandry of several species of livebearing fishes throughout and instructed every student under my supervision in the proper handling of the fish used in their experiments. I instructed each student on how to perform their experiments, how to analyze the data for their final reports or theses, and supported them in academic writing and scientific presentation. One of my student's works was presented as a scientific poster at the 12th Annual Meeting of the Ethological Society in Bonn[1]. I further served as the second referee in evaluating Bachelor's and Master's theses of students at the University of Siegen and co-supervised two undergraduate research internships (ERASMUS+ and DAAD RISE Germany Program 2017).

The experiments described in my thesis were partly performed by students under my supervision as specified in detail for each chapter below. Further, I published several experimental studies in international, peer-reviewed journals together with the help of different co-authors, whose contributions are also given below. The names and affiliations of all co-authors are given at the beginning of the respective chapters. All photographs shown in this thesis were taken by me, unless another photographer is specified.

Chapter 3

In collaboration with Laura Chouinard-Thuly from McGill University (Canada), I wrote a critical literature review on the use of artificial stimuli in animal behavior research with special focus on computer-animated stimuli and virtual reality. We both contributed equally as lead authors to this review and were additionally provided expert opinions and feedback during the peer-review process by Gil G. Rosenthal, Simon M. Reader, Guillaume Rieucau, Kevin L. Woo, Robert Gerlai, Cynthia Tedore, Spencer J. Ingley, John R. Stowers, Joachim G. Frommen, Francine L. Dolins and Klaudia Witte.

[1] Ahmad SB, Gierszewski S, Müller K, Kuhnert KD, Witte K. (2017) More action with interaction? Investigating the effect of interactive virtual 3D-fish on association time in the sailfin molly, *Poecilia latipinna.* 12th Annual Meeting of the Ethological Society, Bonn.

Chapter 5

All validation experiments to demonstrate the usability of *FishSim* were performed exclusively by me. I conceived the study design, analyzed the data and wrote the manuscript, supervised by Klaudia Witte. Klaus Müller contributed to the experimental setup. Klaus Müller, Ievgen Smielik, Jan-Marco Hütwohl and Klaus-Dieter Kuhnert developed the software and provided technical support throughout this study.

Chapter 6

Klaudia Witte and I conceived the study design. Janine Gürke performed the mate-choice copying experiment with live stimulus fish as part of her Bachelor's thesis. The comparative study using virtual fish was done by Shumail B. Ahmad (University of Manchester, UK) as part of an ERASMUS+ undergraduate internship supervised by me. I analyzed the data and wrote the manuscript, supervised by Klaudia Witte. Klaus Müller, Jan-Marco Hütwohl and Klaus-Dieter Kuhnert developed the software and provided technical support throughout this study.

Chapter 7

The mate-choice copying experiment was conducted by Derek Baker (Calgary University, Canada) during a research internship in the DAAD RISE Germany Program, for which my doctorate project was chosen. I supervised Derek Baker in every step during his internship. I conceived the study design, analyzed the data and wrote the manuscript, supervised by Klaudia Witte. Klaus Müller, Jan-Marco Hütwohl and Klaus-Dieter Kuhnert developed the software and provided technical support throughout.

Chapter 8

Klaudia Witte and I conceived the study design. All experiments described in this chapter were performed exclusively by me. I analyzed the data and wrote the manuscript, supervised by Klaudia Witte. Klaus Müller, Jan-Marco Hütwohl and Klaus-Dieter Kuhnert developed the software and provided technical support throughout this study.

Chapter 9

Klaudia Witte and I conceived the study design. Melissa Keil collected the data of the mate-choice copying experiment with live fish described in this chapter as part of her State exam (1. Staatsexamen). I analyzed the data and wrote the manuscript, supervised by Klaudia Witte.

Chapter 1
General Introduction

1.1 Sexual selection theory

In 1871, Darwin introduced his concept of sexual selection with his famous work "*The Descent of Man, and Selection in Relation to Sex*" (Darwin 1871), and laid the foundation for a line of research which until today has not faded in fascinating scientists all over the world. During his observations, Darwin found that some rather conspicuous traits, such as bright colors, courtship displays or courtship song as well as weaponry, could not be explained as being related to an individual's ability to survive but that they must serve a different purpose. In contrast to natural selection, Darwin argued that sexual selection would not act on traits contributing to struggle for survival but in the struggle for access to mates (Darwin 1871). He distinguished between two important processes: intra- and intersexual selection. Intrasexual selection favors traits that provide an advantage to individuals of the same sex in competition for access to mates of the opposite sex. For example, during male-male competition, larger body size, good constitution or elaborate weaponry, can be crucial while competing for females. On the other side, intersexual selection acts on traits that provide an advantage for individuals to increase their attractiveness towards the opposite sex e.g., by advertising superior mate quality. Here, enlarged or brightly colored ornaments, courtship displays, vocalizations or pheromones may play an important role for attracting mates and may hence lead to increased fitness.

Although it has to be kept in mind that both sexes may choose and that sexual selection may act on both sexes likewise (e.g., see Schlupp 2018 for a review on male mate choice), females are mostly considered the choosier sex due to their higher investment in producing offspring and providing brood care (e.g., in mammals) compared to sperm production in males (Trivers 1972; Andersson 1994). From this arises a sexual conflict between males and females in that both sexes aim to maximize their reproductive success and hence their fitness in different ways. Here, a sexual trait increasing the attractiveness of a male towards choosing females would be favored by sexual selection, resulting in copulations with many females and thus in more offspring for that male. Females, on the other side, need to ensure that they choose *the* best quality male.

The overall assumption is, that females are attracted to a trait because it provides information helping to choose the best mating partner. Interestingly, it can be observed that some sexually selected traits are exaggerated or supernormal in a way that they may actually impede an individual's survival but are, nonetheless, preferred by choosing females (e.g., Andersson 1982). At that time, Darwin missed to give an explanation for why and how such traits might evolve; however, he set the baseline for a line of research which, until today, is highly debated and still not completely understood in terms of its mechanisms. Over the last century, different theories on sexual selection providing explanations for the origin and evolution of sexually selected traits as well as mate preferences for these were proclaimed (reviewed by Ryan 1990; Andersson 1994; Andersson and Iwasa 1996; Kokko et al. 2003; Andersson and Simmons 2006).

Ronald A. Fisher was the first who formulated an early sexual selection model to explain a possible venue for the evolution of (exaggerated) male traits based on the given attractiveness of a trait and its benefits for increasing the access to mates and, thus,

increasing reproductive success (Fisherian sexual selection model; Fisher 1930; Pomiankowski and Iwasa 1998; Henshaw and Jones 2019). The model is based on the assumption of a population in which both variation in a given male trait, as well as variation in the female's preference for that particular trait, exist. The model suggests that over time, both the trait and the preference for it become genetically correlated and, hence, heritable. Thereby, sons will inherit their father's favorable trait and daughters the preference for that trait. Such a correlation may result in a self-reinforced process ('runaway process'), with sexual selection e.g., favoring males with larger traits (compared to the population's mean trait value) indirectly also favoring a stronger preference for that trait in females. Over time, both the expression of the trait as well as the preference for that particular trait will thereby increase. This process may also lead to the evolution of exaggerated traits that actually impede male survival. Nevertheless, as long as survival consequences do not prevail, this self-enforced process will continue and still grant males with exaggerated traits a higher fitness. This very same process may also result in a decrease and potential loss of a male trait. For example, Bakker (1993) demonstrated a positive genetic correlation between the expression of a color ornament (redness of breeding coloration) in male three-spined sticklebacks (*Gasterosteus aculeatus*) and the strength of female preference for the very same trait, thereby confirming the model by Fisher. However, while Brooks (2000) showed the same relationship for male ornamentation in the guppy (*Poecilia reticulata*), he also found that offspring survival and maturity were negatively correlated with increasing attractiveness of the ornament.

Based on the idea that sexual selection would not only favor traits that increase an individual's access to mates but which also demonstrate a benefit in terms of survival in that individual (Maynard Smith 1976), later models proposed that females might not only choose males based on their attractiveness alone but also because attractive traits derive from good genetic quality ('good genes hypotheses'). Here the assumption is that the expression of a sexual trait is directly linked to gene quality in a way that the choosing sex will gain indirect benefits for their offspring from a certain mate (see e.g., Moore 1994).

With his controversial 'handicap principle', Zahavi (1975) proposed the possibility, that mate preferences actually represent the preference for a handicap. Bearing a handicap, that is a given sexual trait, poses a kind of survival test to the individual by lowering its chances for survival. An individual with a well-developed trait therefore passed the test and demonstrates, towards choosing females, its capabilities for survival despite having a handicap (compared to males not bearing the trait). In his rationale, Zahavi further argues that the mere existence of a well-developed trait (i.e., handicap) in a phenotype, and hence passing the test, is very likely also representative for good genetic quality without any "*need to assume any special genetic linkage between the marker of quality and the quality of the individual*" (Zahavi 1975). His model, however, ignored that without genetic linkage of the trait's quality with the actual quality of the individual (e.g., his constitution) male offspring inheriting the handicap would still suffer from decrease in survival. In reaction to critics of his model (Davis and O'Donald 1976; Maynard Smith 1976), Zahavi later corrected his 'handicap principle' by postulating that both the expression of a handicap and an individual's phenotypic quality are correlated. He suggested that "*high quality phenotypes and experienced individuals pay less for the cost of the same sized handicap than low quality phenotypes*" (Zahavi 1977). The handicap therefore represents an honest signal, which helps females to distinguish the best quality mate by choosing the most developed trait within a population (Zahavi 1975; Zahavi 1977; but see Kirkpatrick 1986).

Weatherhead and Robertson (1979) proposed their 'sexy son hypothesis' with regard to the evolution of polygynous mating systems, in which the difference in parental investment of each sex is potentially high. In a previous study on redwinged blackbirds (*Agelaius phoeniceus*), Weatherhead and Robertson (1977) had found that male attractiveness was not necessarily correlated with the quality of the male's territory (e.g., harem size, male assistance, resources) which was in contrast to earlier assumptions (e.g., 'Orians-Verner model'; Orians 1969, Verner 1964, Verner and Willson 1966). Weatherhead and Robertson (1979) proposed that females mating with attractive males, albeit them having a lower quality territory (i.e., larger harem and less male assistance), may not benefit from immediate reproductive success but rather from indirect future benefits compensating for that loss. Assuming that attributes describing a male's attractiveness would be inherited to the female's male offspring, their 'sexy sons' would consequently have a higher future reproductive success by attracting more females; resulting in a higher number of descendants for that initial female. Kirkpatrick (1985), however, argued against the 'sexy son hypothesis'. Using genetic modelling, he showed that the evolution of male preferences that do not yield to a female's immediate highest reproductive success could not persist in a population.

With focus on genes that actually affect individual fitness, namely those that grant resistance to pathogens and parasites, Hamilton and Zuk (1982) formulated their 'parasite load hypothesis', which is also referred to as parasite-mediated sexual selection (PMSS; e.g., Ezenwa and Jolles 2008; Buzatto et al. 2019). Based on a survey in North American passerines and associated blood parasites, Hamilton and Zuk (1982) argued that the expression of a well-developed sexual trait (e.g., bright male plumage) reflects an individual's resistance to certain parasites and diseases. They argued that only healthy males can spend their resources in developing elaborate traits, which then represent an honest indicator for parasite resistance and, hence, good genetic quality. Females attracted to such a trait would consequently prefer less parasitized males and would thereby gain an indirect benefit for their offspring, that is, heritable parasite resistance (e.g., Buzatto et al. 2019). Moreover, females would gain a direct benefit by decreasing their own risk of getting in touch with parasites while mating (decreased parasite transmission; see also Zuk et al. 1990). For example, Doucet and Montgomerie (2003) found a relationship for blood parasite infection and plumage brightness in satin bowerbirds (*Ptilonorhynchus violaceus*). Similarly, color brightness of orange bars in the rainbow darter (*Etheostoma caeruleum*) was found to be associated with parasite count (Ciccotto et al. 2014). A more recent evaluation of the model by Hamilton and Zuk, as well as possibilities for testing its implications in light of modern genomic approaches can be found in Balenger and Zuk (2014).

Following a similar rationale but focusing on a more general immunocompetence in the host rather than on specific parasites, Folstad and Karter (1992) proposed the 'immunocompetence-handicap hypothesis'. Their hypothesis concentrates on the endocrinological perspective regarding the development of sexual traits, specifically, with regard to immune-suppressive effects of testosterone. Folstad and Karter (1992) suggested a negative-feedback loop in a way that only the best males would be able to develop elaborate ornamental traits (stimulated by elevated testosterone levels), as well as, at the same time, fight disease (impeded by elevated testosterone levels). Consequently, well-developed sexual traits should be an honest signal for a good immune system in a certain mate. Olsson et al. (2000) found supporting evidence for the ICH hypothesis in male sand lizards (*Lacerta agilis*) by experimentally manipulating their testosterone levels which increased their presumed mating success while suppressing immune function (but see e.g., Desprat et al. 2015 and references within).

A different viewpoint on how current sexually selected traits might have evolved in the first place is described by the 'sensory exploitation hypothesis' (Ryan 1990; Ryan and Rand 1990). Here, the prerequisite is that a random mutation causes the existence of a novel male trait which exploits a pre-existing bias in choosing females. The origin of such a bias can be completely random or derive from factors related to natural selection or specific environmental constraints, e.g., a bias matching a specific color or shape resembling a typical diet (Rodd et al. 2002; Grether et al. 2005; Kolm et al. 2012; Amcoff et al. 2013). If a novel trait matches such a bias, both the rate of sensory stimulation (e.g., greater firing rates of neurons due to higher stimulation of an animal's sensory system) this trait elicits in females and their preference for the trait will be linked (e.g., Magnus 1958; Rowland 1989a; Rowland 1989b; Ryan and Rand 1990). Here, selection will favor those male traits that elicit a higher sensory stimulation in females. To understand selection pressure on certain traits in the study of mate choice, knowledge about the properties of an animal's sensory system is, therefore, essential (Ryan 1990). For example, the well-documented preference for red breeding coloration in male sticklebacks (Ter Pelkwijk and Tinbergen 1937; Semler 1971; Bakker and Mundwiler 1994) is maintained by an increase in female spectral sensitivity for the color red during the breeding season (Cronly-Dillon and Sharmaf 1968).

Based on the sensory exploitation hypothesis, Holland and Rice (1998) proposed their 'chase-away' hypothesis, which becomes relevant when sensory exploitation by males reduces female fitness. Here, male traits that have established due to a sensory exploitation without bearing any direct or indirect fitness benefit for the choosing females, may exaggerate as the result of an antagonistic coevolution. Here, females will get resistant to the trait (their mating threshold increases) and males will exaggerate to overcome the resistance and so on.

All theoretical models introduced above have in common, that their explanations for the evolution of sexually selected traits assume preferences for sexual traits being genetically predisposed and inherited (e.g., Andersson 1994; Bakker and Pomiankowski 1995; Bakker 1999; Andersson and Simmons 2006). However, observation showed that mate preferences can be highly variable and numerous studies to date demonstrated that also non-genetic factors contribute to establish mate preferences in both sexes. Aside from maternal effects (e.g., Mousseau and Fox 1998; Forstmeier et al. 2004), environmental factors (e.g., predator-induced effects: Plath et al. 2019) and/or experience (e.g., age-dependent effects: Atwell and Wagner 2014; Dukas and Baxter 2014), the social environment may greatly affect mate preferences. This gives rise to the possibility for sexually selected traits evolving and spreading within a population via the use of public information and social learning (Westneat et al. 2000; Danchin et al. 2004; Dall et al. 2005; Witte and Nöbel 2011).

1.2 Public information and social learning

Animals constantly need to assess and evaluate various features in their environment to gain access to food sources, breeding habitats and mates but also to avoid predators or other potentially dangerous situations. For this, animals can: (I) extract information from their environment by personal inspection (i.e., using personal information), or they can (II) extract public (or social) information by observing others. Public information can either be acquired by observing the performance or decisions of one or more conspecifics or of heterospecifics sharing the same habitat. The ability to learn from

these observations and interactions of others, to then incorporate this information in one's own decision-making, is termed social learning (Nordell and Valone 1998; Danchin et al. 2004; Laland 2004; Valone 2007; Reader 2016). Thereby, an animal gains access to an additional source of information, apart from its personal information, and information acquisition may become less time consuming. For example, a forager can assess the behavior of its group members to get information about food availability without personally sampling the whole food patch (Clark and Mangel 1986; Valone 1989; Giraldeau et al. 1994; Valone and Templeton 2002).

Public information gained by observing interactions between other individuals is also frequently described as 'inadvertent socially acquired information', since the observed interactions are not deliberately directed to the observer (Dall et al. 2005; see also 'eavesdropping' in Chapter 1.2.2 below). Although, the opportunity to acquire public information is especially high in group-living animals (Valone and Templeton 2002; Danchin et al. 2004; Dall et al. 2005; Valone 2007; Ioannou et al. 2011), social learning is not inevitable linked to sociality but can be observed in non-social species as well (Wilkinson et al. 2010; Webster et al. 2017; Vila Pouca et al. 2020). Moreover, as already stated above, public information is not restricted to being solely transferred between conspecifics but may also be accessed by heterospecifics (Mathis et al. 1996; Avarguès-Weber et al. 2013; Farine et al. 2015; Webster et al. 2017). Overall, the ability to acquire and act on public information in various contexts is prevalent in nature and can be found across the whole animal kingdom (e.g., insects and spiders: Wray et al. 2012; Leadbeater and Dawson 2017; amphibians: Swanson et al. 2007; reptiles: Wilkinson et al. 2010; Kis et al. 2015; birds: Jaakkonen et al. 2015; fish: Laland et al. 2011; Webster et al. 2019; mammals: Rauber and Manser 2018) to even microbes (Ross-Gillespie and Kümmerli 2014).

It was predicted that animals should refer to public information when their personal information is either out of date, unreliable, or expensive to obtain, e.g., under risk of predation (Webster and Laland 2008; Rieucau and Giraldeau 2011; Wray et al. 2012; Smolla et al. 2016; White, Davies, et al. 2017). With regard to assumptions based on theoretical models, Laland (2004) proposed a set of social learning strategies specifying under which circumstances, and from whom, individuals should learn from. Public information is especially beneficial if decisions are hard to make, as in cases in which it is hard to distinguish the value between food sources (Baracchi et al. 2018), when potential mating partners appear to be similar in quality (Witte and Ryan 1998; see also 'mate-choice copying' in Chapter 1.3 below) or when using personal information alone would be more time-consuming (Scott et al. 2019). However, when faced with a pheromone trail (public information) confronting their own navigational memory of a food patch (personal information), experienced foragers of *Lasius niger* ants relied more on their own personal information (Grüter et al. 2011). Therefore, granting more validity to public information over personal information seems not to be advantageous in every situation.

Indeed, utilizing public information over personal information may also come with a cost in cases when the public information gained is not reliable or misleading (as reviewed by Rieucau and Giraldeau 2011). For example, false alarm calls can lead to abandoning a food source in favor of the signaler (Wheeler 2009; Møller 2010; Flower 2011), leading to costs for the deceived individual. Further, an individual may deliberately change its behavior in presence of an observer (a potential rival) to obscure its honest mate choice to reduce the risk of sperm competition (see 'audience effect' in Chapter 1.2.2 below). Using public information to copy the mate choice for a costly male variant can even

result in reduced fitness in the observer (Nöbel, Danchin, et al. 2018; see also 'mate-choice copying' in Chapter 1.3 below).

In the same way as the social environment influences decisions related to e.g., food sources, habitat choice or predator evasion, it may further also shape mate-choice decisions throughout an animal's lifespan, from birth to sexual maturity and beyond. The following chapters focus on aspects of social learning in relation to mate choice.

1.2.1 Sexual imprinting and early social learning

The term '*sexual imprinting*', which was first established by Konrad Lorenz (1937), describes an irreversible process of early learning which takes place during a specific timeframe, i.e., the sensitive phase in early development. Brood care is a prerequisite for sexual imprinting to occur, in which at least one parent (genetic or social parent) cares for the offspring. While having intense contact to the caring parent, the young learn the specific traits of the parent which later as adults serve for sex and species recognition as well as mate choice (e.g., Vos 1995; Witte et al. 2000). Early learning in a more broad sense (apart from the strict definition by Lorenz 1937) also refers to 'sibling effects' that may influence mate preferences even under the absence of parental brood care (Kozak and Boughman 2009; Macario et al. 2017; Rosenthal 2017). For example, female green swordtails who naturally prefer large sworded males, preferred short-sworded males instead when raised together with only short-sworded males (Walling et al. 2008). Overall, sexual imprinting and early learning shaping mate preferences was widely demonstrated using cross-fostering experiments in which offspring is raised with a novel set of (social) parents (e.g., Verzijden and ten Cate 2007) or parents with a novel trait (Witte et al. 2000; Witte and Sawka 2003; Caspers and Witte 2006).

1.2.2 Social learning after sexual maturity

Both imprinting and early social learning set the baseline for mate preferences so that, upon reaching sexual maturity, an animal is generally able to recognize potential mates. At this stage, however, mate choice decisions are by no means fixed (as reviewed by Witte and Nöbel 2011). For example, mate choice in male and female guppies is shaped by age and experience (Rosenqvist and Houde 1997; Jirotkul 1999; Kodric-Brown and Nicoletto 2001; Magurran and Ramnarine 2004) throughout their life. Learning also plays an important role in the acquisition of adult mating decisions. Magurran and Ramnarine (2004) reported that adult male Trinidadian guppies (*Poecilia reticulata*) raised in allopatry from swamp guppies (*P. picta*), did not initially prefer conspecific females over heterospecific females but chose randomly when given the chance. However, when allowed to interact with females from both species, *P. reticulata* males were able to learn to readily distinguish conspecific females within four days.

Particularly when living in social groups, public information about the mate choice of others can greatly influence an individual's mate-choice decisions, apart from their own personal information.

Eavesdropping

Eavesdropping enables an animal (the bystander) to gather information from the interaction of others by picking-up the signaling between a sender and a receiver (McGregor 1993; McGregor and Dabelsteen 1996; Matos and Schlupp 2005). Here, public information can be gathered with only little costs and no risks and without being directly involved in the communication event (McGregor 1993; McGregor et al. 2000). For example, eavesdropping can be a valuable source of information for assessing social hierarchy in a group (Grosenick et al. 2007). Male Siamese fighting fish were found to assess male fighting ability by eavesdropping on fighting neighboring rivals without getting directly involved (Oliveira et al. 1998; McGregor et al. 2001). In the same way, eavesdropping may provide females with information on male quality, while observing male contest behavior (Otter et al. 1999; McGregor and Doutrelant 2000; Johnstone 2001; Mennill et al. 2002). McGregor and Doutrelant (2000) also found that male Siamese fighting fish increased their fighting intensity in the presence of females, showing that, in case the eavesdropper is detected, observed individuals may exhibit so-called audience effects as a result (see below).

Audience effect

The audience effect is defined as a change in the signaling behavior of an individual caused by the presence of one or more bystanders (the audience), who are detected by the interacting individuals and who may or may not extract information from the observed individual's signaling interaction (Plath and Schlupp 2008; Zuberbühler 2008). Audience effects were found to be wide-spread across various contexts (as reviewed by Matos and Schlupp 2005) in both vertebrates (e.g., mammals: Di Bitetti 2005; le Roux et al. 2008; Townsend and Zuberbuhler 2009; birds: Evans and Marler 1991; Ung et al. 2011; Fitzsimmons and Bertram 2013; Kniel et al. 2016; fish: Desjardins et al. 2012; Dzieweczynski et al. 2012; Cruz and Oliveira 2015) and invertebrates (Fitzsimmons and Bertram 2013). For example, cleaner fish (*Labroides dimidiatus*) were shown to increase their levels of cooperation in presence of an audience of potential clients, readily eating ectoparasites instead of cheating and eating a client's mucus (Pinto et al. 2011). Especially when sperm competition risk is high among rivals, as in livebearing fishes where females mate multiply and are only receptive for a short period of time (Parzefall 1973; Meffe and Snelson 1989), audience effects are prevalent (e.g., Ziege et al. 2009; Makowicz et al. 2010a; Makowicz et al. 2010b; Bierbach, Makowicz, et al. 2013; Nöbel and Witte 2013; Auld and Godin 2015; Witte et al. 2018).

Audience effects are particularly interesting when considering that an animal may deliberately change its signaling behavior in presence of an audience to alter the quality of the information extracted by the audience, e.g., to mislead the audience about the observed individual's mate choice (Plath and Schlupp 2008; Plath et al. 2010). This phenomenon was summarized as the so-called 'deception hypothesis' (Castellano et al. 2016; Witte et al. 2018). However, male Atlantic mollies (*Poecilia mexicana*) showed audience effects in presence of rivals only when it matters, i.e., when the rival was sexually active (Plath and Bierbach 2011). Indeed, being observed during sexual interactions may result in mate-choice copying of the audience and, therefore, increases the risk of sperm competition for the observed individual itself (Ziege et al. 2009; Witte et al. 2018; see below).

1.3 Mate-choice copying (MCC)

Mate-choice copying (short: MCC) is defined as a non-independent mate-choice strategy that relies on public information from others. Per definition, an individual's probability of choosing (or rejecting) a mate increases, if one (or more) other 'model' individuals have chosen (or rejected) this mate previously (Pruett-Jones 1992; Witte and Ueding 2003; Witte and Nöbel 2011; see Fig. 1). MCC describes a non-genetic mechanism for the acquisition and evolution of (novel) mate preferences (Servedio and Kirkpatrick 1996; Agrawal 2001; Danchin et al. 2004; Verzijden et al. 2012; Kniel, Dürler, et al. 2015; Witte et al. 2015; Danchin et al. 2018; Varela et al. 2018; MacLaren 2019) that even leads to speciation (Varela et al. 2018; Dion et al. 2019).

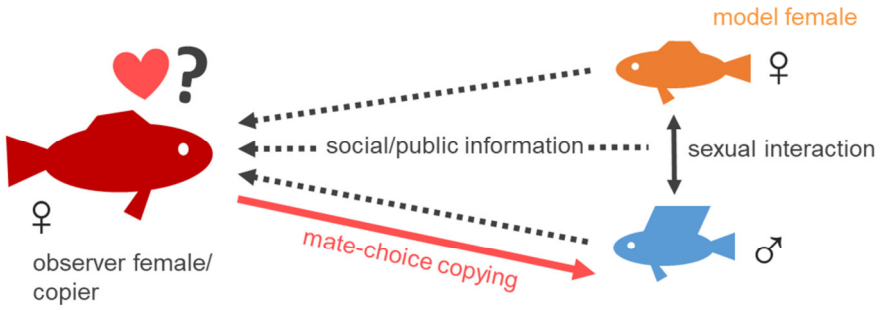

Figure 1. Public information use in mate choice. Instead of evaluating different mating partners on her own (personal information), a female may use inadvertently sent public information (dashed black arrow) from observing a conspecific female (the 'model' female; orange) in her mate choice. Thereby, the observing female extracts information on both the model female herself and her respective mating partner (blue), as well as on the sexual interaction between the pair (solid black two-way arrow). Choosing the same male individual (or phenotype) as the model did previously is termed mate-choice copying (solid red arrow). The same is true for males as the observing individual.

The study of MCC has fascinated scientist over decades since Lee A. Dugatkin (1992) showed for the first time that female guppies (*Poecilia reticulata*) did not choose a mating partner independently but copied the mate choice of conspecific females. Since then, an accumulation of studies confirmed the existence of MCC as an alternative mate-choice strategy in humans (e.g., Waynforth 2007; Little et al. 2015; Luoto and Spriggs 2018; Gouda-Vossos et al. 2018) and various other vertebrates (e.g., mammals: Galef et al. 2008; birds: Galef and White 1998; White and Galef 2000; Kniel, Dürler, et al. 2015; Kniel et al. 2017; fish: Dugatkin and Godin 1992; Grant and Green 1996; Munger et al. 2004; Widemo 2005; Heubel et al. 2008; Frommen et al. 2009; Bierbach, Girndt, et al. 2011), a list that is still continuously growing. Recent work also nicely demonstrates that MCC is not unique to vertebrates but is also deployed by the invertebrate *Drosophila melanogaster* (Dagaeff et al. 2016; Danchin et al. 2018; Monier et al. 2018; Nöbel, Allain, et al. 2018; Nöbel, Danchin, et al. 2018) and presumably also by spiders (Fowler-Finn et al. 2015). Even though MCC was first demonstrated in various laboratory settings, it could later be shown that MCC was also performed by animals in the wild (Witte and Ryan 2002; Alonzo 2008; Godin and Hair 2009), which classifies MCC as a biologically relevant mate choice strategy. Further, despite the majority of studies tested MCC in females, also males were found to deploy this strategy (Schlupp and Ryan 1997; Witte

and Ryan 2002; Bierbach, Kronmarck, et al. 2011). Although differences in MCC usage between both sexes may exist (Widemo 2005; Kniel, Dürler, et al. 2015).

As already stated above in Chapter 1.2, animals are presumed to use public information when the reliance on personal information alone is potentially costly or unreliable. It was assumed that MCC evolved to decrease the costs involved alongside mate choice, that are, time spent to search for and assess different mates as well as increased predation risk while searching (Pomiankowski 1987; Wong and Jennions 2003; Frommen et al. 2009; but see Briggs et al. 1996; Dugatkin and Godin 2010). Also, choosing a mate of lower genetic quality will result in lower quality offspring and, hence, reduced fitness (Pomiankowski 1987). Here, public information can grant the observer a "second opinion by others". Especially, if two (or more) potential mates are very similar in quality, and a "good" choice is therefore difficult to make, the strategy of MCC is presumed to be very beneficial (Witte and Ryan 1998; Danchin et al. 2004). For example, female sailfin mollies (*Poecilia latipinna*) were shown to have a preference for larger males. When presented with two males who were distinctly different in size ("easier" choice situation), females did not deploy MCC (after seeing a model associated with the smaller male) but relied more on their personal information and chose the larger male (Witte and Ryan 1998; Witte and Noltemeier 2002). However, when both males were very similar in size ("harder" choice situation; Witte and Ryan 1998), or when females had seen more model females subsequently interact with the smaller male (potentially rendering her own choice unreliable; Witte and Noltemeier 2002), focal females utilized the public information provided by the model and copied her choice.

As with other uses of public information (as mentioned in Chapter 1.2), the strategy of MCC also bears potential costs for the observer, which consequently may lead to maladaptive decisions (Dubois et al. 2011; Nöbel, Danchin, et al. 2018). For example, an observer may fall victim to the audience effect (see Chapter 1.2.2) which can alter the expression of mate preferences in the choosing individual, due to the risk of sperm competition (Plath et al. 2008). The 'deception hypothesis' assumes that the observer/audience will copy the deceptive mate choice, potentially choosing a lower quality mate as the result (Castellano et al. 2016; Witte et al. 2018). Witte et al. (2018) confirmed for the first time that Atlantic mollies (*Poecilia mexicana*) indeed copied the pretended mate choice of individuals they have previously observed. Further, Nöbel, Danchin, et al. (2018) demonstrated that female fruit flies copied the choice for a mutant male variant, which evidently reduces female fitness. Without the opportunity to copy, naïve females show a natural preference for wild type males. These examples show that MCC can indeed be costly. For the observer, it is therefore necessary to ensure that the extracted information is reliable.

Over the last decade, several studies demonstrated that both quantity and quality of the public information that is being assessed by the observer play an important role in whether the choice of the model is copied or not (as reviewed by Witte et al. 2015; see also Chapter 2.2 for MCC in the sailfin molly). For example, female zebra finches copied the choice of a female model when they had access to both acoustic and visual information on the interaction between the model and the male but not when they could only extract acoustic information (Kniel, Schmitz, et al. 2015). Further, it was shown in humans that women attend to the phenotype of the model resulting in a man being more attractive to observing women when paired with an attractive model partner (Waynforth 2007). Similarly, female zebra finches evaluated the phenotype of the model and copied the choice of wild type model females but not that of model females that were visually manipulated to represent a novel phenotype, by adorning them with a red feather (Kniel

et al. 2017). The quality of a model female herself can, therefore, affect whether focal females copy her choice or not (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Hill and Ryan 2006; Vukomanovic and Rodd 2007; Little et al. 2015), as well as the quantity of public information available for the observer (number of models and duration of observation: Witte and Noltemeier 2002).

Copying can result in the cultural inheritance of mating preferences if not only copy the choice for an individual bearing a specific trait but to do this repeatedly by generalizing across other males who share this trait, which was indeed found to be the case in Japanese quail (*Coturnix japonica*; White and Galef 2000), sailfin mollies (Witte and Noltemeier 2002), guppies (Godin et al. 2005), zebra finches (Kniel, Dürler, et al. 2015) and fruit flies (Danchin et al. 2018). Additionally, sailfin mollies remembered the observed choice of a model and still copy the choice for smaller males up to five weeks later (Witte and Noltemeier 2002), which offers implications for potential long-term effects of MCC. Further, MCC could not only be shown to favor the potential spread of preferences for novel phenotypes (Kniel, Dürler, et al. 2015; Kniel, Schmitz, et al. 2015; MacLaren 2019) but also the avoidance of certain phenotypic traits by copying the rejection of a mate (Witte and Ueding 2003). Information gained from observing the mate choice of others can even override otherwise genetically predefined preferences (Dugatkin 1996; Dugatkin 1998; Witte and Noltemeier 2002; Godin et al. 2005). For these reasons, MCC is considered to have wide implications on the evolution of phenotypic traits and sexual selection (Servedio and Kirkpatrick 1996; Agrawal 2001; Danchin et al. 2004; Verzijden et al. 2012; Witte et al. 2015; Danchin et al. 2018; Varela et al. 2018; MacLaren 2019), which renders the study of its mechanism an important field of research.

An important line of research is to define, what exact aspects of public information are relevant for triggering copying behavior and how these might be intertwined. An observing individual can extract information from different sources simultaneously, either from the model itself, the potential mating partner, and/or their sexual interaction. The main focus of my PhD project, therefore, lay in unraveling these aspects in a series of MCC experiments to increase our knowledge of this fascinating mate-choice strategy. To investigate how variations in public information content may affect MCC, I deliberately manipulated the quality of visual information in experiments using the sailfin molly. In Chapter 1.4 below, I will briefly outline the contents of the following chapters that contribute to my PhD thesis.

1.4 Outline

After the first experimental study on MCC (Dugatkin 1992), livebearing fishes of the genus *Poecilia* have been proven to be a valuable study system for the investigation of the important mechanism underlying MCC. In particular, studies using the sailfin molly contributed valuable insights to our current understanding of MCC. Both male and female sailfin mollies were shown to copy the mate choice of others (Schlupp and Ryan 1997; Witte and Ryan 1998; Witte and Ryan 2002) which makes them an ideal model species for MCC research. Even though several aspects of MCC in sailfin mollies have already been investigated (for detailed information see Chapter 2.2), many questions on the relevance of specific features of visual information (e.g., model female quality and behavioral aspects) still remain unsolved. In parts, this might be due to the fact that some of the remaining questions are hard to study using conventional experimental methods, for example how differences in a model's behavior might affect MCC.

My PhD project was focused on investigating how different aspects of visual public information affect MCC in observing focal female sailfin mollies. Aside from using a classic experimental approach with live fish stimuli, I also took advantage of 3D computer-animated fish stimuli (i.e., virtual fish) to manipulate visual public information provided by either the model female and/or her sexual interaction with a male. Below, I will briefly outline the contents of each chapter:

In **Chapter 2** of my thesis, I will introduce my study species, the sailfin molly. I will describe its morphology, biology and I will summarize its fascinating mating system as a livebearing fish. In this chapter, I will further briefly recapitulate the current knowledge about MCC in this species. At the end of Chapter 2, I will provide information on those fish used in my experiments and their general housing conditions.

In **Chapter 3**, I will review the technical background of the method I used for most of my experiments, that is, 3D computer animation, as well as similar techniques. Here, I will discuss best practices for the use of virtual stimuli as well as possible limitations and constraints that may typically arise. This chapter describes the ground-work on which most of the technical and conceptual considerations concerning the development of *FishSim* were based on.

In **Chapter 4**, I will introduce the Virtual Fish Project. Within my PhD project, I was involved in the development of *FishSim*, which was specifically designed to study MCC in sailfin mollies. Here, I will give detailed information on the biological perspective behind the development of the different tools, incorporated in *FishSim,* that are used to create (*FishCreator*), animate (*FishSteering*) and present (*FishPlayer*) virtual fish stimuli in MCC experiments.

In **Chapter 5,** I will present four different experiments that I performed to validate the usage of *FishSim* as a new method for mate choice experiments with sailfin mollies. For this, I tried to disentangle movement, shape and sex of a virtual fish stimulus to test how these features affect a virtual stimulus' attractiveness towards live focal fish. Based on a positive validation of the method, *FishSim* could be used in different MCC experiments.

The following **Chapters 6 to 9** describe four different studies, in which I systematically manipulated the public information available to observing live focal female sailfin mollies during the observation period of a MCC experiment. Specifically, I concentrated on two different aspects possibly contributing to MCC: (I) the quality of the model female as depicted by her morphology and appearance, and (II) the role of the behavioral interaction between the model female and a prior non-preferred male.

It is assumed that the quality of a model female greatly affects whether her choice is copied or not (see e.g., Dugatkin and Godin 1993; Hill and Ryan 2006). What specific characteristics depict a model female's quality in sailfin mollies is, however, largely unknown. In **Chapter 6**, I investigated whether a virtual model female's body size (in relation to her age) affects MCC in live focal females. In a comparative study using both live fish stimuli and virtual fish stimuli created with *FishSim*, the quality of the model female was manipulated by presenting a model female that was either larger (presumably older) or smaller (presumably younger) than the focal female. I will discuss this chapter in light of the social learning strategy "*copy older individuals*" as proposed by Laland (2004).

In **Chapter 7**, I investigated the role of gonopore pigmentation in model females as a sign for model female quality, presumably providing information of a model female's reproductive status. Here, I manipulated the visual appearance of virtual model females with *FishSim* by either presenting virtual model females with a gravid spot or without a gravid spot to live focal females.

In **Chapter 8**, I will present a first attempt in artificial manipulation of extent and direction of courtship between the interacting pair (virtual model and male) as presented during the observation period of a MCC experiment. So far, it has not been systematically tested whether or not differences in courtship behavior might affect MCC in focal females (but see Witte and Ueding 2003). Manipulation of behavior is almost impossible using conventional methods with live fish stimuli. In this study, I used virtual fish stimuli created with *FishSim* to decipher whose behavior of the interacting pair provides most relevant information for copying to observing focal females. In three different treatments, I altered extent and direction of courtship behavior of the interacting pair to be either: (I) mutual, (II) only female driven, or (III) only male driven.

In **Chapter 9**, I manipulated the behavioral interaction of the pair (model female and male) by experimentally increasing the distance between them. Behavioral interaction between the pair was thereby restrained compared to natural courtship at close proximity. Here, I used live fish stimuli only since the role of distance on MCC was impossible to test using the experimental setup for virtual fish due to constraints resulting from the monitor's dimensions.

In **Chapter 10**, I will finally summarize and discuss my results in light of the relevance of public information content for MCC in sailfin mollies. I will suggest new angles on MCC research for future studies. Additionally, I will evaluate the benefit of using *FishSim* in studies on MCC as well as its general prospects for future studies on other aspects of fish behavior.

In summary, my PhD thesis will contribute new insights to the general understanding of the underlying mechanism of MCC, with special regard to the relevance of public information content that determines if the choice of a model female is copied or not. I hope that the results and discussions presented herein will broaden the understanding of MCC in sailfin mollies and inspire further research on MCC in other species of animals as well. Further, I hope that I will success in presenting a new and powerful method for controlled and standardized testing of MCC in sailfin mollies and other small fish.

Chapter 2
Study species

2.1 The sailfin molly *Poecilia latipinna*

The sailfin molly belongs to the family Poeciliidae (Order: Cyprinodontiformes) and was first described by LESUEUR in 1821 as *Mollienesia latipinna*. Later, a systematic review of the family by Rosen and Bailey (1963) led to the re-classification of the genus *Poecilia*, referring to it as *Poecilia latipinna* from there on.

The family Poeciliidae is a monophyletic group containing nearly 220 species of small Neotropical fish with a geographical distribution focusing on Central and South America (Rosen and Bailey 1963; Meffe and Snelson 1989; Evans et al. 2011). Poeciliids are very tolerant and highly adaptive colonizers that can be found in nearly every habitat and microhabitat ranging from freshwater to saltwater as well as toxic, hydrogen sulphide-rich waters (Meffe and Snelson 1989; Riesch et al. 2014; Gomes-Silva et al. 2019). Also the euryhaline sailfin molly is found to inhabit a wide range of different water bodies from springs, ponds and lakes over rivers, streams and drainage ditches to even the salt marshes (Nunez et al. 2015). With populations found from North Carolina along the Atlantic coast to the Yucatán peninsula, Mexico, sailfin mollies primarily inhabit coastal regions and, therein, shallow and protected areas among vegetation on the edge of the water body (Rosen and Bailey 1963; Hubbs et al. 2008). Sailfin mollies live in loose, mixed-sex shoals that are flexible in time and space and comprise of around 20 individuals (Travis 1994b; Witte and Ryan 1998). They are omnivores and easily adapt to different environments which, combined with its wide popularity in the ornamental fish trade, yielded the sailfin molly its worldwide status as a potential invasive species (Courtenay and Meffe 1989; Felley and Daniels 1992; Nordlie et al. 1992; Koutsikos et al. 2018; CABI 2019; Gomes-Silva et al. 2019). Nowadays, established invasive populations of sailfin mollies can for example even be found in Greece (Koutsikos et al. 2017) or Iran (Esmaeili et al. 2017; Mousavi-Sabet 2018).

The reproductive biology of poeciliids is characterized by internal fertilization and ovo-viviparity, i.e., giving birth to live young (but see *Tomeurus gracilis;* Parenti et al. 2010), which made them widely known as “livebearing fishes” (e.g., Rosen and Bailey 1963; Meffe and Snelson 1989; Evans et al. 2011; see also Chapter 2.1.3 below). Due to its fascinating mating system and the fact that it can be easily kept and bred in laboratory environments, the sailfin molly enjoys increasing popularity among scientists studying sexual selection and its underlying mechanisms (e.g., Ptacek and Travis 1997; Schlupp and Ryan 1997; Witte and Ryan 1998; Gabor 1999; Makowicz et al. 2010b; Nöbel and Witte 2013); alongside with other famous representatives of the genus *Poecilia*, such as the guppy *Poecilia reticulata*, the Atlantic molly *Poecilia mexicana*, and the Amazon molly *Poecilia formosa*.

All sailfin mollies used in the experiments of my thesis were descendants of wild caught fish from three different populations in Texas, USA (Fig. 2). Earlier reports stated all inland Texan populations being native (Burgess 1980), though it was later argued that these communities merely represent introductions to the inland freshwater system (Brown 1953; Hubbs et al. 2008). Fish used in my study originated from the Comal River in New Braunfels (Fig. 2, 1), the Coleto Creek near Victoria (Fig. 2, 2), and the Mustang Islands near Corpus Christi (Fig. 2, 3). Fish from Comal River were caught by Klaudia

Witte in 2007 and were raised in the lab in Siegen since 2008. Both fish from Coleto Creek and the Mustang Islands were caught and raised by a commercial fisheries and hatching company (Goliad Farms, Inc.[2]). The group from Coleto Creek was caught in 1998 and its descendants shipped to Siegen in 2009. The coastal Mustang Island's group was caught and shipped in 2014. In the lab, all fish were kept in mixed-sex shoals, separated by population, as described in Chapter 2.3.

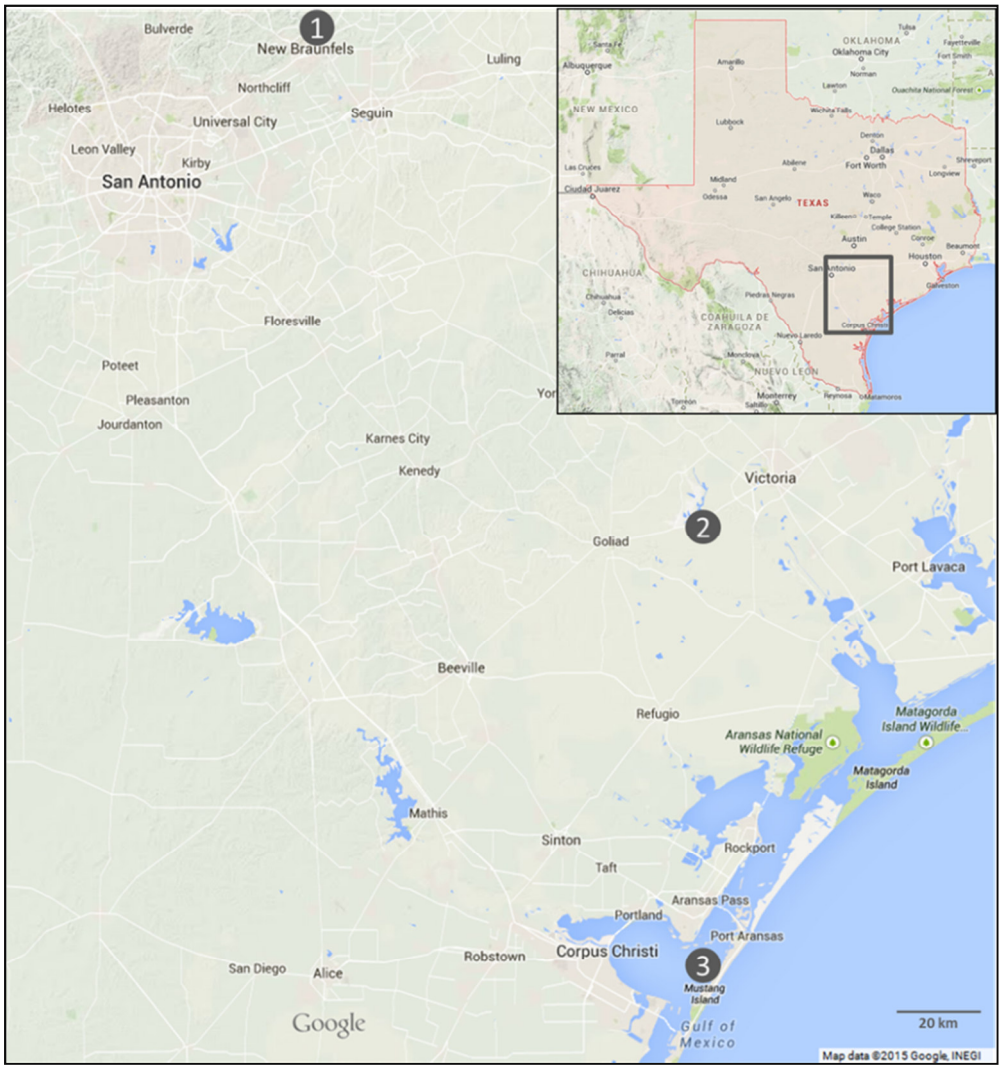

Figure 2. Map of the three sailfin molly catching sites in southern Texas (USA). The map shows the origin of sailfin molly populations used in my thesis: 1) Comal River; 2) Coleto Creek; 3) Mustang Island (modified after Google Maps 2015).

2.1.1 Morphology and appearance

The sailfin molly is a small fish with a light grey to olive-green color. Several rows (typically 5 to 8) of darker brown or orange spots occur along both sides of the body. The head is dorsally flattened with a superior mouth and two bright spots above the snout. Females are quite uniform and inconspicuous in color with a more roundish body shape, although, in the area of the caudal peduncle the body is strongly compressed in both

[2] Goliad Farms website: https://goliadfarms.com

sexes. Similar to other poeciliids, sailfin molly females may show a distinctive dark spot in their urogenital region (Peden 1973; Farr 1980; Sumner et al. 1994; Norazmi-Lokman et al. 2016; see Chapter 7 for more information). In contrast, males typically have a more elongated body shape and exhibit an enlarged, brightly ornamented dorsal fin (the 'sailfin'), and a large colorful caudal fin (Snelson 1985; see Fig. 3 and also compare Fig. 4 in Chapter 2.1.2 as well as Figs. 9 and 11 in Chapter 4.2). The male's dorsal and caudal fins usually express a vivid pattern of black stripes and dots including brightly orange patches and iridescent blue parts. Often, the distal fringe of the caudal fin is black, whereas the sailfin typically shows a broader orange fringe on top. Similar to other poeciliids (Morris et al. 1995; Fisher et al. 2009; Tudor and Morris 2009; Robinson and Morris 2010), males often have dark vertical bars, a melanin-based pigment pattern, on their lateral sides that may either be symmetrical or asymmetrical in number between their left and right side (Parzefall 1969; Schlüter et al. 1998; Fig. 3). Further, and often at stages of high agitation, vertical bars may increase in intensity and males may show a bright yellow-orange coloration on their head and/or throat.

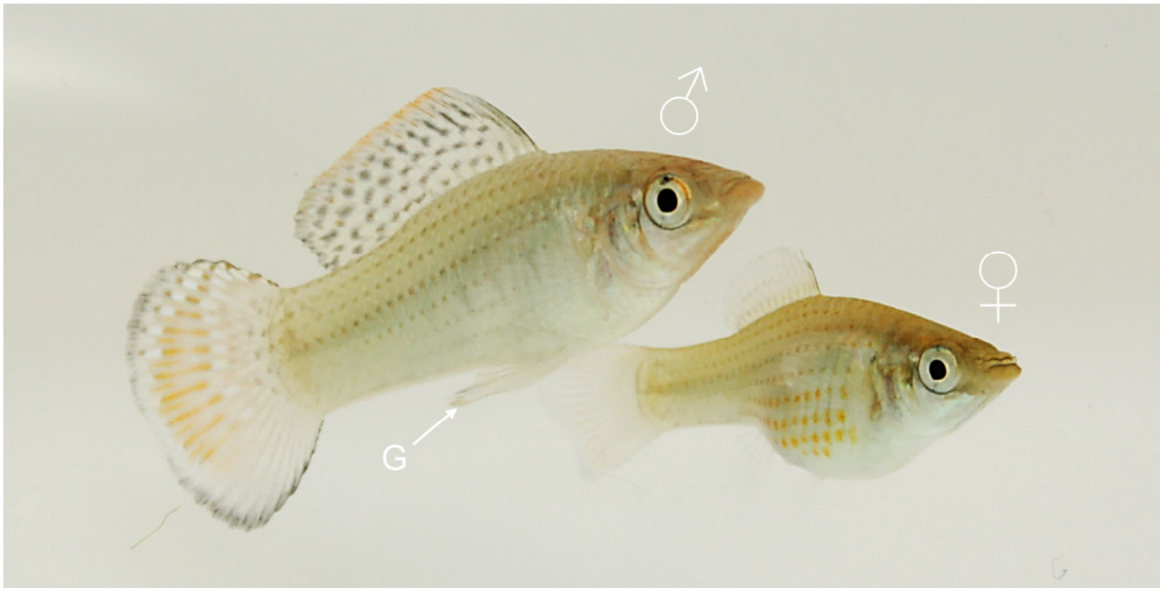

Figure 3: Male (left) and female (right) sailfin mollies (LESUEUR 1821). G = gonopodium.

Male and female poeciliids typically exhibit a sexual size dimorphism with females growing larger than males, which can also be observed to some extent in sailfin mollies (Bisazza 1993). Their maximum standard body length (SL; measured from the tip of the snout till the end of the caudal peduncle) is around 120 mm for adult females and around 100 mm for adult males. A male's definite size is based on a genetic polymorphism and is more or less fixed upon maturation, since, in contrast to females, male growth decelerates from that moment on (Travis 1989a; Travis and Woodward 1989; Trexler et al. 1990; Travis 1994; but see Snelson 1982).

Three different male types were described for sailfin mollies based on their body size at maturity: (I) small dwarf or sneaker males (< 30 mm SL); (II) intermediate sized males (30-45 mm SL); and (III) large courting males (> 45 mm SL; Baird 1974; Luckner 1979; Snelson 1985). Small males are typically very inconspicuous and drab in color, almost resembling females, whereas intermediate males show more variability in fin sizes and

color. In contrast, large males are brightly colored and exhibit greatly enlarged sailfins and dorsal fins (Snelson 1985). Expression of a male's color, however, is also influenced by social status and group composition, that is, the number and type of other males in the respective group (Snelson 1985; Travis and Woodward 1989). In this context, small drab males are considered subordinate within a group, in contrast to dominant larger colorful males (see Snelson 1985 and references within). Large dominant males can frequently be observed to harass and chase away smaller subordinate males (Baird 1974; Luckner 1979; personal observation). The three male types typically also differ in their mating tactics (see Chapter 2.1.2 below) and body size was found to be positively correlated with boldness in this species (Seda et al. 2012). Overall, male size and color were found to be highly variable within and across populations (Snelson 1985). Aside from color, the most distinctive feature telling mature males and females apart is their sexual dimorphic anal fin. In males, the third, fourth and fifth anal fin ray are transformed and fused upon sexual maturation to form an intromittent organ, the so-called 'gonopodium' (Fig. 3; Greven 2011) which enables internal fertilization (Evans et al. 2011; see Chapter 2.1.2 below).

2.1.2 Mating system and courtship behavior

Sailfin mollies have a promiscuous mating system in which both male and female choose their mating partner (Ptacek and Travis 1997; Schlupp and Ryan 1997; Witte and Ryan 2002). As frequently reported for other poeciliids (e.g., Marler and Ryan 1997; Rosenthal and Evans 1998; Aspbury and Basolo 2002; Plath et al. 2004), female sailfin mollies prefer males with larger body size (e.g., Schlupp et al. 1994; Ptacek and Travis 1997; Witte and Ryan 1998; Gabor 1999), larger dorsal fin, and overall larger lateral projection area (LPA; MacLaren et al. 2004). Scherer et al. (2018) reported a positive correlation between male size and offspring quantity, albeit using a very small sample size. Further, females were shown to prefer symmetry in the number of a male's vertical bars (Schlüter et al. 1998).

Male sailfin mollies, likewise, do prefer larger females (e.g., Ptacek and Travis 1997; Gabor 1999) and even produce more sperm when associated with larger compared to smaller females (Aspbury and Gabor 2004). Thereby, larger males showed a stronger preference regarding female size than smaller ones (Ptacek and Travis 1997). The preference for larger females is also shared among other poeciliids (e.g., Dosen and Montgomerie 2004; Plath et al. 2006; Hoysak and Godin 2007; Arriaga and Schlupp 2013) which is likely the result of a strong correlation between female body size and fecundity (e.g., Hughes 1985; Herdman et al. 2004; Marsh-Matthews et al. 2005; Riesch et al. 2009). Accordingly, larger female sailfin mollies were found to carry more eggs compared to smaller ones (Hubbs 1964). Interestingly, the percentage of multiply sired broods was higher in larger females (Travis et al. 1990) and also if variation in female fertility in a population was high (Trexler et al. 1997). Presumably, chances are higher that males who preferably mate with larger females still father at least some of the brood, if those females had previously mated.

Sailfin molly courtship behavior has been studied and described in detail by Parzefall (1969), Baird (1974) and Luckner (1979). Male courtship can be divided into several distinctive behavioral patterns that may vary in order: First, a male approaches and follows a female (Fig. 4A). Frequently, he makes oral or nasal contact with the female's gonopore (gonoporal nibbling; Fig. 4B), presumably to check a female's receptivity indicated by chemical substances (Parzefall 1973; Aspbury et al. 2010; Greven 2011).

Spreading his dorsal fin and caudal fin, he performs lateral displays (mostly next to a female) and sigmoid displays in front of her (Figs. 4C and 4D). During sigmoid displays, his body curvature more or less resembles an S-shape, hence giving the display its name. In an attempt to mate, the male approaches the female from behind and rotates his gonopodium to face anteriorly (gonopodial thrusting), to be inserted into the female's gonopore for internal insemination (see pictures in Greven 2005). Here, the length of a male's gonopodium dictates the distance between male and female necessary for successful copulation. Gonopodial length (relative to a male's SL) is generally associated with a male's mating strategy, that is, males relying on forced copulations tend to have long gonopodia (e.g., *Poeciliopsis* spp., *Heterandria* spp., *Girardinus* spp.), whereas males relying more on courtship prior to copulation tend to have short gonopodia (e.g., *Poecilia* spp., *Xiphophorus* spp.; Rosen and Tucker 1961; Greven 2005; Greven 2011). Overall, gonopodial length is subject to high intra- and inter-specific variation and considered to contribute to both sexual and natural selection (Ptacek and Travis 1998).

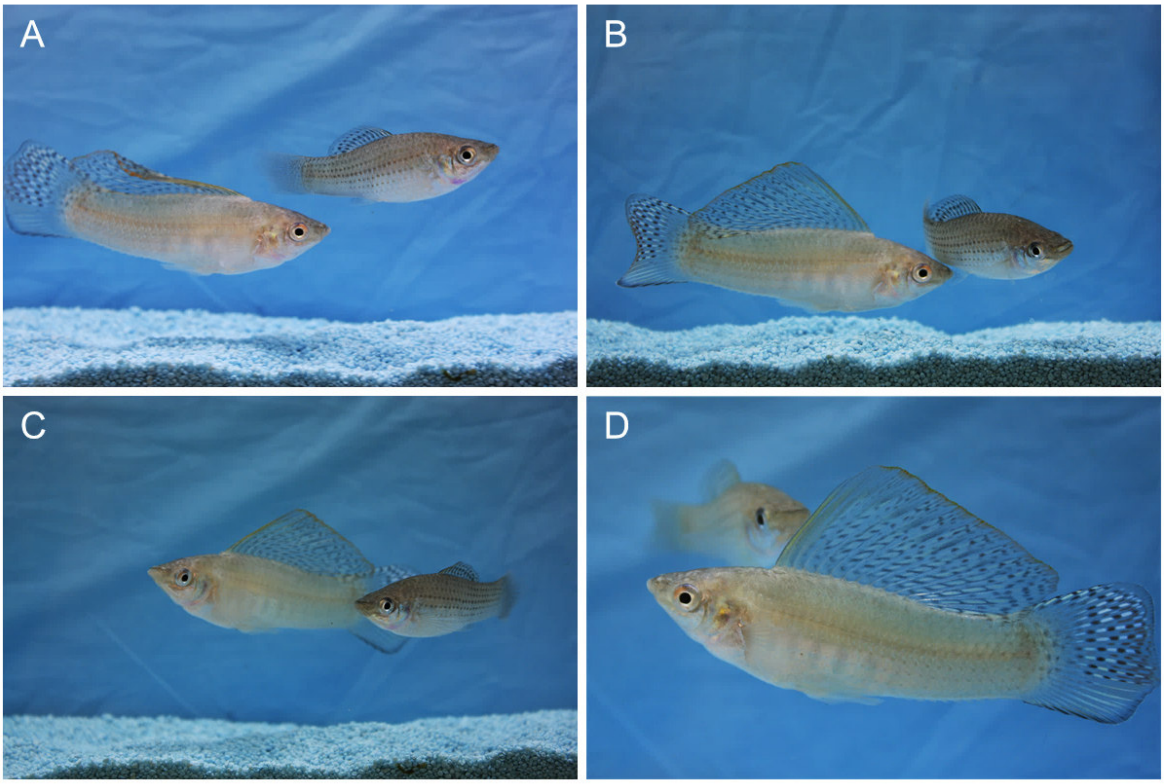

Figure 4. Pictures of sailfin molly courtship behaviour. (A) Male approaching a female. **(B**) Gonopore nipping. **(C)** Lateral display next to the female. Dorsal fin not completely raised. **(D)** Sigmoid display in front of the female.

As already mentioned in Chapter 2.1.1, different mating tactics exist among sailfin molly males (Baird 1974; Luckner 1979; Snelson 1985; Seda et al. 2012; Fraser et al. 2014), a pattern which can also be seen in other fishes (e.g., Ryan et al. 1990; Bisazza 1993; Taborsky 1994). On average, large colorful males tend to rely more on courtship prior to copulation, whereas small and inconspicuous males tend to rely more on sneaky matings, that is, forced-copulations. Intermediate sized males were found to be more flexible in the use of both mating tactics. The above described behavioral patterns of male courtship may vary in both their order and frequency, depending on a male's mating tactic (described in detail by Luckner 1979). Further, a male's absolute size as

well as his relative size within a population were shown to predict the rate at which behaviors are expressed (Travis 1994a). For example, intermediate males may show courtship when associated with smaller males and show sneak copulations when associated with larger males (Travis and Woodward 1989; Fraser et al. 2014). A recent study by Goldberg et al. (2019) on the evolution of male ornaments and courtship behavior postulate that exaggerated fins combined with raising of the dorsal fin as well as sigmoid displays originally evolved in the context of intrasexual competition and, therefore, serve a multi-utility purpose. Indeed, these behaviors are readily observed during aggressive encounters between dominant and subordinate sailfin molly males (Baird 1974; personal observation). When living in sympatry, sailfin molly males are sexually parasitized by the gynogenetic Amazon molly (*Poecilia formosa*), a unisexual and all-female hybrid-species of which sailfin molly and Atlantic molly are considered the parental ancestors (Hubbs and Hubbs 1932; Hubbs 1964; Avise et al. 1991; Schartl et al. 1995). To trigger embryogenesis, Amazon mollies have to mate with either male of the two species, though genetic transfer via sperm does not occur (Schlupp and Plath 2005).

In contrast, the female's behavior is way less pronounced than that of the male and may include aversive behaviors, such as actively fleeing from a male, or staying motionless to show her willingness to copulate. In this case, females will usually tilt their body towards that of the male to facilitate contact between the male's gonopodium and her gonopore for insemination (Baird 1974; Luckner 1979; Greven 2005). In poeciliid species, virgins, post-partum females and females that have been separated from males tend to be more responsive towards male courtship and, consequently, copulation (Baird 1974; Bisazza 1993 and references within). Females are most receptive closely after parturition and, indeed, male sailfin mollies were found to be most attracted towards virgins and post-partum females (Farr and Travis 1986). However, females may generally mate throughout since they are able to store sperm for up to several months (Constantz 1989). Multiple paternities within a brood are, therefore, quite likely (Travis et al. 1990), as is superfetation (i.e., simultaneously carrying two or more broods at different developmental stages; Monaco et al. 1983). Females show facultative matrotrophy, providing nutritive molecules to the embryos to varying degrees via follicular placentae (Trexler 1985; Trexler 1997). After a gestation period of around 30 days they give birth to live young. In ovo-viviparous poeciliids, parturition coincides with ovulation meaning that the young develop and remain inside the mother's body until they are ready to hatch (Greven 2011). Usually, the fry is born without yolk sac (Greven 2011) and is completely relying on its own from then on.

2.1.3 The sailfin molly's visual system

For many organisms, the visual system plays a key role in the detection and evaluation of suitable habitats, food sources, predators and prey, conspecifics and heterospecifics, as well as mating partners. Here, the overall assumption is that an organism's visual system should be tuned to match the visual properties found in its respective habitat. The same also accounts for aspects regarding fine-tuning of the visual system towards visual communication as for example regarding mate preferences during mate choice (e.g., Cummings 2007; Sandkam et al. 2015).

The fish eye conforms to the general anatomy of a vertebrate eye (Hildebrand and Goslow 1988) amended by specific adaptations for vision in aquatic environments, e.g., a spherical lens, as was reviewed in detail by (Fernald 1988). Spectral distribution of light in shallow waters in particular, is not very different from that experienced by terrestrial animals (Lythgoe and Partridge 1989). As a diurnal species that inhabits the surface area of shallow waters with generally high availability of ambient light and good visibility, the sailfin molly's visual capacities can be considered very well established (Caves et al.

2017). Expression of an elaborate courtship display featuring colorful patterns varying in extant between and within the sexes, underlines the importance of visual features for both species recognition and mate choice in this fish (Parzefall 1969; Baird 1974; Luckner 1979; see Chapter 2.1.2 above).

Previous studies showed that females readily discriminate between males based on visual features alone, such as body size (Witte and Ryan 1998; MacLaren et al. 2004), dorsal fin size (MacLaren et al. 2004), overall lateral projection area (LPA; MacLaren et al. 2004), and different color traits (Schlupp et al. 1999; MacLaren 2017; MacLaren 2019). Schlüter et al. (1998) demonstrated that females even seem to recognize whether the number of vertical stripes on a male's left and right side were symmetrical or asymmetrical, preferring the former. Moreover, MacLaren (2006) showed that females were able to discriminate between different male sizes up to a distance of 68 cm. Sailfin mollies were shown to use visual cues from public information when choosing between potential mates (see Chapters 1.2, 1.3 and 2.2) even at distance (Gierszewski, Keil, et al. 2018; see Chapter 9), which requires deliberate observation of others and notice of even subtle changes in behavior. Heubel and Schlupp (2006) demonstrated that if visibility was altered by environmental factors, such as turbidity, sailfin molly mate choice was significantly affected, underlining the importance of their visual sense for this task. This relationship was also demonstrated in guppies (Long and Rosenqvist 1998), whose color vision is specifically tuned towards mate preferences (Sandkam et al. 2015).

A prerequisite for color vision is the existence of photoreceptors containing different visual pigments that are sensitive to different wavelengths of the light's spectrum (Fernald 1988). Körner et al. (2006) investigated the visual pigments in female sailfin mollies and measured their spectral sensitivities. They possess four different cone types, three of them sensitive to wavelengths also visible to the human eye, and one type which is also sensitive to ultraviolet (UV) light (see Körner et al. 2006 for absorption maxima of rods and cones). In contrast to us humans (Boettner and Wolter 1962), many animals are capable of perceiving UV light (e.g., invertebrates: Menzel 1979; Thoen et al. 2014; birds: Cuthill et al. 2000; fish: Losey et al. 1999; Siebeck 2014), meaning that they possess visual pigments sensitive to light at wavelengths below 400 nm. Even near-infrared (750-1400 nm) and infrared light (>1400 nm) can be perceived by some fish (*Pelvicachromis taeniatus*: Meuthen et al. 2012; *Danio rerio*: Hartmann et al. 2018) and other organisms (e.g., snakes: Goris 2011).

Palmer and Hankison (2015) found UV-reflective structures across the bodies of both male and female sailfin mollies via spectrophotometric measurements and demonstrated their importance for female mate choice. In their study, females significantly preferred males viewed under full spectrum ('UV+' condition) compared to males whose UV patterns were made invisible using optical filters ('UV-' condition). Whereas males did not discriminate between females with or without visible UV (UV+/UV-) patterns. Similarly, the significance of UV patterns for mate choice was demonstrated in other poeciliid species as well (e.g., guppies: Kodric-Brown and Johnson 2002; Smith et al. 2002; White et al. 2003; swordtails: Cummings et al. 2003), as well as sticklebacks (Rick et al. 2006). UV contrast in the abdominal region of male sticklebacks was found to be positively correlated with body condition (Rick et al. 2004), underlining the possibility of UV patterns giving away information about mate quality towards the observer. If this is also the case in sailfin mollies has, to my knowledge, never been tested.

Despite their demonstrated ability to perceive and evaluate UV patterns (Palmer and Hankison 2015), previous studies using video playback on monitor screens (unable to emit UV light; see Chapter 3) showed that those patterns were no prerequisite for female sailfin mollies to distinguish between videotaped conspecifics (Witte and Ueding 2003; Witte and Klink 2005; Heubel et al. 2008). Here, focal females discriminated between video male stimuli based on visible movement and RGB colors (as emitted from screens;

see Chapter 3) alone. Further, Makowicz et al. (2010b) supported the effectiveness of video playback to study questions related to mate choice in this species and showed that focal sailfin molly males perceived video male stimuli as competitors. Combined, these studies show that a different mode of presentation (video vs. live stimulus) did not impair the perception and use of visual public information by observing live focal sailfin mollies (MCC: Witte and Ueding 2003; Heubel et al. 2008; audience effect: Makowicz et al. 2010b), which was an important requirement for my thesis. Overall, different video and computer techniques have been deployed in various studies on mate choice in poeciliids over the last decades, demonstrating a general good responsiveness towards artificial stimuli presented on monitor screens within the family (e.g., Körner et al. 1999; Landmann et al. 1999; Nicoletto and Kodric-Brown 1999; Trainor and Basolo 2000; Fisher et al. 2009; Rosenthal and Ryan 2010; Butkowski et al. 2011; Bierbach, Jung, et al. 2013; Polverino et al. 2013).

2.2 The sailfin molly as a model organism for the study of mate-choice copying

Since Dugatkin (1992) first demonstrated MCC in the guppy, scientists tried to decipher its underlying mechanism (see Chapter 1.3 for detailed information on MCC). Then, in 1994, Schlupp and colleagues first described MCC in the sailfin molly (Schlupp et al. 1994), which then rapidly advanced as a popular model organism for the study of MCC, together with other poeciliid species (e.g., Bierbach, Kronmarck, et al. 2011; Zimmer et al. 2013; Auld and Godin 2015).

As group-living animals, sailfin mollies have ample opportunities to observe their conspecifics in their day-to-day life. Indeed, both sexes were shown to utilize public information from conspecifics (Schlupp and Ryan 1997; Witte and Ryan 2002) and heterospecifics (Schlupp et al. 1994; Heubel et al. 2008) for their mate-choice decisions, not only in the context of MCC but also regarding the audience effect (Makowicz et al. 2010b; Nöbel and Witte 2013). Sailfin mollies were shown to copy the choice of others both in the laboratory and in their natural environment (Witte and Ryan 2002), a finding that validated MCC as a biologically relevant mate-choice strategy in this species.

Over the last decades, experiments using sailfin mollies led to several important new discoveries underlining the complexity behind this fascinating strategy. Generally, MCC is especially beneficial if two potential mates are very similar in quality (i.e., body size; Witte and Ryan 1998). Sailfin mollies are sensitive towards differences in public information quantity. When they had access to a larger amount of public information favoring the smaller of two males, females neglected their genetically based preference for larger males (Witte and Noltemeier 2002). The study also confirmed that MCC has long-term effects on the preference of certain male phenotypes, since females still favored smaller males after a period of five weeks. Here, females generalized the learned male phenotype and chose other small males than they had previously observed with a model, which is an important prerequisite for the cultural inheritance of mate preferences (see also Chapter 1.3). On the other hand, female sailfin mollies also remembered the observed choice for a specific male individual even after one day (Witte and Massmann 2003). Using the sailfin molly, it was further shown that MCC may favor the spreading of a novel male trait (MacLaren 2019). Together with the fact that sailfin mollies also copied the rejection of a mate (Witte and Ueding 2003), these findings combined bear important implications for sexual selection and the evolution of traits in this species and animals in general.

Based on information presented in this chapter, the sailfin molly can be considered the ideal study organism to investigate MCC. With the help of the sailfin molly, I will present new insights into MCC to broaden common knowledge on this fascinating mate-choice strategy by applying new and old ideas with different conceptual approaches and modern state-of-the-art research techniques.

2.3 Fish housing and experimental rooms

All fish used in the experiments of my thesis were descendants of wild caught mollies as stated in Chapter 2.1 and were kept, bred and tested in two different rooms belonging to the Witte Lab at the Institute of Biology at University of Siegen, between 2014 and 2018. Experimental procedures as well as fish handling were in line with the German Animal Welfare Act (Deutsches Tierschutzgesetz) and approved by the internal animal welfare officer Dr. Urs Gießelmann, as well as regional authorities (Kreisveterinäramt Siegen-Wittgenstein).

2.3.1 The fish room

The fish room is specialized for the maintenance and rearing of small fish under constant rearing conditions. Here, we kept all fish in mixed-sex shoals (up to 40 individuals) in large housing tanks (80 cm x 35 cm x 40 cm). All housing tanks were positioned in two rows of a large shelf at the longer side of the fish room (Fig. 5A, SH). Here, we separated fish according to their species and population. Besides sailfin mollies, other species were kept in the fish room, including guppies, Atlantic mollies and zebrafish (*Danio rerio*).

Illumination was provided by fluorescent light tubes (OSRAM L58W/965, OSRAM GmbH, Germany). Throughout, we maintained a light/dark cycle of 14/10 hours daily. The water used for fish housing was treated by a reverse-osmosis system and then mineralized (Preis Diskus Mineralien, Preis-Aquaristik KG, Germany) to meet the requirements for livebearing fish, with a conductance of about 250 µS/cm. Treated water was stored in two large water reservoirs à 400 Liters each (Fig. 5A, R) to perform regular water changes (about 20 % per tank). Overall air temperature of the fish room was regulated by an air-conditioning system to heat water temperature to 25 ± 1 °C. In each tank separately, water was aerated and filtered using an external gravel filter (HYDOR Prime 20, HYDOR Srl, Italy) connected to a UV-C clarifier (JBL AquaCristal UV-C 5W, JBL GmbH & Co. KG, Germany). Additionally, tanks were equipped with gravel substrate and plants (e.g., *Cryptocoryne crispatula*) to increase animal welfare. We added several smaller separation tanks (40 cm x 25 cm x 40 cm) to the shelf in which smaller groups of fish (up to 15 individuals) could be separated prior to experiments. Housing conditions and water parameters inside separation tanks were kept to match those of the larger housing tanks as closely as possible. Here, we used internal filters (JBL CristalProfi i60, JBL GmbH & Co. KG, Germany), due to the smaller tank dimensions.

Overall, fish were kept under a constant feeding regime of flake food (NovoBel, JBL GmbH & Co. KG, Germany) and frozen brine shrimp and red mosquito larvae alternately twice a day. On weekends, we only fed flake food once a day. Food was vitamized on a regular basis using a liquid supplement (Atvitol, JBL GmbH & Co. KG, Germany).

The fish room also served to conduct some of the experiments that I will present in my thesis. Thereby, testing always started after the daily cleaning routine was finished, usually at around 10 to 11 am in the morning. For the whole duration of an experimental trial, nobody (except for the experimenter) was allowed to enter the fish room. Overall, a once established experimental setup was not changed or its position moved until the whole experiment was finished. However, curtains were opened (Fig. 5B) or removed (Fig. 5C) after testing. Chairs and tables with operational computers used by the experimenter were also removed but their position marked visually on the floor to maintain a constant distance to the test tank.

During my thesis, four different experimental setups were used to study MCC in sailfin mollies. Here, I will provide a quick overview of where each experiment was performed; however, specific features of each individual setup will be described in detail within the respective chapters. Overall, experimental setups were arranged to resemble those used in prior studies on MCC in fish (e.g., Schlupp and Ryan 1997; Witte and Ryan 1998; Auld and Godin 2015).

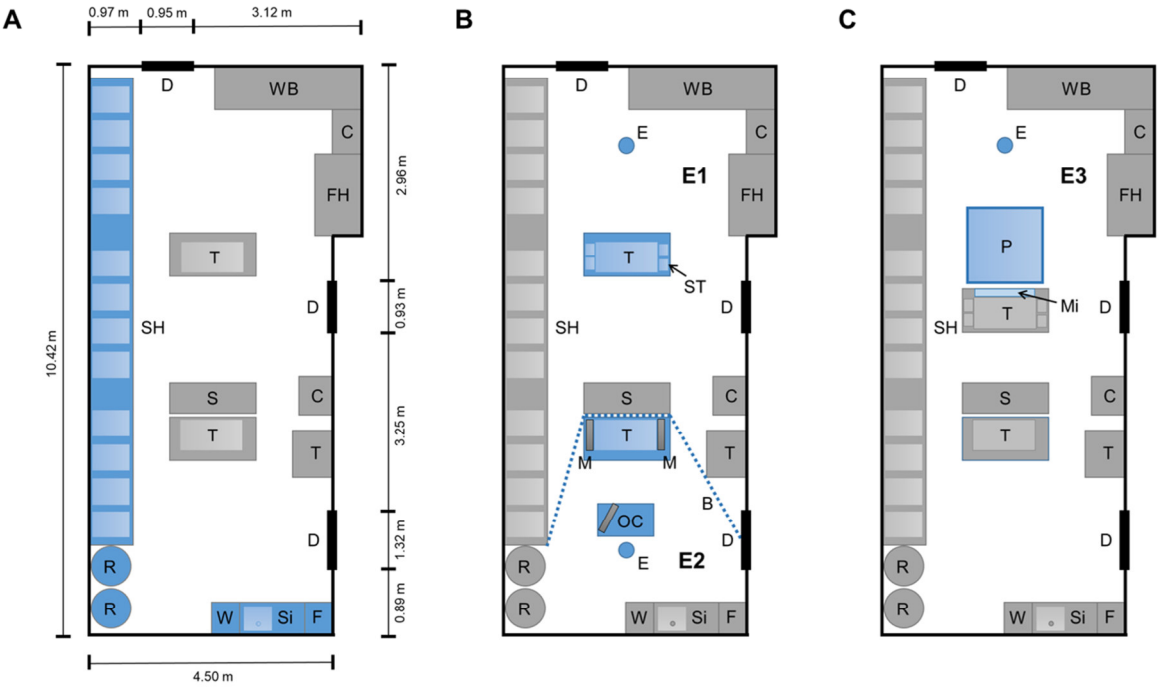

Figure 5: The fish room. (A) Schematic top-view of the fish holding room. (B) Top-view showing the position of setups E1 (Chapter 6) and E2 (Chapters 6 and 7) inside the fish room. (C) Top-view of setup E3 used in Chapter 9. B = visual barrier (curtain) depicted by a dashed line, C = small cabinet, D = door, E = experimenter, F = fridge and freezer for food storage, FH = fume hood, M = LCD monitor, Mi = mirror, OC = table with operating computer and a monitor, P = children's pool, R = water reservoir, S = shelf, SH = shelf with holding tanks, Si = sink, ST = small stimulus tanks, T = table with test tank, W = washing machine, WB = work bench. Important features are presented in blue color. Dimensions may not be perfectly to scale.

Experimental setups E1 and E2 (Fig. 5B) were used to investigate the role of the size of the model female on MCC as presented in Chapter 6. Here, we did a comparative MCC experiment either testing focal fish with live fish stimuli, presented in small stimulus tanks adjacent to the test tank, (Fig. 5B, E1) or using virtual fish stimuli created with *FishSim* instead of live stimulus fish (Fig. 5B, E2). For this, we replaced the small stimulus tanks

containing live fish with LCD monitors presenting virtual fish stimuli (Fig. 5B, ST and M). Further, setup E2 was also used to perform the study on how a gravid spot affects the quality of a virtual model female as presented in Chapter 7. In Chapter 9, however, we followed a new approach to study the effect of distance on information transmission for MCC. For this, we needed a larger distance between fish stimuli and transferred our testing procedure to a children's pool instead (Fig. 5C, E3; see also Fig. S5 in Appendix 6).

2.3.2 The virtual fish lab

In addition to the fish room, a second room entitled as "the virtual fish lab" was used to undertake some of the experiments described in my thesis (Fig. 6). Here, we built a more complex technical setup specialized for the use of *FishSim* combined with a real-time 3D tracking system comprised of two cameras (see Fig. 6A, C1 and C2, and Fig. 6B) developed by Müller et al. (2014; see Chapter 4 for a description of the software). Since the tracking system required uniform lighting, the test tank was framed by an aluminum rack containing several LED stripes (12 V, 6500 K; Fig. 6B). By this, focal fish could be observed and/or tracked during experiments using an operation terminal opposite to the test tank (Fig. 6A, OC).

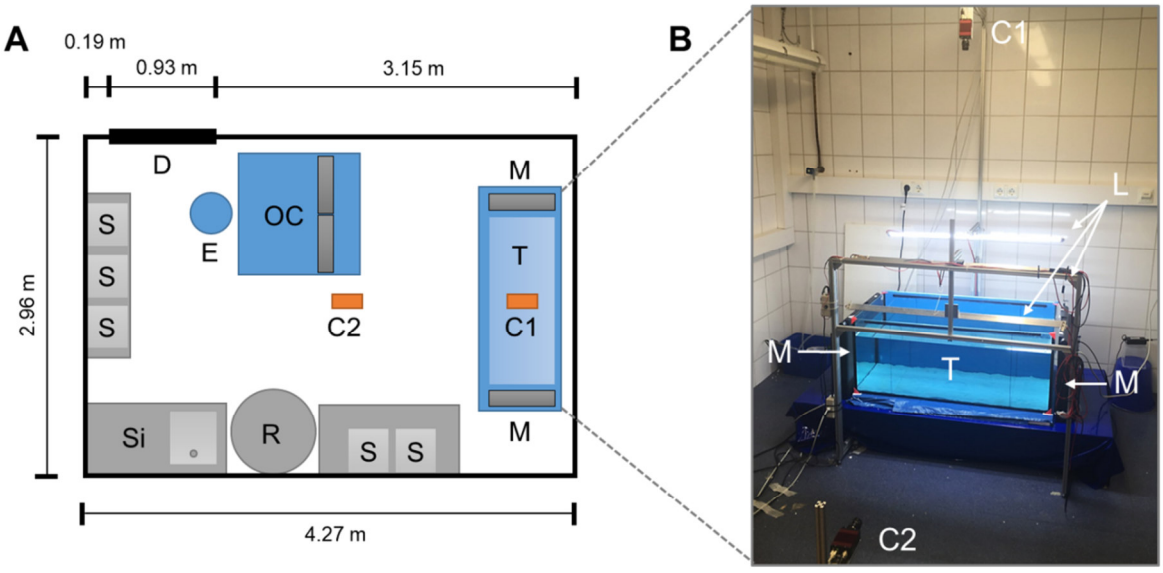

Figure 6: The virtual fish lab. (A) Schematic top-view of the features inside the virtual fish lab. The experimental setup used in Chapters 5 and 8 is shown in color. D = door, C1 = top camera, C2 = front camera, T = test tank, M = LCD monitors, OC = table with operating computer and two monitors for observation, E = experimenter, S = small separation tank, Si = sink, R = water reservoir. Important features are presented in blue color. Dimensions may not be perfectly to scale. (B) Photograph of the test tank (T) with two adjacent monitors (M), top (C1) and front camera (C2) as well as the aluminum rack for LED illumination (L).

The virtual fish lab was also used to acclimate test fish towards their new surroundings (compared to those in the fish room). Here, fish were kept in small separation tanks (40 cm x 25 cm x 40 cm; in Fig. 6A, S) resembling those used in the fish room. Since the test tank in the virtual fish lab was filled with blue-colored sand (NANO Bodengrund,

COLORSTONE, Rudolstadt, Germany; color: sky-blue, grain size: 0.8-1.2 mm), separation tanks were also equipped with the same sand so fish could get accustomed to it prior to testing. We used small heaters (Tetratec HT 50, Tetra GmbH, Melle, Germany) in each separation tank to maintain temperature. Overall, housing conditions and water parameters were kept to match those of the fish's housing tanks as close as possible (e.g., identical feeding regime and light/dark cycle). Similar to the fish room, the virtual fish lab had access to a 400 liters reservoir (Fig. 6A, R) with demineralized and treated water to perform regular water changes.

The virtual fish lab served as the experimental room for all validation experiments that I performed to validate the usability of *FishSim* for my planned experiments on MCC (see Chapter 5). In this study, however, I made several adjustments to the experimental setup, i.e., presentation of virtual/video stimuli on either LCD screen, CRT screen, or as live fish swimming in small tanks. These adjustments are not visualized in Figure 6 but are described in detail in Chapter 5.3.4. Furthermore, I performed all experiments studying whether the behavioral interaction between the model female and the stimulus male affects MCC in the virtual fish lab (Chapter 8).

The following chapters of my thesis are written as manuscripts based on four peer-reviewed papers (Chapters 3, 5, 7, 9), which have been published in different scientific journals as stated separately at the beginning of each chapter. Chapters 6 and 8 describe manuscript drafts intended for future submission. Since all manuscripts need to be comprehensive in themselves, recurrent descriptions and definitions may occur. Please note that I adapted the format and layout of published papers to the general layout of my thesis, resulting in minor differences due to: (I) editorial changes of each journal; (II) editorial changes I made regarding the use of American English throughout; and (III) adjustments of some figures and tables to match the style of my thesis. To be consistent throughout my thesis, I changed the term “preference test” and its abbreviations “P1” and “P2” in Chapter 9 into “mate-choice test” and “M1” and “M2” respectively. Further, I re-labelled the numbering of all figures, tables, as well as supplementary materials (listed as appendices) according to their order of appearance within my thesis. All papers presented in my thesis have several co-authors whose contributions are stated in the ‘Author contributions’ section (pages 1-2) for each chapter separately. Additionally, author names and affiliations are given at the beginning of each chapter.

Chapter 3

Technical and conceptual considerations for using animated stimuli in studies of animal behavior

Laura CHOUINARD-THULY[a,†], Stefanie GIERSZEWSKI[b,†], Gil G. ROSENTHAL[c,d], Simon M. READER[a], Guillaume RIEUCAU[e], Kevin L. WOO[f], Robert GERLAI[g], Cynthia TEDORE[h], Spencer J. INGLEY[i], John R. STOWERS[j,k], Joachim G. FROMMEN[l], Francine L. DOLINS[m] & Klaudia WITTE[b]

[a]Department of Biology, McGill University, 1205 Docteur Penfield, Montreal, Quebec, Canada H3A 1B1, [b]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein Str. 2, Siegen 57076, Germany, [c]Ecology & Evolutionary Biology, Texas A&M University, 3258 TAMU College Station, TX 77843, USA, [d]Centro de Investigaciones Científicas de las Huastecas "Aguazarca", Calnali, Hidalgo, Mexico, [e]Department of Biological Sciences, Florida International University, 3000 Northeast 151 Street, North Miami, FL 33181, USA, [f]SUNY Empire State College, Metropolitan Center, 325 Hudson Street, New York, NY 10013-1005, USA, [g]Department of Psychology, University of Toronto Mississauga, 3359 Mississauga Road, Mississauga, Ontario, Canada L5L 1C6, [h]Lund Vision Group, Department of Biology, Lund University, Solvegatan 35, Lund 22362, Sweden, [i]Department of Biology, University of North Carolina at Chapel Hill, CB#3280, Coker Hall, Chapel Hill, NC 27599, USA, [j]Research Institute of Molecular Pathology IMP, Vienna Biocenter VBC, Dr. Bohr-Gasse 7, Vienna 1030, Austria, [k]loopbio Gmbh, Hauptstrasse 93, Kritzendorf 3420, Austria, [l]Department of Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, Wohlenstrasse 50a, Hinterkappelen 3032, Switzerland, and [m]Department of Behavioral Sciences, University of Michigan-Dearborn, 4901 Evergreen Road, Dearborn, MI 48128, USA

[†]The authors contributed equally to this work.

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3.1 Abstract

Rapid technical advances in the field of computer animation (CA) and virtual reality (VR) have opened new avenues in animal behavior research. Animated stimuli are powerful tools as they offer standardization, repeatability, and complete control over the stimulus presented, thereby "reducing" and "replacing" the animals used, and "refining" the experimental design in line with the 3Rs. However, appropriate use of these technologies raises conceptual and technical questions. In this review, we offer guidelines for common technical and conceptual considerations related to the use of animated stimuli in animal behavior research. Following the steps required to create an animated stimulus, we discuss (I) the creation, (II) the presentation, and (III) the validation of CAs and VRs. Although our review is geared toward computer-graphically designed stimuli, considerations on presentation and validation also apply to video playbacks. CA and VR allow both new behavioral questions to be addressed and existing questions to be addressed in new ways, thus we expect a rich future for these methods in both ultimate and proximate studies of animal behavior.

3.2 Introduction

Recent advances in the technical development of computer animations (CAs) and virtual reality (VR) systems in computer sciences and film have primed the technologies for adoption by behavioral researchers with a variety of interests. CAs are computer graphically generated stimuli which, in contrast to video playback, allow full control of stimulus attributes and can be pre-rendered or rendered in real-time. VRs are also computer-generated stimuli but are rendered in real-time and display perspective-correct views of a 3D scene, in response to the behavior of the observer. CA and VR are powerful alternatives to live or real-world stimuli because they allow a broader range of visual stimuli together with standardization and repeatability of stimulus presentation. They afford numerous opportunities for testing the role of visual stimuli in many research fields where manipulation of visual signals and cues is a common and fruitful approach (Espmark et al. 2000). Historically, researchers have tried to isolate the specific cues (e.g., “key” or “sign” stimuli: Tinbergen 1948; see overview in Gierszewski et al. 2017) that trigger a behavioral response, but this is difficult to accomplish with live stimuli or in an uncontrolled environment (Rowland 1999). In contrast, researchers can use CA and VR to create visual stimuli with single traits or trait combinations that are difficult or impossible to achieve using live animals without surgical or other manipulations (compare Basolo 1990 with Rosenthal and Evans 1998). It is also possible to present phenotypes of animals that are encountered only very rarely in the wild (e.g., Schlupp et al. 1999), to present novel phenotypes (e.g., Witte and Klink 2005), or to vary group composition or behavior (e.g., Ioannou et al. 2012; Gerlai 2017). With CA and VR, researchers can further allow stimuli to be shaped by evolutionary algorithms and create entire virtual environments (Ioannou et al. 2012; Dolins et al. 2014; Thurley et al. 2014; Dolins et al. 2017; Thurley and Ayaz 2017). Finally, CA and VR allow “replacement” and “reduction” of animals used for experimentation, as well as “refinement” of experimental design, which is important for both practical and ethical reasons and thus addressing the requirements of the “3Rs” (Russell and Burch 1959; ASAB 2014). Yet, despite the demonstrated achievements of these techniques over the last few decades and their promise for behavioral research, relatively few researchers have adopted these methods (see Supplementary Table S4, Appendix 2). This may be due to technical and methodological hurdles in using computer graphics in behavioral research. Here, we aim to address these difficulties and to discuss technical and conceptual considerations for the use of animated stimuli to study animal behavior.

Stimulated by the symposium on “Virtual Reality” at the Behaviour 2015 conference in Cairns, Australia, and inspired by a workshop consensus article on considerations for video playback design (Oliveira et al. 2000), here we bring together researchers with varied backgrounds in animal behavior, experimental psychology, and animal visual systems to discuss and build consensus on the design and presentation of CA and VR. We also offer recommendations for avoiding pitfalls, as well as some future research opportunities that these techniques provide (see also Rosenthal 2000; Baldauf et al. 2008). Even though we focus on CA and VR, many considerations discussed below also apply to the use of video playback in animal behavior experiments. With the abundance of novel conceptual and technical applications in this fast developing field, we re-consider limitations and constraints of using CA and VR, and discuss the utility of these methods, and the type of questions they may be able to address in animal behavior and related disciplines (see also Powell and Rosenthal 2017). This review is divided into three sections: (I) how to create animated stimuli, (II) how to present them to nonhuman animals, and (III) how to validate the use of CA and VR regarding the perception of test

subjects. A flowchart outlining the most important conceptual and technical questions can be found in Fig. 7. We indicate in bold important technical and conceptual terms used in the text, and provide definitions in the glossary (Table 1, below).

Table 1. Glossary: definitions for technical and conceptual terms used in the text, ordered alphabetically.

2D animation/ 2D animated stimulus:	two-dimensional animated stimulus
3D animation/ 3D animated stimulus:	three-dimensional animated stimulus
Computer animation (CA):	visual presentation of a moving computer-graphically generated stimulus, presented on a screen to an observer. The stimulus is either animated in 2D (x-, y-axis) or 3D (x-, y-, z-axis) virtual space. CA is usually open-loop and pre-rendered. Viewing perspective on the animated stimuli is not necessarily correct for a moving observer.
CFF:	critical flicker-fusion frequency (in Hz). Lowest frequency at which a flashing light is perceived as constantly glowing. Important parameter to consider when using CRT monitors for stimulus presentation.
Closed-loop:	the visual stimulus responds to specific actions (movement or behavior) of the observer (c.f. open-loop where the visual stimulus is independent of the actions of the observer).
CRT monitor:	cathode ray tube monitor. No longer in general production.
Frame rate/frames per second (fps):	commonly refers to image frequency in CAs and video; describes the number of single images (frames) that are displayed in 1 s (fps). Perception of fluent animations depends on the capabilities of the observer's visual system as well as lighting conditions. Frame rate is also frequently called IPR.
Game engine:	software framework used to develop video games. Typically provides 2D and 3D graphics rendering in real-time and incorporates options for interaction (e.g., input from video game controller).
Gamut (color):	the range of colors that can be presented using a given display device. A display with a large color gamut can accurately show more colors than a display with a narrow gamut.
Geometric morphometrics:	a method for analyzing shape that uses Cartesian geometric coordinates rather than linear, areal, or volumetric variables. Points can be used to represent morphological landmarks, curves, outlines, or surfaces.
Interpolation (in keyframing animation):	process that automatically calculates and fills in frames between two set keyframes to generate continuous movement
Keyframing:	saving different x, y, (z) positions/postures/actions of a stimulus to specific times in the animation and letting the software generate the in- between frames to gain a smooth change of positions.
Latency (lag):	response time;
Latency (display):	describes the difference in time between the input of a signal and the time needed to present this signal on screen.
Latency, closed-loop (in VR):	time delay taken from registering a change in the position of the observer, and that change being reflected on the display to ensure viewpoint correct perspective.
LCD monitor:	liquid crystal display monitor.
Mesh/polygon mesh:	the representation of an object's 3D shape made of vertices, edges, and faces. The mesh provides the base for a 3D model.
Open-loop:	see "closed-loop"
Plasma display:	a type of flat panel display that uses small cells of electrically charged gas called plasmas. No longer in general production.
Pseudoreplication:	"[...] defined as the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent." (Hurlbert 1984). In terms of CAs, this problem arises when measurements of test animals are gained by presenting the identical stimulus (or a pair of stimuli) over and over again, while neglecting natural variation of, for example, a presented trait or cue (see McGregor 2000). Responses toward the presentation of such stimuli cannot be considered as statistically independent replicates.

… continued overleaf …

Rendering:	final process of transferring a designed raw template or raw model into the final 3D graphic object by the animation software.
Pre-rendered animation:	rendering of the animated scene or object conducted prior to an experiment and the final output was saved as a movie file for presentation.
Real-time rendered animation:	rendering of the animated scene or object is conducted continuously during the experiment in real-time as response given to input from an external device like a video game controller or subject position data provided by a tracking system. Real-time rendering needs considerably more sophisticated hardware to process constant data flow.
RGB (red, green, blue) color model:	color space in which every color is simulated by different proportions of red, green, and blue primaries. Fundamental for color presentation on screens and devices, with each pixel of an image composed of three RGB values.
Rig:	a mesh can be rigged with a virtual skeleton (a rig of bones and joints) to provide realistic movement for animating the object. Through the process of "skinning" the rig is interconnected to the mesh, providing deformation of the 3D shape while moving certain bones.
Rotoscoping:	an animation technique in which animators trace over video footage, frame by frame, to create a realistic animation sequence. In the context of behavioral studies, rotoscoping could be used to create realistic behavioral sequences (e.g., mating display) based on real-life videos of behaviors.
Texture:	the visualized surface of a 3D model. Animators can "map" a texture onto a 3D polygon mesh to create a realistic object. See also "UV map".
Uncanny valley:	After a hypothetical modulation by Mori (1970) that was original developed for robots, the uncanny valley predicts that acceptance of an artificial stimulus increases with increased degree of realism until this graph suddenly declines very steeply (into the uncanny valley) when the stimulus reaches a point of almost, but not perfectly, realistic appearance. The uncanny valley then results in rejection of the artificial stimulus.
UV map:	a flat plane generated by "unfolding" the 3D model into single parts of connecting polygons, like unfolding a cube. This 2D plane is described by specific "U" and "V" coordinates (called UV, because x, y, and z are used to describe the axes of the original 3D object). In some cases, UV maps are created automatically within the animation software, while in other cases they can be manually created according to specific needs. UV maps are used to assign textures to a model.
Virtual animal/ stimulus:	a CA of an animal/stimulus designed to simulate an artificial counterpart (hetero/conspecific, rival, predator) toward a live test animal.
Virtual reality (VR):	CAs of stimuli and/or environments that are rendered in real-time in response to the behavior of the observer. The real-time responsiveness of VR may include behavioral responses to specific actions as well as perspective-correct adjustments of viewpoint, changes in viewing angle of the stimulus while the observer is moving. The first allows for true communication between the observer and the virtual stimulus, and the second means that the observer and the virtual stimulus share the same space. VR hence simulates physical presence of the observer in the virtual environment.

3.3 Creation of an animated stimulus

In this section, we discuss the creation of animated stimuli. We suggest that the animal's visual system, if known (otherwise see "validation" section in Chapter 3.5), its biology, as well as the research question must drive the decisions about the technological components and the type of animation (2D, 3D, or VR) needed (see Fig. 7). Although it might be sufficient for certain studies to present a simple, moving 2D shape to elicit a response, in other contexts a highly realistic animation may be required. The term "realistic" is itself ambiguous and limited by what humans can measure. Realism can be considered the sum of many visual cues including; "photo realism", "realistic movement patterns", "realistic depth queues" through perspective correctness, and other visual features the experimenter believes salient for their animal. Information on evaluating this correctness is found in the "validation" section in Chapter 3.5. Information on software to create stimuli can be found in the supplementary materials (Supplementary Table S5, Appendix 2).

Depending on the aims of the study, the animated stimulus can be created as a CA in 2D, 3D, or as a VR. In VR, but not CA, a correct perspective is maintained even when the subject animal moves. While both 2D and 3D animation can simulate movement of virtual animals, the visual perspective of the simulation as seen by the watching animal (the observer), will not necessarily be correct. Indeed, a pre-rendered animation is only perspective-correct for one observer position and will appear incorrect to the observer when at other positions. However, the degree of this difference, and whether it is of consequence to the behavior being studied, depends on the animal, the question, and the testing setup. For example, the difference in perspective (between the actual observer position and the position for which the animation was rendered) is small when the distance between the virtual and observing animal is large.

A 3D animation would be particularly useful, although not necessarily required, when presenting spatially complex visual displays involving movement in the third dimension (front–back axis), such as courtship behavior (Künzler and Bakker 1998), or when questions about spatial features are addressed (Peters and Evans 2007). However, complex 3D animations might not be required in all cases. Indeed, 2D animations might be sufficient for testing conspecific mate preference (Fischer et al. 2014), conspecific grouping (Qin et al. 2014), or social responses to morphological parameters (e.g., fin size; Baldauf et al. 2010). VRs can be used to track an animal's movements in this environment (Peckmezian and Taylor 2015) and are thus particularly useful for investigating spatial cognition (Dolins et al. 2017; Thurley and Ayaz 2017).

CAs can be pre-rendered or real-time rendered. In a pre-rendered animation, each frame of the animation is exported from the software before the experiment, and joined to produce an animated video file that can be played during experiments. Hence, motion and behavior of the virtual animal are pre-defined and non-interactive. Real-time rendered animation allows the motion and behavior of the virtual animal to be determined in real-time during the experiment, by receiving feedback from a tracking software or, input given by controllers or sensors manipulated by the test animal or the experimenter. Real-time rendering is one requirement for VR, as the viewpoint of the test animal depends on changes in head and/or body position (Thurley et al. 2014; Peckmezian and Taylor 2015) or by input given to a joystick (Dolins et al. 2014).

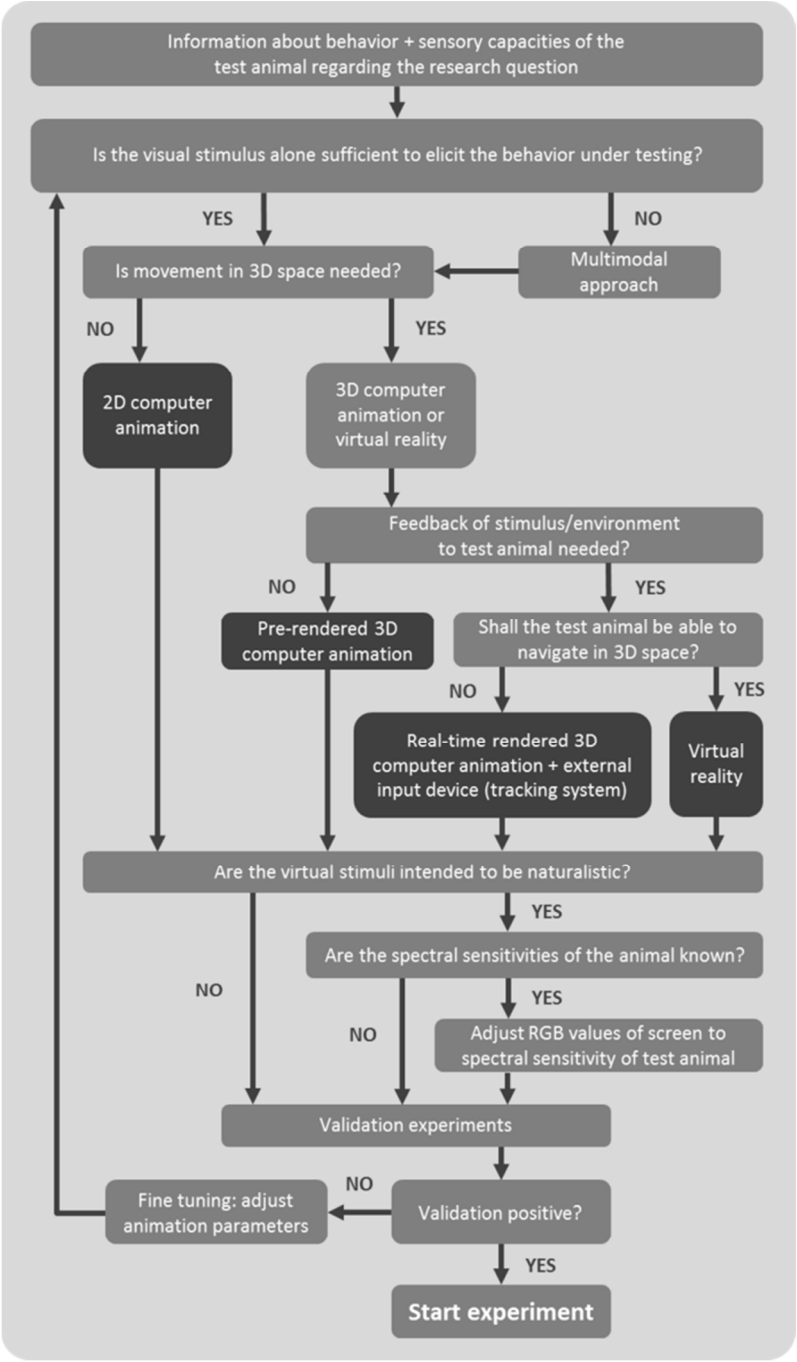

Figure 7. Simplified workflow with the most important conceptual and technical questions that have to be raised when creating and using CA or VR.

3.3.1 2D techniques

2D animations present a virtual animal stimulus that only moves up and down (y-axis), and/or left and right (x-axis) on the screen. Such animations are limited in how well they can simulate motion of the virtual animal between the front and back of the scene (z-axis), or correctly simulate the animated animals' rotation. 2D animations might be sufficient when motion is absent or confined to a 2D plane and orientation or perspective can be neglected. 2D animations are less complex to create, and are particularly appropriate when the stimulus being simulated is in itself spatially and temporally simple.

Simple 2D animations can be created using digitally modified photographs of animals assembled to construct movement via defined movement paths. In a number of recent studies, test animals were presented left-right/up-down swimming movements of 2D fish stimuli animated from images in Microsoft PowerPoint (Amcoff et al. 2013; Fischer et al. 2014; Levy et al. 2014; Balzarini et al. 2017), or other image editing software such as GIMP (Baldauf et al. 2011; see Supplementary Table S5, Appendix 2). Keyframed animation, which has been applied to the study of courtship and agonistic behavior of jumping spiders *Lyssomanes viridis*, can be created using the program Adobe After Effects (Tedore and Johnsen 2013; Tedore and Johnsen 2015). Details on image editing software are available in Supplementary Table S5 (Appendix 2).

3.3.2 3D techniques

Producing 3D animations requires more sophisticated software, but offers the flexibility to create stimuli that move freely in three dimensions (x-, y-, and z-axis). Movement patterns in 3D may appear more realistic to subjects than with only two dimensions. Moreover, even though 3D animations can be drawn with some 2D editing programs, 3D animation software offers special graphical interfaces to deal with the 3D geometry of objects, making it easier to present different angles of a stimulus and postural changes than in 2D software (note that most 3D software can also produce 2D animations; see more details in Supplementary Table S5, Appendix 2). With 3D animations, it might also be possible to simulate interactions with animated objects or between several animated stimuli (such as animated animal groups) more realistically than in 2D animations, especially if these interactions involve postural changes in depth. Animations in 3D are thus particularly useful to portray complex physical movement patterns of animals (Künzler and Bakker 1998; Watanabe and Troje 2006; Wong and Rosenthal 2006; Van Dyk and Evans 2008; Parr et al. 2008; Campbell et al. 2009; Nelson et al. 2010; Woo and Rieucau 2012; Woo and Rieucau 2015; Gierszewski et al. 2017; Müller et al. 2017). Similar to 2D animation, 3D techniques vary in complexity. All 3D animations require the stimulus to be created as a mesh in three dimensions, featuring a rig and texture (see the “shape” section on page 36). Various software is available to create a 3D stimulus (see Supplementary Table S5 and Box S1, Appendix 2). Once the 3D stimulus is created, it has to be animated, that is, movement patterns have to be assigned to the stimulus, to finalize the animation (discussed in the “motion” section on page 37ff.).

3.3.3 VR techniques

VR is the presentation of visual stimuli that are perspective-correct from the observer’s body orientation and position, and which continuously adjust to changes in body position in real-time. This continuous rendering allows more realistic depth cues and movement in 3D. Usually, VRs are used to simulate virtual environments and can facilitate investigations of spatial cognition, for example, navigation (Dolins et al. 2014; Thurley et al. 2014; Peckmezian and Taylor 2015; Dolins et al. 2017; Thurley and Ayaz 2017). In these contexts, VR also allows for greater ecological validity than some traditional experimental methods, allowing natural environments to be realistically mimicked while also allowing experimental manipulation.

3.3.4 Components of an animated stimulus

An animated stimulus is assembled by three components: its shape, its texture, and its motion. Additionally, the scene in which it is presented is important as well. These principles apply equally to the creation of animated animals to be used in CA or in VR.

Shape

The shape is defined by the outer margins of the animated stimulus. This is the contour in 2D animations. For 3D animations, there are three distinct ways to create the virtual animal's shape, which is made of a mesh and subsequently rigged with a skeleton. The first, simplest, and most popular method is to shape the mesh based on high-quality digital photographs of the subject taken from different angles. These pictures are imported into the animation software and the mesh is built by "re-drawing" the body shape in 3D. The second method is to use landmark-based geometric morphometric measurements taken on a picture to define and shape the virtual animal model. This requires slightly more skill and sophisticated software. For fish it is relatively easy to do in software such as *anyFish* 2.0 (Veen et al. 2013; Ingley et al. 2015; see Supplementary Box S1 and Table S5). A third and probably most accurate method is to construct an exact replica by using computed tomography (CT) scans or 3D laser scans of anesthetized or preserved animals, or digitized scans of sections of animals. For example, Woo (2007) used a high-quality 3D laser scanner to construct a precise virtual model of a taxidermic jacky dragon lizard *Amphibolurus muricatus*, while Künzler and Bakker (1998) used digitized scans of sections of the three-spined stickleback *Gasterosteus aculeatus*. Although this approach has the advantage of creating an extremely realistic virtual animal, it requires either a scanner of high quality or technical abilities for sectioning. This method allows precise reconstruction of existing individuals, and it is not clear whether recreating the exact shape to such an extent is essential for eliciting a proper behavioral response (Nakayasu and Watanabe 2014). For research questions where displaying a specific individual is of interest, such as whether chimpanzees *Pan troglodytes* yawn contagiously more to familiar individuals (Campbell et al. 2009), 3D laser scans and CT scans could be particularly beneficial.

Texture

After determining the general shape of the virtual animal, its appearance has to be finalized by adding a texture or surface pattern to the object. Strictly speaking, the texture refers to the structure and coloring given to a 3D mesh. The most common and simplest technique is to use a high-quality photograph of an exemplar animal as a texture. Therefore, texturing describes the process of mapping a 2D photograph onto a 3D surface. For texturing, the 3D model is represented by a flat plane, referred to as a UV map, which is generated by "unfolding" the 3D model into 2D single parts of connecting polygons described by specific coordinates, like unfolding a cube. The UV maps can be either created automatically or manually according to specific needs. The 2D photograph is then placed onto the map, for example, by using image editing programs, to match the coordinates given in the UV map. The applied texture will then appear on the 3D model after rendering. With this technique, a body can be textured as a whole, or single body parts can be texture-mapped separately, which is more accurate and typically most appropriate for more complex morphologies. Additionally, certain body

parts can be defined to be more rigid than others or altered in transparency or surface specifications to resemble muscles, fins, feathers, hair, or other biological structures.

Textures can be manipulated in a variety of ways using common image editing software (e.g., Adobe Photoshop, GIMP). Human-specific measures of color (hue/saturation) of the whole animal or of only a single body part can be altered (e.g., throat color in three-spined sticklebacks in Hiermes et al. 2016; darkness of operculum stripes of *Neolamprologus pulcher* in Balzarini et al. 2017). It is also possible to paint different colored markings directly onto the virtual animal's mesh by hand using a pen tool (e.g., egg spot in *Pseudocrenilabrus multicolor;* Egger et al. 2011), or to relocate and transfer specific markings or body parts from one texture onto another. For example, the "clone stamp" tool in Photoshop was used to test for the relevance of vertical bars for mate choice in *Xiphophorus cortezi* (Robinson and Morris 2010). Finally, textures from body parts of different individuals can be used to create combinations of traits and phenotypes that would not be found in nature.

Locomotion and behavior

A major advantage of using animated stimuli in animal behavior experiments is the ability to manipulate movement and behavior in a standardized way, which is only marginally possible in live or videotaped animals. Evidence is mounting that spatiotemporal patterns are often important for visual recognition. In fishes, attention toward animated stimuli relies greatly on the movement of the stimulus (Baldauf et al. 2009; Egger et al. 2011). For instance, movement that closely mimicked an animal's natural behavior, referred to as "biological motion" (see Johansson 1973 for an analysis model), elicited a closer association to focal fish (Abaid et al. 2012; Nakayasu and Watanabe 2014). Biologically relevant movement has been found to increase association time of the test animal with animated stimuli regardless of the shape of the stimulus presented in reptiles and fish (Woo and Rieucau 2015; Gierszewski et al. 2017). In addition to the movement of the animated stimulus through the active space, the correct syntax of behavior is important for signal recognition in the jacky dragon (Woo and Rieucau 2015). Motion of the objects includes patterns related to physical movement and displacement in the scene. For example, a bird's movement when flying would involve parameters related to wing flapping, the displacement of the bird through the space, and some breathing motion of the body. We suggest looping some of these patterns, for example the wings flapping, throughout the animation.

Regardless of whether one uses video playbacks, 2D, 3D animation, or VR, the illusion of movement is created by presenting a series of still images of the stimulus at different positions. The number of frames per second of an animation needs to be adjusted depending on the tested species' abilities for motion perception, to ensure that the focal animal does not perceive single frames but a smooth motion. Hence, an animation displayed at a low frame rate (lower than the species-specific minimum fps needed for continuous motion perception) will be perceived as juddering or in the worst case as discontinuous series of single images. The higher the frame rate and the smaller the distance, the smoother a certain movement or behavioral pattern can be displayed; hence, fast motion needs more fps than slower motion. For humans, an approximate minimum frame rate of 15 fps is sufficient for continuous motion perception, and in cinematic movies 24 fps are common (Baldauf et al. 2008). Typical fps are derived from industry standards encoding formats such as NTSC or PAL, and a widely used and validated frame rate for fish is 30 fps, although species and individuals vary within each

family. Higher frame rates might be needed for other animals that possess highly sensitive motion perception, such as some birds (see the “display parameter” section on page 44ff. for additional information). Indeed, Ware et al. (2015) recently showed that male pigeons *Columba livia* significantly increased courtship duration toward a video of the opposite sex when the frame rate increased from 15 to 30, and from 30 to 60 fps. Moreover, the tested animals only tailored the courtship behavior to the individual presented in the 60 fps condition, suggesting that with each frame rate different behavioral cues may have been available to be assessed by the tested pigeons. The frame rate has to be determined before creating the motion of the animal, as an animation containing 120 frames will last 2 s when rendered at 60 fps and last 4 s when rendered at 30 fps, and speed of the moving animal will be twice as fast in the former than in the later.

The first and perhaps the technically simplest way to encode motion is to keyframe the position and posture of the object every few frames and let the software interpolate the position of the object between keyframes. Most 3D animation software provides the possibility to assign a virtual skeleton to the 3D model. Moving the skeleton results in naturalistic postural changes of the different body parts that can be tweaked by the experimenter (Müller et al. 2017). Generally, the higher the adjusted frame rate the smoother the interpolated movement between two set keyframes.

A second way for the experimenter to control movement of an animated animal through a space is the “video game” method, in which the experimenter controls the movement through the use of a game remote controller (Gierszewski et al. 2017; Müller et al. 2017). In this case, some behavioral patterns could also be looped or defined by rules (turn tail when turning left). This method requires the use of a game engine (Supplementary Table S5, Appendix 2, for examples of game engines) or custom-made software. The movement can be recorded for later display, but this method could also be used for real-time rendering if the experimenter steers the animated animal during the trial.

The third method presented is similar to the “video game” method and only applies to real-time rendering. It is rarely used, but offers the most opportunities for future research. The position or behavior of the animated stimulus is determined based on input from software tracking the test animal (Butkowski et al. 2011; Müller, Gierszewski, et al. 2016). With this approach, real-time rendered (following the live subject’s position) and pre-rendered (showing a specific action, e.g., fin raising) animations can also be combined in a way such that the test animal’s actions trigger sequences taken from a library of pre-defined behavioral patterns. Here, as well as with the “video game” method, the virtual animal’s movement through the virtual space is realized using algorithms comprised of defined movement rules that account for, for example, object collision and movement speed. For example, Smielik et al. (2015) analyzed fish movement from videos using polynomial interpolation to develop an algorithm which transfers the movement onto a virtual 3D fish skeleton (Gierszewski et al. 2017; Müller et al. 2017). Such algorithms can also be used to let software determine a movement path independent from external input (Abaid et al. 2012).

A fourth way to specify an animal’s motion is through a rotoscoping method, where the movement of a real animal is assigned to a virtual one (Rosenthal and Ryan 2005). This method allows the investigation of specific behavior, and could be useful for investigating individual differences. For movement through space, the path can be extrapolated from tracking the movement of a live animal, or from a video. This can be automated for some animals using tracking software. If multiple points are consistently tracked over time

(Nakayasu and Watanabe 2014), their position can be used to map the path and postural changes onto the animated object. Similarly, a live animal's movement can be recorded using optical motion capture where sensors are directly placed onto a behaving real animal's body and movement patterns are captured in 3D (Watanabe and Troje 2006).

Any of these techniques can produce moving stimuli that can potentially be used in behavioral experiments. To verify whether the movement generated in the animation corresponds to the live animal on which it is based, optic flow analyses (analysis of image motion) can be performed, as described by Woo and Rieucau (2008) and New and Peters (2010). Optic flow analyses validate generated movements by comparing motion characteristics of the animation, particularly characteristics of velocity and acceleration, to movement patterns gained from videos taken from a behaving live animal. This method is particularly useful when used to verify animal visual displays.

The scene: background and light

In addition to the virtual animal, computer animators must create the surrounding environment referred to as the scene. In the case of 2D animations, most commonly a single background color is used or stimuli are animated on a background image taken from the natural environment (e.g., coral reef: Levy et al. 2014). It is advisable to test the effect of the background color on the test animal, as some colors might produce behavioral changes (e.g., sailfin mollies *Poecilia latipinna* avoid screens with a black background; Witte K, personal communication). Whether the animation is produced in 2D or 3D on a computer screen, both animation styles are represented on a 2D surface. However, there are possibilities to make the three-dimensional effect more obvious (Zeil 2000) such as creating an environment with reference objects for size and for depth perception (e.g., plants: Baldauf et al. 2009; pictures of artificial structures known by the test animals: Künzler and Bakker 1998; Zbinden et al. 2004; Mehlis et al. 2008). Depth and size cues in an animation might be provided by illusionary effects (e.g., occlusion of objects and texture gradients), since various animals have been shown to respond to visual illusions in a manner similar to humans (Nieder 2002). All standard animation software provides different options for light sources that can be placed to illuminate the virtual environment. Usually, there are options to change number, angle, position, filtering, color, and intensity of the light source so it might be possible to simulate illumination as found in natural environments (e.g., the flickering of underwater light, diffuse scattering of light, or reflection). Illuminating a scene is also a prerequisite for adding realistic shadows to improve the illusion of 3D space (see Gierszewski et al. 2017), a feature also implemented in *anyFish* 2.0 (Ingley et al. 2015; see Supplementary Box S1 and Table S5, Appendix 2).

3.3.5 Combined traits and multimodal stimuli

CAs enable controlled testing of combinations of traits and their effect on behavior in numerous contexts. For example, Künzler and Bakker (2001) studied mate choice in sticklebacks and presented live females with virtual males differing in throat coloration, courtship intensity, body size, or a combination of these traits. Live females showed a stronger preference when more traits were available to assess the quality of virtual males. Tedore and Johnsen (2013) created different 2D stimuli of jumping spiders that were comprised of combinations of face and leg textures taken from different sexes or

different species to investigate visual recognition in the jumping spider *L. viridis*. To widen the scope of research applications even further, we emphasize that it is also possible to present a visual stimulus together with a cue derived from a different modality to investigate interactions of multimodal stimuli (see Fig. 7). It is possible to add auditory signals (Partan et al. 2010), olfactory cues (Tedore and Johnsen 2013), or even tactile information such as vibrations used by some spiders for communication (Uetz et al. 2015; Kozak and Uetz 2016), or lower frequency vibrations detected by the lateral line of fish (Blaxter 1987). These cues can be altered and presented either in accordance with, or in contrast to, the visual input (see e.g., Kozak and Uetz 2016). Hence, the effect of multimodal signals and priority levels of ornaments and cues for decision making can be tested in a more standardized and controlled way than would be possible with stimuli coming from live subjects. For example, water containing olfactory cues has been employed during the presentation of virtual fish to investigate kin discrimination and kin selection in three-spined sticklebacks (Mehlis et al. 2008) and in the cichlid *Pelvicachromis taeniatus* (Thünken et al. 2014), while Tedore and Johnsen (2013) investigated male spiders' responses to virtual females with or without the presence of female pheromones in *L. viridis*.

Experimenters have to carefully consider the spatial arrangement and temporal order of presentation of stimuli if multiple cues are combined for testing, as the synchronicity of different cues can greatly affect the perception of and response to such stimuli. Kozak and Uetz (2016) combined video playbacks of male *Schizocosa ocreata* spider courtship behavior with corresponding vibratory signals to test cross-modal integration of multimodal courtship signals. They varied spatial location and temporal synchrony of both signal components and found that females responded to signals that were spatially separated by >90° as if they originated from two different sources. Furthermore, females responded more to male signals if visual and tactile information was presented in temporal synchrony rather than asynchronously.

3.3.6 Creating VR

The term "virtual reality" was first applied to a specific set of computer graphics techniques and hardware developed and popularized in the 1980s. These early systems modulated projection of the environment based on body movements of the video game player (Krueger 1991). Today there are many types of VR systems available, some for example requiring the user to wear head mounted display goggles, while others use projectors and tracking to allow the user to move freely. In order to create an immersive experience, all VR systems share two common criteria with the original; the display responds to the behavior of the observer (so-called closed-loop), and the visual stimulus presented is perspective-correct.

Subsequently, creating a VR requires the support of software to generate perspective-correct representations of 3D objects on a 2D display (projector screen or monitor), and the hardware support for tracking a moving observer in the virtual environment using custom-made or commercial tracking systems (Supplementary Table S5, Appendix 2). The necessity of an immediate update of the simulated environment in response to the behavior of the observer makes VR systems more technically challenging than open-loop CA.

Most VR setups feature display screens that cover a substantial part of the test animal's field of view (e.g., panoramic or hemispherical) on which the computer generated virtual

environment is presented, often using a projector. VR setups usually need an apparatus to mount the live animal in front of the screen, and a tracking system to relay the changes in position and orientation to the system (Fry et al. 2008; Stowers et al. 2014). Animals may be immobilized (Gray et al. 2002) or partly restricted, but should be able to move or change their orientation and position (Thurley et al. 2014; Peckmezian and Taylor 2015). There are multiple techniques available, such as treadmills, to track the movement of animals through a virtual space, and the efficiency of such techniques may vary depending upon the species tested (see Supplementary Table S4, Appendix 2, for a list of examples). Creating virtual environments can be done using common 3D modeling software (Supplementary Table S5, Appendix 2), but their integration into a complete VR setup can be complex. Therefore, we only briefly discuss this topic to highlight its significance when using virtual stimuli. Further details and discussion may be found in Stowers et al. (2014) who review different VR systems for freely moving and unrestrained animals of several species, in (Thurley and Ayaz 2017) who review the use of VR with rodents, as well as Dolins et al. (2017) for a review on VR use with nonhuman primates.

3.3.7 Pseudoreplication

A frequent concern about auditory or visual playback studies (video, 2D/3D animation, VR) is pseudoreplication. As proposed by McGregor (2000), using many variations of a stimulus or of motion paths to cover a wide range of phenotypic variation is the most reliable way to solve this problem. Unfortunately, designing various animated stimuli can be time-consuming. Furthermore, it may not be possible to determine how much variation in artificial stimuli and on which phenotypic trait is needed to address the issue of pseudoreplication. Nevertheless, by presenting identical copies of a stimulus exhibiting variation only in the trait of interest (which is exactly the power of CAs), we can clarify that a difference in behavior most probably results from this exact variation of the tested trait (Mazzi et al. 2003). Instead of creating a single replicate of one individual (exemplar-based animation), many researchers design a stimulus that represents an average phenotype based on population data (parameter-based animation; Rosenthal 2000). This can be achieved by displaying a virtual animal with mean values for size measured from several individuals. The software *anyFish*, for example, incorporates the option to use a consensus file of a study population to create a virtual fish on the basis of geometric morphometrics obtained from several individuals (Ingley et al. 2015). Therefore, it is a straightforward extension to create consensus shapes based on different sets of individuals in order to create a variety of stimuli. It is worth noting that the presentation of an averaged individual still measures the response to a single stimulus and could produce strange artifacts when this stimulus is then used to create groups of individuals, as to the test animal it may be very unusual to have a group composed of several identical individuals. The ideal solution is therefore to create at random different models whose parameters fit within the range seen in natural populations to create such groups.

3.4 Displaying the animated stimulus

When it comes to presenting the animated stimulus, we are confronted with the issue that readily available display technologies are specifically designed for the human visual system, which may differ considerably from animal visual systems. Hence, there are some important considerations that we have to address to ensure that test animals perceive animated stimuli in a manner needed to test a certain hypothesis.

3.4.1 Animal visual systems and their implications for the presentation of animated stimuli

The visual systems of animals rely on the reception of light by different classes of photoreceptors that are each activated by a limited range of wavelengths. Color is encoded by the nervous system as the ratio of stimulation of different photoreceptor classes. Since color is encoded by the relative magnitude of three data points for trichromats like humans, a wide range of colors can be simulated in the eyes of humans by combining various intensities of three spectrally distinct (red, green, and blue) phosphors in screens, the RGB color model. The range of colors (gamut) that can be displayed with accuracy by screens increases with each phosphor's specificity to the cone class it is designed to stimulate. Therefore, the spectral peak of each phosphor is very narrow, and tailored to stimulate human photoreceptors as independently as possible. The default RGB color model of human devices, monitors, cameras, and computers, is not well suited for many animals. While we discuss color more thoroughly below, it is important to note that in non-human animals the number of photoreceptor classes most commonly varies from two (dichromats) to four (tetrachromats), but that some animals such as the mantis shrimp can have up to twelve (Thoen et al. 2014), and the number of photoreceptors might further be sex-specific (Jacobs et al. 1996). It is also notable that the characteristics of an animal's photoreceptor classes, specifically their peak wavelength sensitivity and the range of wavelengths they respond to, sometimes differ within species (e.g., butterflies; Arikawa et al. 2005) and also differ from those of the human receptors for which our hardware is built.

Color is probably the most contentious aspect of CAs, because many animals have different numbers of photoreceptor classes spanning both the UV and visible spectrum, with spectral sensitivities centered differently to humans. This means that RGB phosphors will often not be aligned with another animal's photoreceptor spectral sensitivity curves in a way such that their different photoreceptor classes can be independently stimulated, and it might not be possible to accurately simulate color even for non-human trichromats. This constraint has led to a broad literature on color representation and solutions for this issue in video playbacks and images, which are also applicable to CA and VR studies (see Fleishman et al. 1998; Fleishman and Endler 2000; Tedore and Johnsen 2017).

Accurately representing color is thus difficult and testing hypotheses on color even more so, especially if the test animal's spectral sensitivities are unknown. In the case where the visual system of the tested species is not well described, one option is to render the stimulus in grayscale. The caveat is that gray rendered by an RGB system may look like different brightness of a certain color for some species, and stimuli might appear in a "redscale", for example, instead of a grayscale. This may become problematic if the perceived color has any relevance for the specific animal. When the animal's spectral sensitivity is unknown and cannot be assumed from related species, and when the

research question is not specifically about the effect of color, for simplicity we suggest adjusting RGB values to look as natural as possible to the human eye. In the case where the spectral sensitivity of the organism is well described, or can be estimated from related species, it is sometimes possible to simulate accurate color representation for the study species. Tedore and Johnsen (2017) provide a user-friendly tool that calculates the best-fit RGB values for the background and every specified color patch shown in an animation, for presentation to di-, tri-, or tetrachromats. If the experimenter wishes to test hypotheses on coloration by using stimulus presentation on any display device, then these calculations are essential for the relevance and interpretation of the experimental results.

We also recommend calibrating screens for color every two weeks, not only for those who do specifically test for the effects of color, but also for those who are not manipulating color or testing for its effects. This is due to the fact that RGB colors may drift naturally within just two weeks. Calibration is important to make sure that the color presented by the screen does not change. Some monitors and operation software come with a built-in colorimeter (three-filtered light measurement device) and calibration software, but this is rare. Purely software- or web-based calibrations, which the user conducts by eye, are available, but will not produce identical results across calibrations within, and especially not between, display devices. Proper monitor calibration requires a device containing a colorimeter which takes quantitative and repeatable measurements, such as those manufactured by X-Rite or Datacolor. Such devices are relatively inexpensive, with the highest-end models currently costing less than 250 USD.

In humans, most of the light below 400 nm (UV) is blocked by the ocular media (cornea, lens, vitreous fluid; Boettner and Wolter 1962). In contrast, a large number of both vertebrates and invertebrates have ocular media that are transparent in the UV portion of the spectrum, and have photoreceptor spectral sensitivities peaking well below 400 nm (Marshall et al. 1999; Briscoe and Chittka 2001; Hart and Vorobyev 2005). It is still generally impossible to simulate strongly reflective UV patterns using an RGB screen since RGB phosphors do not emit UV light. While this does not necessarily invalidate results, one should keep this limitation in mind when interpreting responses to live animals versus CAs. UV light plays an important role in visual communication in many species (e.g., Lim et al. 2007; Siebeck 2014). Therefore, it is likely that some information will be lost when this spectral channel is excluded which could confound the subjects' responses.

Many animal visual systems have polarization sensitivity (Wehner 2001). This is particularly common in invertebrates, which in terrestrial habitats, use polarized light for celestial navigation or to localize water sources, and in underwater habitats, to detect open water or enhance contrast between the background and unpolarized targets. There is evidence that some vertebrates have polarization sensitivity as well (e.g., anchovies: Flamarique and Hawryshyn 1998; Flamarique and Hárosi 2002). Moreover, some animals have polarized patterns on the body that may be used for the detection or recognition of potential mates, or possibly in mate choice (e.g., stomatopod crustaceans, Chiou et al. 2008; cuttlefish, Shashar et al. 1996). The extent to which polarized patterns of reflectance on the body have evolved as signals to communicate information to receivers in the animal kingdom is poorly known. Like UV light, this cue is difficult to control and manipulate in CAs or VR. Unfortunately, the most common type of display on the market, the LCD display, is highly polarized. Below, under Display parameters, we discuss alternatives to LCD displays if polarized light is a concern for the species under study. Please also see the section "emission of polarized light".

3.4.2 Display parameters

Since animal visual systems are extremely variable, decisions on monitor parameters must be determined depending on species-specific priorities. We can use various types of display devices (e.g., TV screens, computer monitors, notebooks, tablet PCs, smartphones, projectors) to present animated stimuli. Projectors can be used for the presentation of animations (Harland and Jackson 2002) and they are usually used for the presentation of virtual stimuli and environments in VR setups (Thurley and Ayaz 2017).

Display technologies are rapidly evolving and numerous characteristics must be considered and balanced in choosing an appropriate display method. Display characteristics to consider include temporal resolution and flicker (e.g., refresh rate, response time, backlight flicker), spatial resolution, color representation and calibration, brightness, display size, viewing angle, screen polarization, screen reflectance, active versus passive displays, and compatibility with different computer interfaces, as well as practical considerations such as cost, weight, robustness, and continued availability. Trade-offs between display characteristics are common (see Baldauf et al. 2008). For example, high temporal resolution may mean compromises in terms of spatial resolution or color representation. Moreover, commercially available displays and software designed for humans may not be optimized for accuracy of representation but instead other characteristics, such as reducing user fatigue or enhancing object visibility.

For presentation, we highly recommend presenting a life-sized replica of the animated stimulus to enhance realism for the test animal. The choice for a particular device might be influenced by this consideration. For example, a 72-inch monitor was required to present chimpanzees a realistic image size (Dolins et al. 2014) while Harland and Jackson (2002) used an array of different lenses and filters to ensure a life-sized (60.1 mm) rear projection of a small 3D jumping spider *Jacksonoides queenslandicus*.

Display devices typically derive from two distinct designs, CRT or LCD screens. Although authors (e.g., Baldauf et al. 2008) favored in older articles the use of CRTs over LCDs, we reevaluate this preference in light of the advancement of LCD technology and the decreasing availability of CRT screens. Although CRTs were preferred for color display, viewing angle properties, and interpolation, many LCD screens are now built with IPS (in plane switching) that at least decreases viewpoint dependencies regarding luminance and color. The IPS technology produces low deformation of image color with shifting viewing angle, which may be important if the test animal is likely to move during the presentation. However, this will be less important in the case of a short-duration display to a stationary test animal, such as chimpanzees watching videos of yawning (Campbell et al. 2009). Plasma displays are also favored when distortion with viewing angle might be a problem (Stewart et al. 2015), although these screens are decreasingly manufactured. Although most monitors are designed to display roughly the same range of color, there are several LCD monitors on the market with wide gamut specifications. A wide gamut monitor is able to display colors outside the standard color space of commercial displays. Such a monitor may be useful for experimental tests involving highly saturated colors.

Emission of polarized light

It is important to note that LCD screens and projectors emit polarized light, which may interfere with the test animals' response if the species is sensitive to polarized light. However, this may be neglected if the animal's polarization sensitivity is restricted to dorsally directed photoreceptors, as these receptors are not being stimulated by the RGB display. If polarization sensitivity is a concern, a polarization scattering film can be applied to the display to minimize the issue. Otherwise, plasma screens, CRT monitors, and Digital Light Processing or CRT projectors emit little polarized light. If still unsure what monitor might be best suitable for presentation, it might be worth considering directly comparing different monitor types in an experiment to see if the species under question shows a more reliable response to a certain monitor type (Gierszewski et al. 2017). For each monitor, the temporal and spatial resolution parameters should be examined.

Temporal resolution

The important values that describe temporal resolution are the monitor's refresh rate (in Hz), the animation's frame rate, which is determined at the animation's design stage, and the latency. In earlier studies using CRT monitors, a critical parameter was the monitor's refresh rate. On a CRT screen each pixel is ON only for a short time and then is turned OFF while the cathode ray is serving the other pixels, resulting in flickering of the screen. Flickering was a particular issue with animals whose critical flicker-fusion (CFF) frequency values exceeded that of humans or the refresh rate of standard CRT screens (e.g., Railton et al. 2010). An animal's CFF is the threshold frequency at which a blinking light will switch from being perceived as flickering to being perceived as continuous, that is, non-flickering. CFFs are highly variable among species (for a list see Woo et al. 2009; Healy et al. 2013). Although a high CFF would not affect motion perceptions per se, a visible screen flicker may inhibit the perception of smooth motion by masking movement of a stimulus by variations in illumination, like movement seen under strobe light (Ware et al. 2015). The frame rate (see the "Locomotion and behavior" section on page 37ff.), also called image presentation rate (IPR), is crucial to simulate continuous movement and should be adjusted to exceed the test animal's CSF value (critical sampling frequency; see Watson et al. 1986), the rate needed to render sampled and continuous moving images indistinguishable. CSF is dynamic and may vary with stimulus characteristics and the visual environment (e.g., lighting conditions) during stimulus presentation (Watson et al. 1986; Ware et al. 2015).

In LCD screens, pixels are constantly glowing (although the backlight may flicker at rates of about 150–250 Hz), and therefore the refresh rate-related flickering issue is absent. LCD screens still have a refresh rate that refers to the rate at which it samples the information to be displayed, and is generally around 60 Hz. This means that a screen set at 60 Hz displaying an animation rendered at 30 fps shows each image in the animation twice. As much as possible, hardware and software should be aligned in their temporal characteristics.

Display latency or display lag describe the difference in time between the input of a signal to the display and the time needed for this signal to be shown on the screen. This is particularly important for closed-loop applications like VR, as the time between the VR being rendered and it being seen by the animal should be as low as possible.

Manufacturers do not always provide accurate information on a display's latency but there are ways to calculate it (see the "measuring display latency" section on page 47).

Spatial resolution

Understanding the display properties of screens is important as the trend to use newer yet less standardized devices such as tablet PCs or smartphones for behavioral research increases. The important measures that describe spatial resolution in screen specifications are: screen resolution, pixel density, and pixel spacing. Screen resolution refers to the total number of pixels that are displayed (width x height, e.g., full HD resolution of 1920 x 1080 pixels). Since screens differ in size, pixel density and spacing must also be considered. Pixel (or screen) density describes the number of pixels or dots per linear inch (ppi, dpi), and equals screen width (or height) in pixels divided by screen width (or height) in inches. Low-density screens have fewer ppi than high-density screens, and hence objects displayed on low-density screens appear physically larger than when displayed on high-density screens. Pixel spacing (in mm) describes the distance between neighboring pixels, which is low in high-density screens. The problem of pixelation affects animals when their visual acuity greatly exceeds that of humans. Animals with higher visual acuity than the resolution of the display device will view the stimulus as composed of many square pixels or even individual red, green, and blue phosphors rather than organically flowing lines and spots. However, even for animals with lower or average visual acuity, pixelation may occur when the subject is positioned too close to the screen. For animals with high visual acuity, or in situations where they are positioned close to the screen (e.g., jumping spiders, Tedore and Johnsen 2013), we recommend the use of high-density devices with the smallest possible pixel spacing. Fleishman and Endler (2000) demonstrated how to calculate the minimum distance needed between the experimental animal and the screen to ensure that the animal cannot resolve individual pixels.

3.4.3 Interactivity of the animated stimulus

Currently, the most effective form of interaction between a live animal and a virtual stimulus so far has been the implementation of VR systems in research that enables real-time interaction between the animal and a virtual environment (Dolins et al. 2014; Stowers et al. 2014; Dolins et al. 2017; Thurley and Ayaz 2017; Stowers et al. 2017).

CA stimuli rarely enable interaction between the animated stimulus and the experimental test subject. Typically, the behavior of an animated animal is predefined and it will not respond to the test animal, which may greatly reduce ethological relevance of the stimulus and thus the validity and interpretability of the experiment and its results. In contrast, VR or real-time rendered animated stimuli enable interaction and are considered a promising advantage for the future. However, they require more complex software or the creation of a user interface that would allow the experimenter to change the animated animal's behavior. Tracking software (see Supplementary Table S5, Appendix 2) can provide real-time information on the position of the live test animal in 2D or 3D space, which can then be used to determine the position of the animated stimuli accordingly, based on predetermined rules. Recently, feedback-based interactive approaches were successfully used with robotic fish (Landgraf et al. 2014; Landgraf et al. 2016), but as far as we know, there have only been a few studies that implemented

some degree of interaction in video playback and CA studies. Ord and Evans (2002) used an interactive algorithm for the presentation of different video sequences showing aggressive and appeasement displays of male jacky dragons, depending on behavior of an observing lizard. Sequence presentation was not completely automatic as the experimenter had to indicate the beginning of a display of the live animal by a key press. Butkowski et al. (2011) combined video game technology and the BIOBSERVE tracking software (see Supplementary Table S5, Appendix 2) to enable a rulebased interaction between live female swordtail fish *Xiphophorus birchmanni* and an animated male conspecific and heterospecific fish. Animated males were programmed to automatically track the horizontal position of live females and to raise their dorsal fins, an aggressive signal to rival males, depending on the proximity of the female. Müller, Gierszewski, et al. (2016) developed an easy to handle method for fully automatic real-time 3D tracking of fish, the sailfin molly *P. latipinna*. The system enables interaction, with the virtual fish stimulus following the live test fish and performing courtship behavior, and it was already successfully tested in practice. Ware et al. (2017) also successfully manipulated social interaction in courtship of pigeons.

3.4.4 Real-time rendered animations and VR-specific considerations

To date, the total system latency (sometimes called lag) has been identified as a critical measurement of VR performance (see Supplementary Table S6, Appendix 2). This would also apply for real-time rendered animations, if the experiment and the test animal require a timely response. In humans this latency has been stated as one of the most important factors limiting the effectiveness of VR, and should be less than 50 ms, with 20 ms as ideal, and <8 ms being likely imperceptible (MacKenzie and Ware 1993; Ellis et al. 1997; Miller and Bishop 2002).

Measuring display latency

One common and thorough approach to quantify total closed-loop latency is to use the VR apparatus tracking system to measure the position of an object in the real world, and then project a virtual representation of that same object at the previously measured object position. If the real-world object moves at a known and constant velocity then the difference in position between the virtual and real object can be used to estimate the total closed-loop latency (Liang et al. 1991; Swindells et al. 2000). A simplified estimate (lacking the contribution of the tracking system to the total time) can be achieved by having the user manually change the virtual world and filming how long it takes the result to appear (see http://renderingpipeline.com/2013/09/measuring-input-latency/, on how to measure latency with video footage). Both estimates require using an external high speed imaging system, and can therefore sometimes be difficult. Other approaches attempt to use the system to estimate its own latency, by showing specific patterns on the display and recognizing those patterns using the same tracking cameras used to estimate the object position (Swindells et al. 2000).

Commercial display devices such as monitors and projectors will often be the single largest contributor to total closed-loop latency, contributing between 15 and 50 ms in an average system. When selecting a display device for VR systems, it is important to be able to measure display device latency directly, so one may purchase the best performing display device within one's budget. Display latency can be measured using

custom hardware, for example, with a video signal input lag tester (www.leobodnar.com/shop). Comparative display latency can be measured using a combination of hardware and software (see websites for information on how to measure latency: tftcentral.co.uk, tft.vanity.dk). It is important to always measure display latency as the numbers provided by the manufacturer may only refer to the lag of the display panel itself, and may not include the additional signal or image processing induced lag, which takes place in the display or projector electronics. Any processing done by the monitor or projector, such as built in color correction, contrast enhancement, or scaling of the image should be disabled, as such processing takes time and thus increases display latency. If unsure or unable to measure, displays will often have a “game mode” which should generally have the lowest latency.

3.5 Validating the animated stimulus

After creating the animated stimulus and deciding how to display it, it should be validated thoroughly for at least every test species since perception and recognition might be species and even individual specific (see also Powell and Rosenthal 2017).

A widely used first validation test is to compare the attention given to the CA and an empty counterpart (e.g., blank background image) by presenting them simultaneously. This effectively tests whether the animation is generally perceived by the test animal and whether it attracts attention, but does not determine whether the subjects perceive the animation as intended. In several studies using a two-choice paradigm, poeciliid fish were attracted to an animated fish and preferred to spend time with the animated fish over the empty background (Morris et al. 2003; Culumber and Rosenthal 2013; Gierszewski et al. 2017). Zebrafish *Danio rerio* significantly reduced their distance to the screen when presented with an animated fish shoal versus a blank background (Pather and Gerlai 2009).

To validate that the subjects perceive the animation similarly to real animals, comparing behavior of test animals when presented with a CA, live animals, and video playback is a crucial step in the validation of a stimulus, and is becoming a gold standard (Clark and Stephenson 1999; Qin et al. 2014; Gierszewski et al. 2017). Fischer et al. (2014) describe a detailed validation of a CA to test visual communication in the cichlid *N. pulcher*.

Another approach to validate animated stimuli is to perform a classical conditioning experiment, which can reveal if test animals are able to perceive and discriminate between specific features of the animated stimuli. This approach might particularly be useful to test a preference for a certain trait that is represented by only subtle changes in morphology or differences in texture. Here, test animals are provided with the opportunity to learn to associate, for example, food with an animated stimulus during a learning phase. Afterwards, the test animals have to discriminate between the learned stimulus and another stimulus (differing in the expression of a trait or the texture) in a binary choice experiment. Such a conditioning experiment was performed successfully to investigate whether sailfin molly females perceive an artificial yellow sword attached to live males on videos presented on TV monitors in mate choice experiments (Witte and Klink 2005).

Although conducting the above tests is generally sufficient to validate an animation, one could additionally compare the performance of CAs generated with different methods

(2D, 3D, VR) or containing different characteristics. As the knowledge of which visual characteristics are essential for recognition does not exist a priori for many species, testing which visual features can be simplified by comparing animations with different levels of complexity would provide a more detailed understanding of the communication and recognition systems.

Depending on the research question, control tests confirming different discrimination abilities should follow to complete the validation process. It might be required to show that test animals successfully discriminate between animated conspecifics differing in size, age, sex, familiarity, etc., and that they distinguish between animated heterospecifics or also predators. Using this method, Gerlai et al. (2009) confirmed that the animated image of a predator could be used to elicit a significant stress response in zebrafish. Fischer et al. (2014) demonstrated that cichlids were able to gain information from presented animated conspecifics, heterospecifics, and predators by adjusting their aggression and aversive behavior accordingly.

To validate the significance of results obtained with CA methods, one can perform identical experiments with animated and live animals. Early studies in guppies found that video playbacks of the stimulus and the use of live stimuli yielded similar responses while the former increased efficiency by removing temporal variation (Kodric-Brown and Nicoletto 1997). Amcoff et al. (2013) trained female swordtail characins *Corynopoma riisei* on red and green food items to induce a preference for the same colored male ornament. This preference was demonstrated for live and 2D computer animated fish. However, since in many cases CAs are employed to specifically investigate variation in traits or behavior that are hard or impossible to reproduce with live animals, this approach might not be practical for many studies. Therefore, following the above described validation tests of comparing an animated stimulus to a blank counterpart, live animals, and video playbacks should be considered sufficient to validate usage of CAs. Depending on the research question, the ability to discriminate between sex and/or species, and/or discrimination of different sizes (e.g., of animal size, trait size) should additionally be investigated prior to testing.

3.5.1 Validation of VR

Once the VR system is developed, it is possible to validate its performance against its real-world equivalent by testing if the test animals respond to the virtual environment as if it were real. This necessarily requires finding a strong behavioral response in the real world that can be recreated in VR. Once such a behavior is found, one approach to validation is, for example, to parametrically manipulate certain aspects of the VR system, including the system latency, thus presumably changing the system's realism and the subject's response. As the latency approaches zero (real-world), the difference in the behavior under question elicited in the experimental subject in the VR versus the real-world context should approach zero. If the difference between VR and real-world at minimum latency is already sufficiently small then one can argue that, by this behavioral definition, the VR system is accurately simulating the real world. Relatedly, the tracking method and suitability of the VR setup should be carefully investigated and fine-tuned to the focal species (see Bohil et al. 2011 for more details on VR). Especially as some tracking methods may require partial immobilization of animals (Thurley et al. 2014). Beyond ethical issues, such manipulations can lead to decreased ecological validity from the test animal, and decreased realism regarding the VR. Whether a particular tracking

method is appropriate for the species tested should therefore also be validated (examples for species already used in Supplementary Table S4, Appendix 2).

When interpreting validation results for VR it should be kept in mind that the immersion into highly realistic, yet imperfect, virtual environments might frighten or alarm non-human animals. Here, it is also important to consider the uncanny valley phenomenon, described in humans by Seyama and Nagayama (2007). For nonhuman animals, this phenomenon has so far only been described in long-tailed macaques *Macaca fascicularis*. Steckenfinger and Ghazanfar (2009) found that long-tailed macaques preferred to look at unrealistic synthetic monkey faces as well as real monkey faces, when compared with realistic synthetic monkey faces. Implications of the uncanny valley for other non-human animals can currently only be guessed at and should be the subject of future research (see also Alicea 2015). In general, considerations regarding the uncanny valley phenomenon can be transferred to any artificial and hence virtual stimulus (CA and VR) that is designed to be highly realistic.

If a validation is negative, for example, that the behavior of a test animal is not congruent to that found in nature, every parameter that was prior set to a CA or VR has to be evaluated and if needed to be adjusted until the validation leads to a positive result (see Fig. 7). This might especially be the case when a species is tested with CA or VR for the first time and nothing is known on how and if the animal will respond to a newly created virtual stimulus.

3.6 Conclusions and future directions

CA and VR are useful and promising methods for studying animal behavior. That said, regardless of their potential, virtual stimuli may not be the ideal choice for all research questions involving visual stimuli. Even with external resources and support, creating CAs and VRs can be extremely time-consuming. This investment will be particularly worthwhile if CAs and VRs enable a set of studies, if methods and stimuli can be reused by multiples researchers or laboratory, or if the required stimulus is hard to obtain using live animals. Moreover, the results obtained using CAs and VRs may not be generalizable to real-world situations, as they typically only present the visual modality (but see the “combined traits and multimodal stimuli” section on page 39). For example, one cichlid species’ response to mirrors may not be indicative of aggressiveness in all species (Desjardins and Fernald 2010; Balzarini et al. 2014), a consideration that might apply to CA and VR as well. We would, thus, advocate care in the interpretation of findings until further ecological validations are conducted. Their implementation, the degree of realism required, and the choice between CA and VR, will depend on both technical and conceptual considerations. Systematic experimental analysis of animal behavior will be required to determine whether stimuli are ethologically relevant, and which method of presentation is required in the context of the research question. For many research questions, relatively simple stimuli and setups may be all that are needed, but this remains an empirical question.

CAs have, up to now, been used more often than VRs in animal behavior studies (see Supplementary Table S4, Appendix 2), partly because of the higher technical demands for implementing a VR setup, and the cost of implementing the movement tracking systems. This preference also reflects the idea that it may not be necessary to employ a VR for all questions. CAs have mostly been used to investigate questions of perception and recognition, as well as aspects of visual communication and signaling, notably the

manipulation of individual traits to assess their role in mate choice. In contrast, VR has primarily been used to investigate cognitive mechanisms, especially regarding spatial navigation (see Supplementary Table S4, Appendix 2). VR offers valuable opportunities to study how environmental cues are used in navigation, and how navigation is affected by surgical or pharmacological manipulation of neural substrates. VR systems hence represent a promising technique for future neuroscientific research, and questions of navigation, but CAs seem appropriate to answer most questions of communication and signaling. Animated stimuli have been used in all major taxonomic animal groups that rely on visual communication (see Supplementary Table S4, Appendix 2), but fish are the group most often tested using CAs, while VRs most often are used with insects or mammals. This may be explained partly by the investment in VR systems for biomedical research in rodents, which further increases the technical knowledge and tools available for implementation.

For the use of CA, future directions should address the issue of non-interactivity as this still represents one of the major limitations when using animated animals. Ongoing improvement in tracking systems, that also function in 3D (e.g., Straw et al. 2011; Müller et al. 2014), may help to create interactive animated stimuli in the future (Müller, Gierszewski, et al. 2016). So far, animated stimuli have predominantly been used in choice experiments and their possible use in other popular testing paradigms has mostly been neglected. And yet, animated stimuli are also very well suited to be observers, bystanders, or demonstrators in experiments that investigate higher-order aspects of the social interactions of a species (e.g., Witte and Ueding 2003; Makowicz et al. 2010b). Regarding VR, the majority of current systems necessitate partial or complete immobilization of the tested animal and this might limit the use of these systems much more than the complexity of the programs needed for implementation, as subjects might not be able to show their full behavioral repertoire. Future directions should hence promote the development of free-ranging VR systems that do not restrict natural behavior.

Even if CA and VR have not yet reached their peak of innovation and accessibility, current technical advances already provide opportunities for sophisticated design and presentation of animated stimuli. Software applications for both beginning and advanced users can be found and the increase of professional freeware (see Supplementary Table S5, Appendix 2) also facilitates an inexpensive implementation of animated stimuli in research. Numerous possibilities for creating animated stimuli with varying complexity can be used to address questions concerning visual communication and spatial cognition. Further technical advances are expected, following the increasing popularity of VR in mobile gaming applications, and its use in robotic and remote surgery. Insofar as this affects animal VR one should expect to see market pressures encouraging the sale of low latency display devices. The trends highlighted by the current use of CA and VR in animal behavior research, and the prospect of technical advances imply that a major barrier for increased use of VRs and CAs may reside in the technical hurdles of building and validating a new system. As such, the creation of shareable systems (e.g., *anyFish* 2.0, see Box 1 in the Supplementary Material, Appendix 2), open-source or freeware, how-to guides, etc. to assist in building the systems would be invaluable in improving the accessibility to virtual research techniques in the future.

We hope that our review will inspire future research and the continuous development of more advanced techniques that hence lead to novel insights into animal behavior.

3.7 Acknowledgements

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Chapter 4
The Virtual Fish Project

The Virtual Fish Project was a collaboration of scientists from both the Institute of Biology and the Institute of Real-Time Learning Systems of University of Siegen, starting in 2012. From the beginning of 2014, my role in the Virtual Fish Project included in-depth involvement in the conceptual design of simulation software for computer-animated three-dimensional (3D) sailfin mollies (i.e., virtual fish). For this, I provided advice on the biology and behavior of the study species, the sailfin molly, as well as advice on experimental design and its implications for software development. The development of the general technical framework, as well as programming, 3D modelling and animation were performed by Klaus Müller, Jan-Marco-Hütwohl and Ievgen Smielik, supervised by Prof. Dr. Klaus Dieter-Kuhnert. The aim of my thesis was then to use this simulation software to manipulate public information in experiments on MCC with live sailfin mollies (*Poecilia latipinna*). I provide a detailed description of my study species in Chapter 2.

Here, I will introduce *FishSim* Animation Toolchain (short: *FishSim*) which was developed within the scope of the Virtual Fish Project. Whereas this chapter is more focused on the biological background and justification of the different steps during software development, the technical aspects are described in the peer-reviewed article by Müller et al. (2017), of which I was a co-author. Focusing on the biological perspective of the project, I distinguish seven different and important key stages within the Virtual Fish Project (see Fig. 8), which I will describe below. Throughout, the success of the Virtual Fish Project required close collaboration and constant feedback between both project partners.

4.1 Stage 1: Technical and conceptual considerations

Over the last decades, the use of computer animated stimuli in animal behavior research greatly increased in popularity due to its advantages concerning the standardized and non-invasive creation and manipulation of artificial animal stimuli. To date, scientists have access to various techniques and tools for creating computer animations (see Chapter 3 for a review). From simple 2D animations, that typically derive from a picture of a stimulus moving in only two dimensions (e.g., Tedore and Johnsen 2015; Balzarini et al. 2017), 3D animations, which enable more realistic and complex physical movement (e.g., Campbell et al. 2009; Nakayasu and Watanabe 2014), scientists may choose what technique best fits their research question. Even VR designs that simulate a 3D environment, where live animals may navigate, exist and are, since, constantly refined (Peckmezian and Taylor 2015; Van De Poll et al. 2015; Thurley and Ayaz 2017). However, biologists are mostly lacking the expertise necessary for programming or sophisticated software development, therefore, the need for more standardized and easy-to use research tools arose. For example, *anyFish* (Veen et al. 2013; Ingley et al. 2015) was developed to offer scientists a tool for 3D animation of small fish. Similar software was created for the study of zebrafish behavior (Qin et al. 2014) and even custom-made solutions for VR systems for freely moving animals exist to date (Stowers et al. 2017).

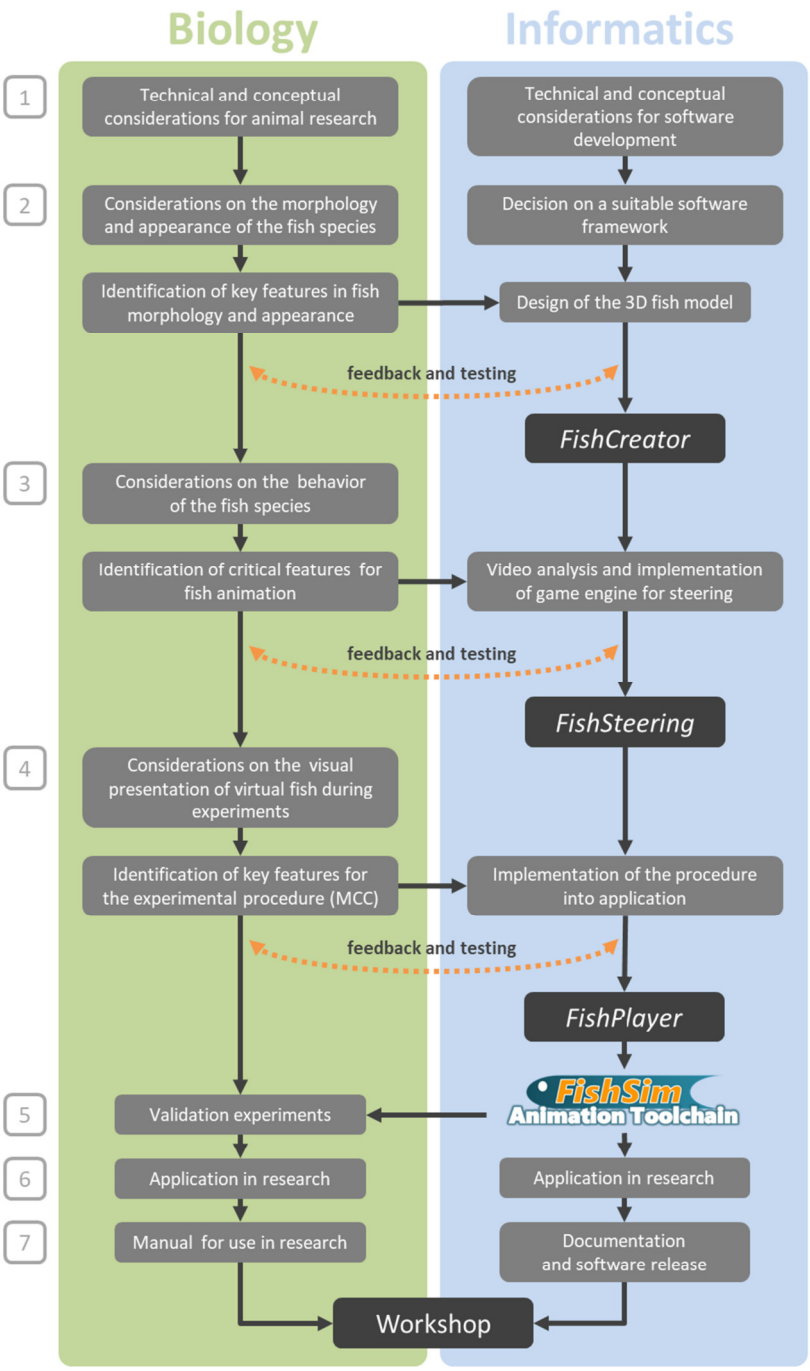

Figure 8. Flowchart describing the key stages (1-7) within the Virtual Fish Project.

The main goal of the development of *FishSim* was, therefore, to create an easy-to-use research tool for the specific study of mate-choice copying in sailfin mollies, in a standardized and controlled way (Stage 1, Fig. 8). During software development, we followed the technical considerations for the use of computer-animated stimuli in animal behavior research, as proposed by Baldauf et al. (2008), Chouinard-Thuly, Gierszewski, et al. (2017; Chapter 3 in this thesis), and Woo and Rieucau (2011; see also Oliveira et al. 2000). Further, our work was greatly influenced by prior studies using computer animation with poeciliids (Nicoletto and Kodric-Brown 1999; Rosenthal et al. 2002; Kingston et al. 2003; Fisher et al. 2006; Fisher and Rosenthal 2007; Butkowski et al.

2011; Verzijden and Rosenthal 2011; Culumber and Rosenthal 2013; Veen et al. 2013) and other fish (Künzler and Bakker 1998; Baldauf et al. 2009; Gerlai 2017). Most of the considerations on the design and implementation of *FishSim* described in the following paragraphs were, however, primarily focused on (I) the biology and behavior of sailfin mollies, and (II) the performance of experimental protocols as described for MCC experiments. Since we were not only interested in how morphological aspects of visual stimuli contribute to MCC but also differences in behavior, we considered it obligatory to simulate naturalistic sailfin molly courtship which, consequently, required computer animation in 3D and, hence, virtual sailfin mollies modelled in 3D. Based on long-term considerations, we further decided to follow an interactive approach with the possibility to have real-time rendered animations.

Since we were aiming for a very sophisticated and innovative animation system, we decided to base the general software framework of our toolchain on the robot operation system ROS (v. fuerte)[3] which provides a high degree of modularity and flexibility. A robot operation system generally consists of sensors, manipulators, controllers, and human machine interfaces that have to work together, even if distributed over different computers. Here, a middleware like ROS enables communication between these different components. As a result, we were not only able to replay sequences of computer animated fish stimuli on multiple screens simultaneously but we also integrated a real-time 3D tracking system (Müller et al. 2014; Müller, Smielik, et al. 2016). Tracking was used for automatic measurement of behavior (e.g., association time) and video-recording during experiments and it set the foot for the implementation of interactive computer animation (Müller, Gierszewski, et al. 2016; Müller et al. 2018). Similarly, Butkowski et al. (2011) developed a system for interactive animations to study mate choice in green swordtails (*Xiphophorus hellerii*). Interactive movement of their virtual fish stimuli, however, only functioned in 2D. Further, we added an external input device (i.e., a video game controller) used for animating ("steering") the virtual fish, as well as an operations terminal for the experimenter to operate the toolchain during experiments.

Overall, we developed four different tools to (I) create virtual 3D sailfin mollies (*FishCreator*); to (II) animate virtual sailfin mollies using a video game controller (*FishSteering*); and to (III) play-back prior created animated sequences on computer monitors during experiments (*FishPlayer*). Further, (IV) *FishSim* is the central tool for visualization of virtual stimuli and their movement. For this, *FishSim* is based on the free and open-source computer game engine Irrlicht (v. 1.81)[4]. By this, *FishSim* combines all tools in an easy-to-use framework for the application in research and provides everything needed to create, animate and present virtual sailfin mollies to live focal fish during experiments. I will describe *FishCreator*, *FishSteering*, and *FishPlayer* in more detail in the following sections of this chapter.

4.2 Stage 2: Design of virtual sailfin mollies - *FishCreator*

With regard to the general biology of sailfin mollies, which I described in Chapter 2, the first step was the design of virtual 3D replicas of live male and female sailfin mollies. We aimed to create 3D fish showing natural dimensions in morphology combined with a photorealistic quality of their appearance (color and pattern) when presented to live focal fish during experiments (Stage 2, Fig. 8). For this, we assessed the variability of morphological features characterizing sailfin mollies. Aside from general descriptions of sailfin molly morphology found in the literature (e.g., Snelson 1985), we performed

[3] ROS website: www.ros.org
[4] Irrlicht website: http://irrlicht.sourceforge.net/

detailed measurements of several male and female sailfin molly individuals. We did this to get a better idea of their dimensions and to identify important morphological features, which should later be customizable in the virtual fish stimuli. The most important questions were: What are the key features identifying male and female sailfin mollies? Which features are most variable across individuals? Which features are key to the study at hand, that is, answering questions on MCC in sailfin mollies?

To obtain body measurements for a generic 3D virtual fish template, we photographed free-ranging male and female individuals laterally (Canon EOS 600D, F/5.6, 1/250 s, ISO 200, focal distance 55 mm) in a small tank (40 cm x 40 cm x 12 cm) with light background. Illumination was provided by two neon tubes (ORSAM L58W/965) above the tank and an external camera flash (Nissin Speedlite). Digital pictures were then imported as RAW format using the tool UFRaw[5]. We used the image editing tool GIMP (v 2.8)[6] to adjust for white balance and illumination. Afterwards, we determined the standard length (SL; body size from snout to the beginning of the caudal fin) of each individual in millimeter using scale paper. Here, males (n = 22) had a mean SL of 38.2 ± 7.1 mm and females (n = 23) had a mean SL of 39.2 ± 5.3 mm.

Additional morphological measurements were done digitally using the free picture analysis tool ImageJ[7], according to a predefined measuring scheme for male (Fig. 9) and female (Fig. 11) fish. For picture analysis in ImageJ, the scale for each individual picture was set on basis of the prior measured SL of the respective live fish in millimeter. Thereby, the length of a respective measuring point (MP) was marked using the "line selection" tool and equals the number of pixels marked. The number of pixels is then transferred into millimeter in relation to the prior set scale (here: number of pixels per millimeter). The area of a respective MP (e.g., MP12 = dorsal fin area; Fig. 9) was marked using the "polygon selections" tool and calculated in square millimeters. For each MP in males and females, we calculated the coefficient of variation [%CV = (standard deviation MP / mean MP) x 100], to compare the relative variation between measuring points differing in either units and/or mean value. The higher the %CV value, the higher the variation within a given sample.

Measurements obtained for all males and females are shown in Tables S1 and S2 (Appendix 1) and Figures 4 and 6 respectively. Overall, our results support observations and morphological descriptions for sailfin mollies found in the literature (see also Chapter 2.1.1). MPs describing the general body shape of both male and female fish did not show considerable large differences in variation (all below 25 %; compare Figs. 10 and 12). As expected for males, we found the highest %CV (above 25 %) for MPs describing properties of a male's dorsal fin (MP9, MP10, MP12), the caudal fin area (MP15), as well as the number of lateral stripes (MP17R, MP17L; Fig. 10). It is well known that different reproductive strategies exist among males of a population (e.g., dwarf males), which are primarily represented by differences in general body size as well as dorsal and caudal fin size and body coloration (see e.g., Luckner 1979; Fraser et al. 2014; Chapter 2 in this thesis). For females, we found the highest %CV for dorsal fin area (MP12), caudal fin area (MP15), as well as pelvic fins (MP18, MP19). However, we consider variation in pelvic fins being due to the fact that females often kept their pelvic fins close to their body which made their dimensions difficult to measure. Considerably, the area of the gravid spot (MP20; Fig. 11) showed the most distinct variation between females, which may be partially due to their asynchronous reproductive cycle (Sumner et al. 1994; see also Chapters 2 and 7). Overall, male and female measurements defined important implications for the following virtual fish design.

[5] Download UFRaw: http://ufraw.sourceforge.net/
[6] GIMP website and download: https://www.gimp.org/
[7] Download ImageJ: https://imagej.nih.gov/ij/

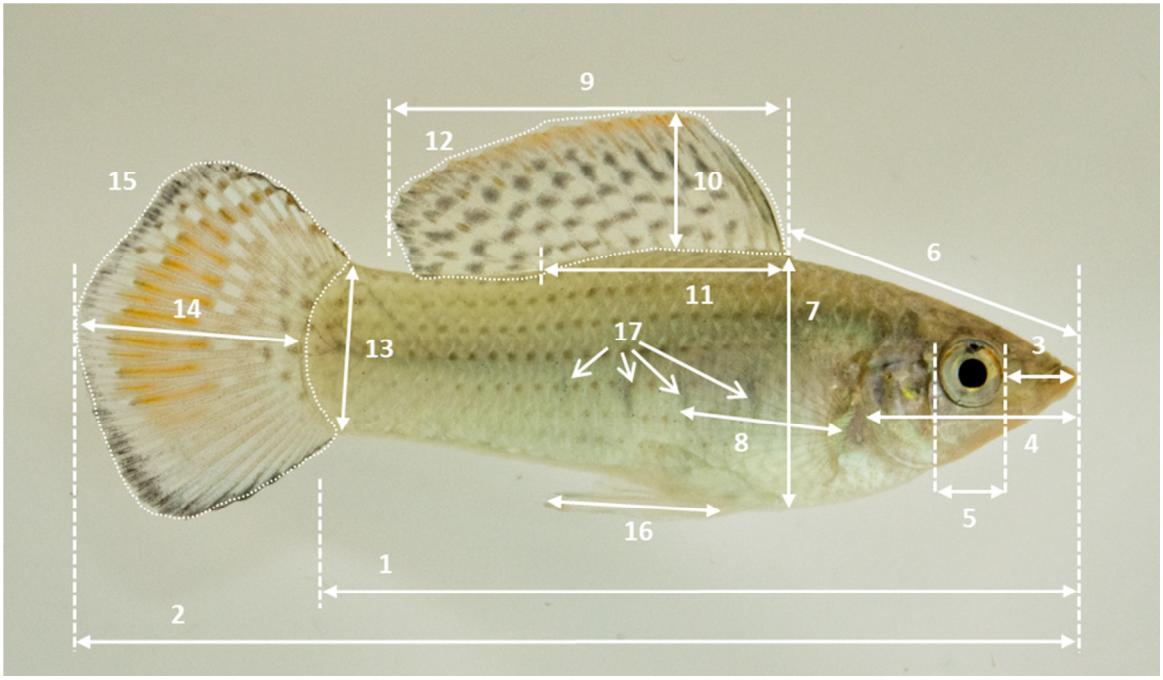

Figure 9. Measuring scheme for male sailfin molly photographs. Given are measuring points (MP) calculated for a male sailfin molly: (1) Standard length, (2) total length, (3) tip of snout to eye, (4) snout to operculum, (5) eye diameter, (6) snout to dorsal fin, (7) widest part of the body, (8) longest spine of pectoral fin, (9) maximum length of dorsal fin, (10) maximum height of dorsal fin, (11) length of dorsal fin base, (12) dorsal fin area, (13) width of caudal fin base, (14) maximum length of caudal fin, (15) caudal fin area, (16) gonopodium length, (17) number of vertical bars (L = left side, R = right side). Markings only serve as a means of illustration and may not be perfectly accurate.

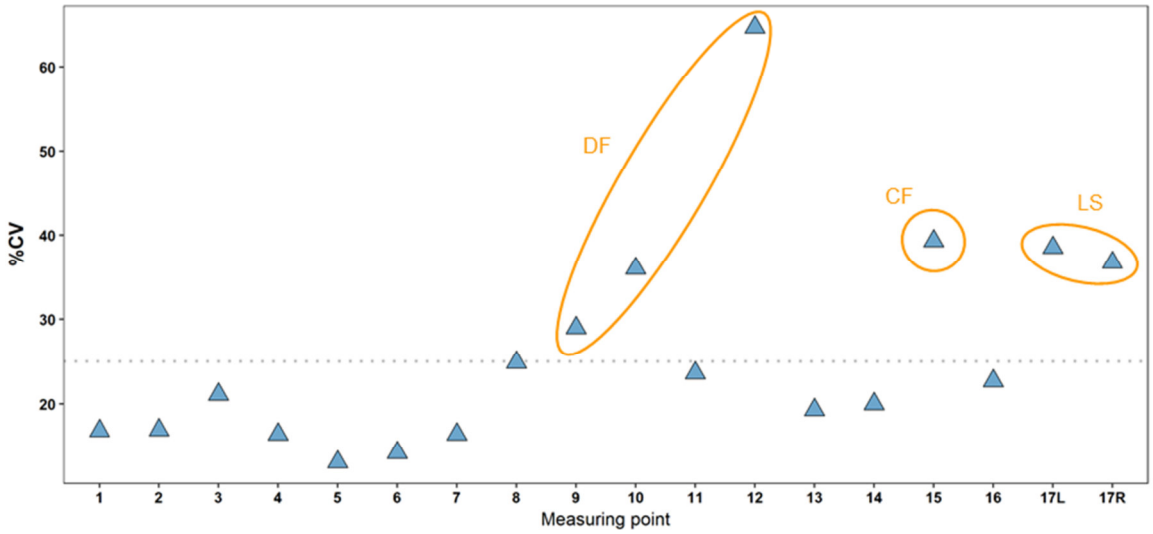

Figure 10. Coefficient of variation (%CV) for each measuring point obtained for male sailfin mollies. Measuring points (MP) depicted in the x-axis correspond to those described in Figure 9. N = 22 for each MP. DF = dorsal fin, CF = caudal fin area, LS = number of lateral stripes.

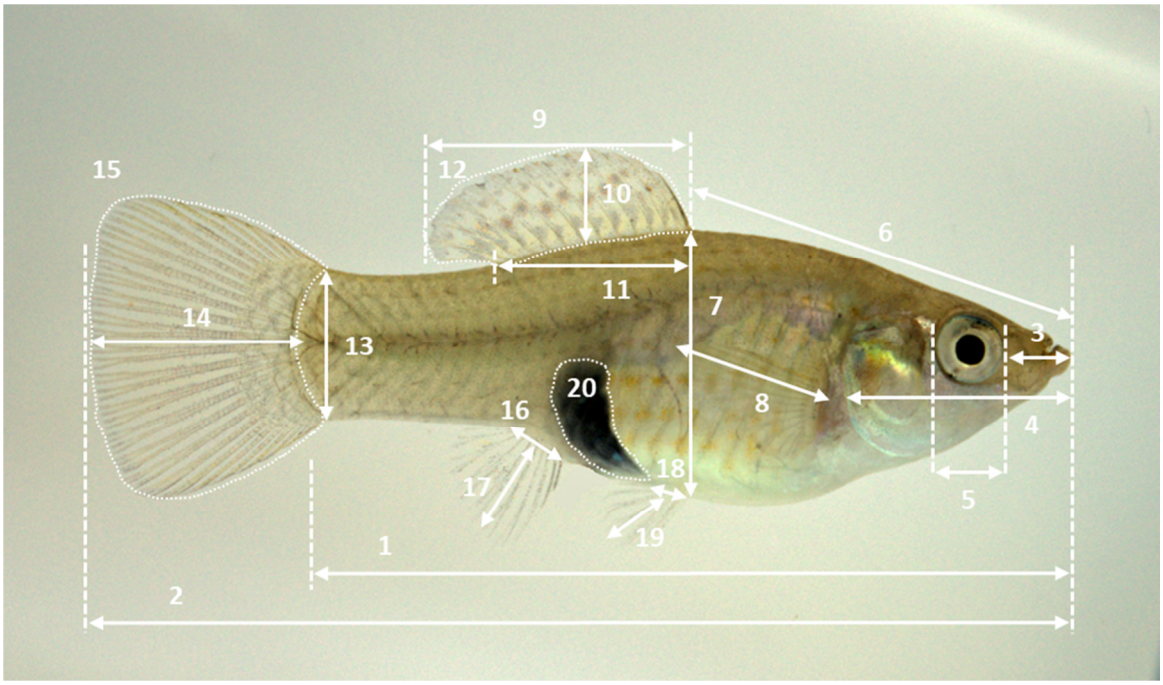

Figure 11. Measuring scheme for female sailfin molly photographs. Given are measuring points (MP) calculated for a female sailfin molly: (1) Standard length, (2) total length, (3) tip of snout to eye, (4) snout to operculum, (5) eye diameter, (6) snout to dorsal fin base, (7) widest part of the body, (8) longest spine of pectoral fin, (9) maximum length of dorsal fin, (10) maximum height of dorsal fin, (11) length of the dorsal fin base, (12) dorsal fin area, (13) width of caudal fin base, (14) maximum length of caudal fin, (15) caudal fin area, (16) length of the anal fin base, (17) longest spine of anal fin, (18) length of the pelvic fin base, (19) longest spine of pelvic fin, (20) gravid spot area. Markings only serve as a means of illustration and may not be perfectly accurate.

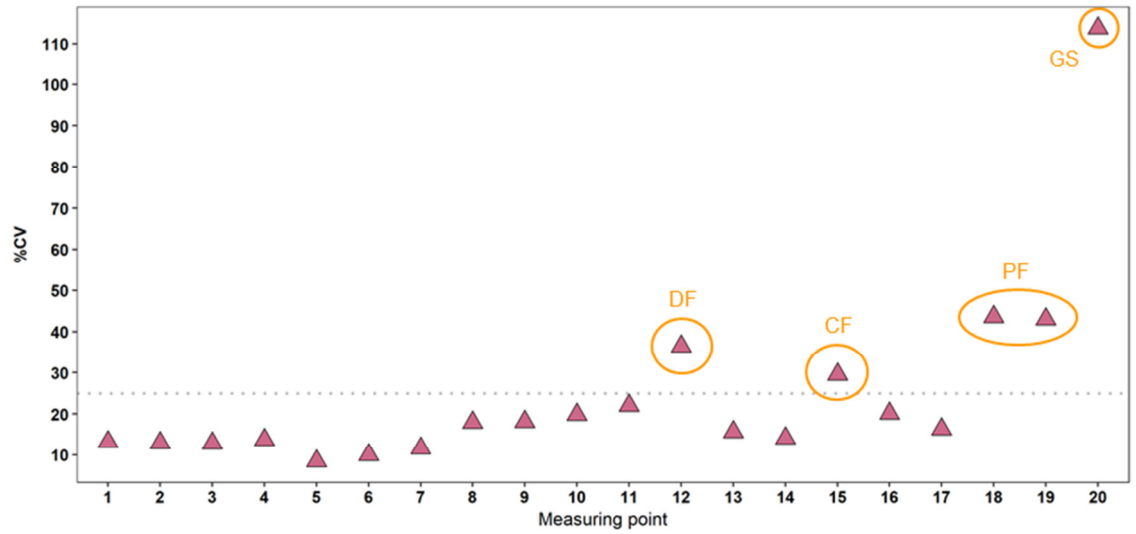

Figure 12. Coefficient of variation (%CV) for each measuring point obtained for female sailfin mollies. Measuring points (MP) depicted in the x-axis correspond to those described in Figure 11. N = 23 for each MP. DF = dorsal fin area, CF = caudal fin area, PF = pelvic fin, GS = gravid spot area.

Based on our findings described above, we created two generic 3D fish using the free 3D graphics modelling program Blender[8] (v. 2.70a; Fig. 13A-B). Due to the sexual dimorphism shown in males and females, we created one generic 3D fish for each sex separately. First, we created a 3D virtual wire mesh representing the general body shape of one exemplar male and female individual (Fig. 13B, Step 2). We then equipped the wire mesh with an outer skin and a virtual skeleton used for later animation of the 3D fish (Fig. 13B, Step 3). Further, we used the identical set of digital photographs, as used for measuring males and females, to create textures for UV-mapping of the generic 3D fish templates (Fig. 13B, Step 4). A UV-map represents a 2D image of the corresponding 3D model, similar to virtually unfolding a cube into a flat plane. In the UV-mapping process, those 2D images are wrapped around the 3D mesh of the virtual fish to give it color and pattern. Since we found no prominent differences in variation of MPs describing the general shape of the fish body, we decided to not separate the fish body into several pieces but textured it as a whole. Moreover, we only used textures showing the left side of a fish's body and mirrored its pattern onto the right side of the virtual fish. On the one hand, this procedure facilitated texture mapping from a technical standpoint, on the other hand, Schlüter et al. (1998) had demonstrated that female sailfin mollies show a preference for males with symmetrical vertical bars on the side of their body. With regard to the fins, we found high variation for dorsal and caudal fin properties in both sexes. Therefore, we decided to have those fins as separate textures during customization. Since overall fin size was highly variable, especially in males, we created oversized UV-maps for dorsal and caudal fins (Fig. 13C). The experimenter, therefore, has the possibility to experimentally enlarge or diminish the textures for both fins even beyond its natural range. Using GIMP, we selected and cut the fin and body parts from each picture and fit them onto the UV-maps of the corresponding fin and body parts of the generic 3D male and female respectively (Fig. 13D). The result was a whole catalogue of various male and female textures that could be used to create different virtual fish for the use as visual stimuli in experiments.

We created the tool *FishCreator* to provide a graphical user interface (GUI) which gives the experimenter different options for customization of the generic 3D fish. The fish species and its sex can be chosen and the fish can be scaled in size. Here, the virtual fish may be scaled in its length (i.e., total length as correspondent to MP2 in Figs. 9 and 10), its height, as well as its width (Fig. 13E). The actual size of the virtual fish in an animation (e.g., in millimeter), however, has to be determined by measuring the virtual fish's size as displayed on the respective monitor screen, since size may depend on a monitor's display properties. Total length and standard length of a stimulus are important parameters describing stimuli used in experiments and customization options are important to be able to create different virtual fish representing natural variation in size. Moreover, Witte and Ryan (1998) found that male size is an important predictor for MCC in female sailfin mollies, who generally prefer larger males (Schlupp et al. 1994; Marler and Ryan 1997). For application of *FishSim* in MCC experiments, the option to vary a virtual male's body size was, therefore, essential. Furthermore, in Chapter 6 in this thesis, I used this function to test the effect of a virtual model female's body size on MCC in live focal female sailfin mollies.

Being able to change the appearance of the virtual fish (e.g., color and pattern) was another essential feature we accounted for during software development. Options for customization in *FishCreator* include the changing of textures of different parts of the generic 3D fish (body and fins). For this, the experimenter may choose from the catalogue of textures and may, thereby, change the appearance of the virtual fish (Fig. 13F). By this, we are able to experimentally account for the high variation found in e.g., male coloration, male dorsal fin size or their number of lateral stripes. Using a picture editing tool like GIMP, textures of a virtual fish may further be artificially

[8] Blender website and download: https://www.blender.org/

manipulated to investigate certain research questions, such as how variation zebrafish lateral stripes affects shoaling decisions (Rosenthal and Ryan 2005). In Chapter 7 of my thesis, I performed an experiment in which I deliberately manipulated female textures showing either a gravid spot or no gravid spot to test its possible role in the context of MCC. Overall, we designed *FishCreator* to offer various options to prevent pseudoreplication in experiments (Hurlbert 1984). Virtual stimuli may even be designed at random and size measurements can be adjusted to represent mean values of populations, as proposed by Rosenthal (2000).

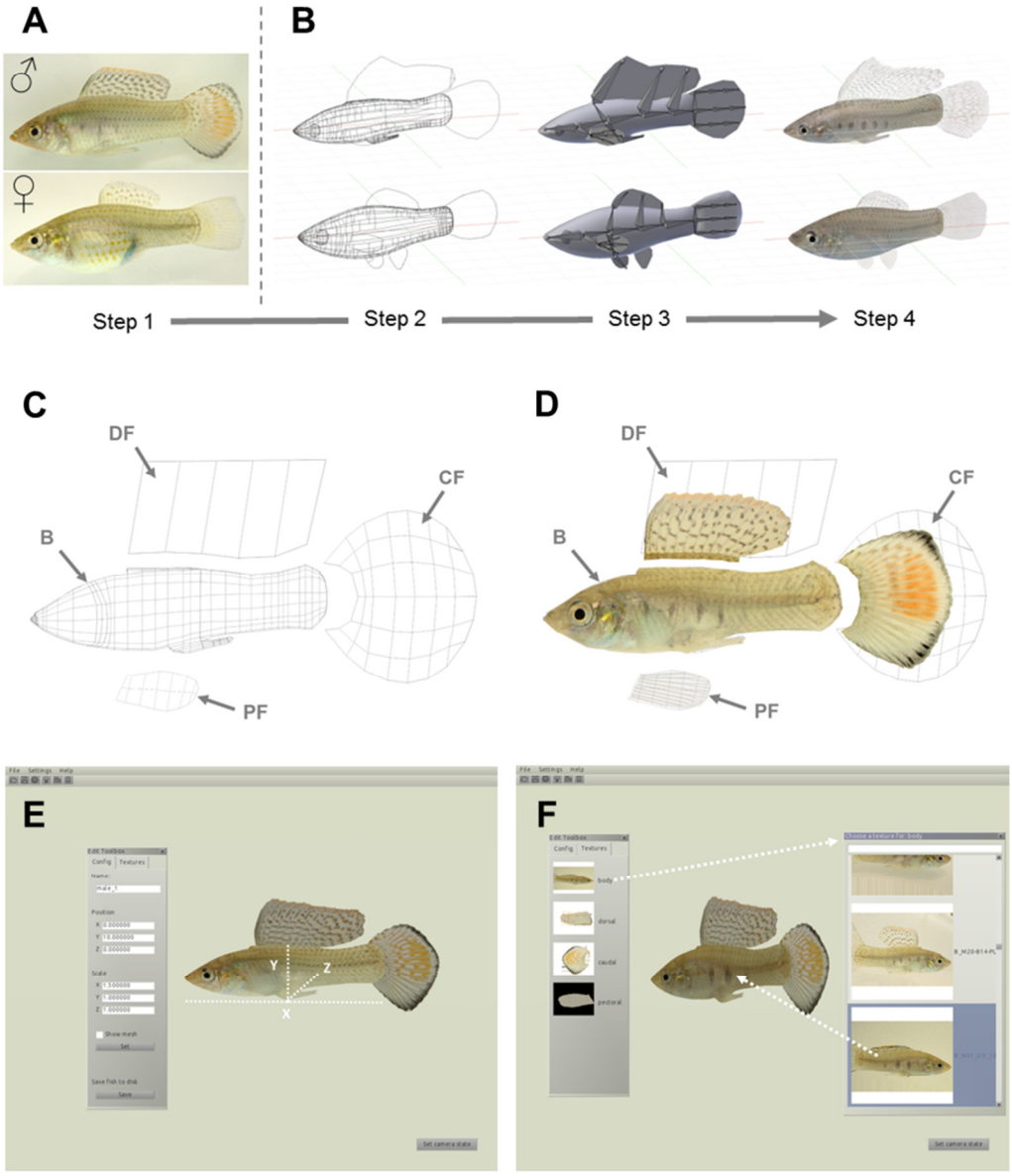

Figure 13. Visualization of the functions underlying virtual fish design with *FishCreator*. (A) Lateral exemplar photographs of male and female sailfin mollies used for 3D fish creation (Step 1). **(B)** Steps illustrating 3D fish modelling with Blender. The 3D wire mesh of body and fins for male and female each, as derived from lateral photographs (Step 2). Object view of the 3D fish showing the inner skeleton for animation purpose (Step 3). Textures for body and fins wrapped around the 3D fish to add color and pattern (Step 4; modified after Gierszewski et al. 2017). **(C)** UV-maps used for texturing of the generic virtual male. UV maps are a 2D representation of the 3D mesh (B, step 2). **(D)** Cut body and fin textures of an exemplar male individual adjusted to fit the corresponding UV-maps for the respective body and fin parts. **(E)** Scaling options for dimensions (X = length; Y = height; Z = width) of a virtual male fish given in *FishCreator*. **(F)** Changing the body texture of a more compressed version of the virtual male fish in *FishCreator*. Available textures are automatically taken from the *FishSim* database and mapped onto the visible 3D fish. B = body, CF = caudal fin, DF = dorsal fin, PF = pectoral fin.

4.3 Stage 3: Animation of virtual sailfin mollies - *FishSteering*

An important step in software development was the implementation of movement of the virtual fish (Stage 3, Fig. 8). Prior studies showed that movement was of key importance for the attraction towards virtual stimuli in live fish (Baldauf et al. 2009; Egger et al. 2011). Moreover, movement of virtual stimuli that more closely mimicked the natural behavior of an animal resulted in a closer association of live focal fish (Abaid et al. 2012; Nakayasu and Watanabe 2014; Nakayasu et al. 2017). For an observing focal animal, the correct syntax of movement patterns may even be more important than the general appearance (realistic or stylized) of a virtual stimulus (Woo and Rieucau 2015). Our aim was to create an innovative and easy-to-use steering tool, by which the experimenter would be able to create movement of the virtual fish as realistic as possible. Moreover, we wanted to be able to manipulate the behavior of presented stimuli in a controlled way during experiments, which is almost impossible in live animals.

Aside from simulating general swimming kinematics as described for fish (Videler 2012), we were specifically interested in simulating sailfin molly courtship behavior. Sailfin molly courtship consists of different behavioral patterns that were previously described by (Parzefall 1969; Baird 1974; Luckner 1979) and which are predominantly performed by males. Males exhibit an elaborate courtship display which typically includes: (I) Approaching and (II) following a female, (III) lateral displays (dorsal and caudal fins raised) as well as (IV) sigmoid displays in front of or next to the female, (V) gonopore nipping, (VI) gonopodial thrusting and (VII) copulation (see also Chapter 2.1.2 in this thesis). In contrast, females do not actively court males but may deliberately approach them. Females may further indicate their willingness to copulate by stopping in front of a male and slightly rotating their urogenital region up and sideways to facilitate internal insemination (Baird 1974; Luckner 1979).

Important implications for virtual sailfin molly animation are that these behavioral patterns not only require movement in 2D (x- and y-coordinates) but in 3D (x-, y- and z-coordinates) as well. For this, we equipped our generic 3D fish with a virtual skeleton (Fig. 13B, Step 3 and Fig 14C). The virtual skeleton consisted of several bones that were positioned to ensure natural movement of the fish body in general (bending) as well as raising/lowering the dorsal fin and rotation of the male's gonopodium in particular. Even though movement of dorsal fin and gonopodium is mostly relevant during male courtship, we kept the virtual skeleton identical in both males and females. Each virtual bone is interconnected to its surrounding wire mesh of the 3D fish and may affect this area when moved. We deliberately defined each bone's area of influence (so-called 'weight-painting') and thereby adjusted movement of the virtual fish to closely match sailfin molly behavior.

To obtain data on sailfin molly behavior, we first performed a thorough analysis of video recordings of live fish swimming in a small tank as described in detail by Smielik et al. (2015; Fig. 14A). Live fish were tracked and information on their body posture while swimming was extracted using computer vision algorithms (Fig. 14B). Movement of the live fish's body was segmented in relation to the size of each virtual bone and then transferred onto the virtual skeleton of the 3D fish to generate natural movement during animation (Fig. 14C). To manipulate bones and, hence, the virtual skeleton for animation, we created the tool *FishSteering.* With this, the experimenter can create a swimming path for each virtual fish based on a semi-automated steering mode controlled via video-game controller (Sony Playstation DualShock 3, Sony Computer Entertainment Inc., Japan). Input to the controller, for example pressing a button, is then translated into movement of a respective bone (or a set of several bones) by a predefined algorithm in real-time (see Table S3, Appendix 1).

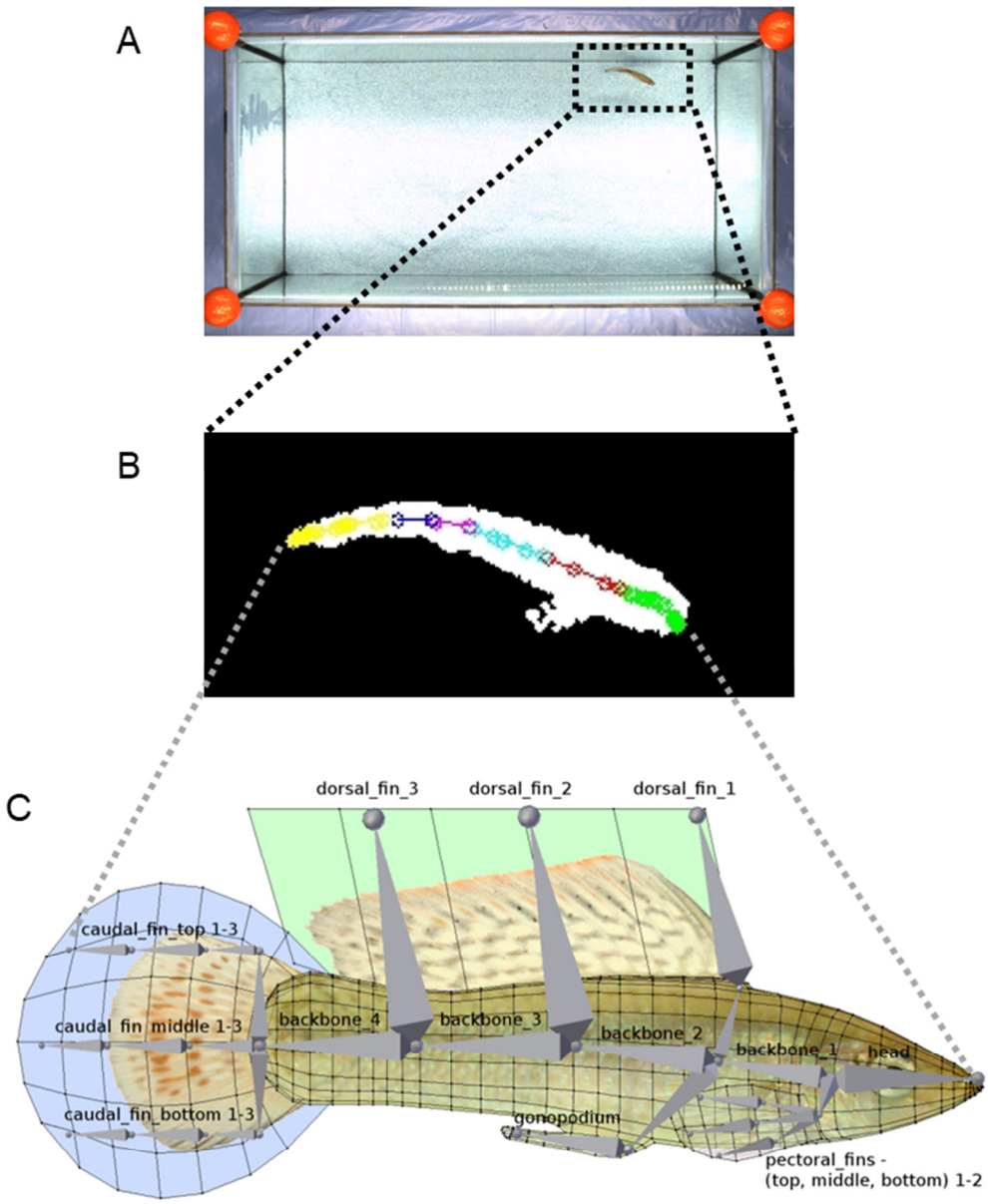

Figure 14. Visualization of sailfin molly movement underlying the animation process in *FishSteering*. **(A)** Exemplar picture of the tank used for video analysis of a swimming male as seen from above. **(B)** Fish silhouette as derived by background segmentation during video analysis. White represents the fish body and black describes the background. Each line segment corresponds to one bone of the virtual skeleton depicted in (C) below: green = head, red = backbone 1, light-blue = backbone 2, purple = backbone 3, blue = backbone 4, yellow = caudal fin bones 1-3 (modified after Smielik et al. 2015). Bones of the dorsal fin, pectoral fins and gonopodium are not illustrated. **(C)** Generic 3D sailfin molly male showing mesh, textures and the virtual skeleton. A single gray cone describes a bone of the virtual skeleton used to create movement (modified after Müller et al. 2017).

We further used realistic data obtained on swimming speed to fine tune movement speed of virtual fish in relation to the input given to the controller. In a subsequent editing step, the prior created swimming path is replayed and the experimenter may add single movements (e.g., dorsal fin or gonopodium) by pressing the respective buttons to finalize the animated sequence. The duration of each animated sequence may be adjusted as needed for the respective experimental procedure. For simulation of courtship behavior,

we included the option to animate more than one virtual fish. Here, swimming paths of each virtual fish have to be created one after another. A once created swimming path can be replayed with various different virtual fish (of the same species and sex) to either control for behavioral differences but also provide the option to vary stimulus appearance. Animations were always rendered at a frame rate of 60 fps which is considered sufficient for the visual system of most fish (Fleishman and Endler 2000; Oliveira et al. 2000).

To increase the overall realism of animated sequences, we created a 3D virtual tank environment, resembling the test tank of the focal fish (Fig. 15). Virtual fish were, therefore, always simulated to be swimming in a small tank to simulate depth. Additionally, we included a 3D stone as reference object (as in Mehlis et al. 2008). Further, virtual fish were subject to occlusion effects and would cast shadows (Zeil 2000; Veen et al. 2013).

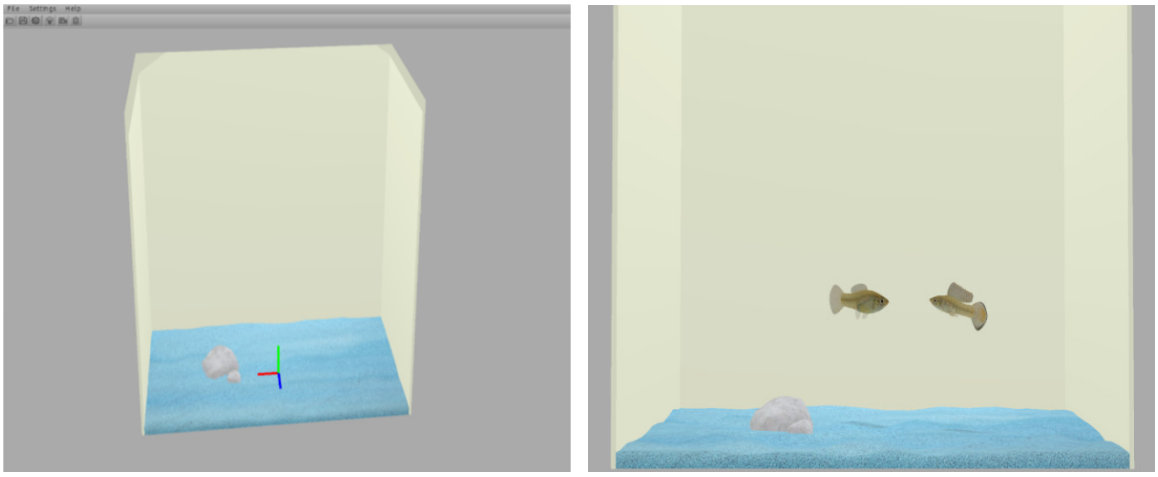

Figure 15. Virtual tank environment. Editing mode in *FishSim* (left) and viewing mode (right) as seen by live focal fish on the monitor screen. A stone object and shadows of both fish on the ground provide depth cues to observing live fish.

4.4 Stage 4: Presentation of virtual sailfin mollies in experiments - *FishPlayer*

With our study aim at hand, the most important consideration was regarding the feasibility of computer animations in experimental procedures as described for testing MCC in sailfin mollies (see e.g., Witte and Ryan 1998; Witte and Noltemeier 2002). For this, it was necessary to adapt the classic experimental procedure to the use with animations presented on computer monitors, including all important experimental stages: (I) First mate-choice test, (II) observation period, and (III) second mate-choice test (see Chapters 6.3.3 and 6.3.4 for more information on the MCC experiment). For this purpose, we developed *FishPlayer* as a tool for visual presentation during experiments (Fig. 8, Stage 4). All prior created animated sequences with *FishSteering* can be loaded into *FishPlayer* for the presentation on computer monitors. Though, we focused its design to be specific for the use in dichotomous test situations (i.e., mate-choice tests), *FishPlayer* may be used to present animations on up to three different computer monitors simultaneously. Thereby, *FishPlayer* may be configured to match the properties of different monitors, for example regarding a display's resolution.

FishPlayer allows for a flexible arrangement of each animation as single entries in a playlist. Each playlist entry typically consists of an animated scene including one or more virtual fish and a so-called bag-file including the recorded swimming path. *FishPlayer* then connects both files and replays the swimming path using the respective fish stimuli. Each playlist is bound to one monitor screen. Therefore, it is possible to either show identical or different animations on each monitor separately. For example, during mate-choice tests, it is possible to present two different male stimuli simultaneously. Thanks to the flexible and innovative playlist structure of *FishPlayer*, it possible to adopt the general experimental procedure of an MCC experiment in a very user-friendly way. Each experimental step is represented by the different entries of a playlist and their order may be adjusted during the course of the experiment (Fig. 16). Further, it is possible to add a "pause" entry which is typically used when live fish have to be handled during the experiment and no animation is shown, e.g., when focal fish are placed inside a cylinder. Once started, *FishPlayer* runs each playlist automatically from top to bottom. By this, the general experimental procedure may be standardized across experiments.

4.5 Stage 5: Validation of *FishSim*

A central responsibility of my work within the Virtual Fish Project was the validation of *FishSim* as a new research tool to study questions related to MCC in sailfin mollies (Fig. 8, Stage 5). Upon introduction of a new method, especially with computer animation, it is necessary to validate whether the study animals perceive the animation similarly to real animals. They should not behave aversive but rather be attracted towards animated stimuli and show behavior similar to that observed in situations with live stimuli. Here, both perception as well as recognition of virtual stimuli may be species specific and dependent on a focal animal's visual system (Chouinard-Thuly et al. 2017; Powell and Rosenthal 2017; see Chapters 2.1.3 and 3.4 in this thesis).

Similar to other studies validating the use of computer animation in studies with fish (Clark and Stephenson 1999; Baldauf et al. 2009; Pather and Gerlai 2009; Fischer et al. 2014; Qin et al. 2014; Scherer et al. 2017), I performed four different experiments in which I presented virtual stimuli created with *FishSim* in two-choice experiments to live sailfin mollies. Among other things, I analyzed associative behavior of focal fish towards virtual fish compared to that towards video-recorded fish and live fish stimuli. I will describe my validation experiments and their results in detail in Chapter 5 in this thesis. Furthermore, the comparative study I will show in Chapter 6 served as an additional validation to demonstrate the successful adoption of *FishSim* to perform the experimental procedure as described for MCC experiments.

Figure 16. Playlist entries for an MCC experiment with *FishPlayer*. (Top) Playlist entries for the first mate-choice test of an MCC experiment. **(Bottom)** Playlist entries for the observation period and subsequent second mate-choice test (modified from Gierszewski, Baker, et al. 2018). M1.1 = first part of the first mate-choice test, M1.2 = second part of the first mate-choice test; M2.1 = first part of the second mate-choice test, M2.2 = second part of the second mate-choice test, O = observation period, P = pause.

4.6 Stage 6: Application of *FishSim* in research on MCC

Resulting from a positive validation for the use of *FishSim* in studies on sailfin molly mate choice (see Chapter 5) I performed several experiments to investigate various aspects of public information use in the context of MCC. These studies represent the heart of my thesis and are described in detail in Chapters 6 to 9.

In each study, I benefitted from different key features of our toolchain regarding the ability to visually manipulate quality and quantity of public information. I then experimentally tested the effect of each manipulation regarding the expression of MCC in live focal females. In Chapter 6, I will describe how we used the specific functions of *FishCreator* to manipulate the quality of a virtual model female by varying the dimensions of the generic 3D fish. We then presented either a large or a small virtual model female during the observation period in experiments. For this study, we used a comparative approach with live fish stimuli and virtual fish. Further, in Chapter 7, I will describe how we manipulated textures used to create virtual model females, in whether they showed a gravid spot or not. Here, we wanted to know whether a gravid spot indicates model female quality to observing live focal females. Aside from manipulations of morphology and appearance, we were further able to manipulate the behavior of virtual stimuli using the specific functions of *FishSteering*. In Chapter 8, I will describe a first attempt in testing how manipulation of courtship dynamics, expressed by an interacting pair (virtual model female and a virtual male), affects MCC in observing focal females.

Additionally, in Chapter 9, I will describe a second experiment manipulating the interaction of the observed pair of model female and male, however, without the application of *FishSim*. In the final synopsis of my thesis (Chapter 10), I will inter alia evaluate *FishSim* as a new method to study MCC and give an outlook on future applications.

4.7 *FishSim* goes public

The development of *FishSim* as an open-source project, led to the decision to make the software public and accessible for scientists around the world. Even though the development of *FishSim* was specific for the study of MCC in sailfin mollies, we tried to keep the structure of *FishSim* as flexible as possible to allow scientists to include their own fish templates. As to date, *FishSim* includes four other species templates: the Atlantic molly *Poecilia mexicana*, the guppy *Poecilia reticulata*, the three-spined stickleback *Gasterosteus aculeatus* and the cichlid *Haplochromis* spp. We released the software for download from the sharing platform Bitbucket[9] in October 2017. Using the knowledge gained during my research, I wrote a detailed manual on how to use *FishSim* in research and on how to best handle its different tools. The user manual is available for download together with *FishSim* from Bitbucket.

To provide first-hand experience and assistance for the use and implementation of *FishSim* in research, we organized the three-day international workshop “Discovering *FishSim* in 3 days - new software for computer-animated 3D fish stimuli for innovative research in animal behavior” at University of Siegen[10]. The workshop program was divided into theoretical and practical sessions during which I introduced participants to best-practices for the use in research and helped them used the software. I was granted a funding award from the German Zoological Society e.V. for the organization of this workshop.

[9] Download *FishSim* Toolchain: https://bitbucket.org/EZLS/fish_animation_toolchain/wiki/Home
[10] Workshop website: https://virtualfishproject.wixsite.com/fishsim

To facilitate new implementation in research for other scientists, we published a detailed step-by-step protocol (including a demonstration video) describing the correct use of the different tools of *FishSim* in an MCC experiment (Gierszewski, Baker, et al. 2018). This publication was based on the study I describe in Chapter 7.

Over the course of the Virtual Fish Project, I was engaged in several science communication and outreach events to present my work to the broad and scientific public and to promote the use of computer animation in research. In 2015, I co-organized a symposium and workshop with the topic "*Virtual reality – Computer animations as a tool in animal behavior research*"[11] during the 34th Ethological Conference in Cairns, Australia, together with Klaudia Witte and Laura Chouinard-Thuly (former McGill University, Canada). By this, we wanted to gather scientists working in the field of animal behavior using computer animation and virtual reality, to exchange expertise and advice on new methods and applications in research. Wide interest and popularity resulted in the publication of a special issue on the same topic in the international journal *Current Zoology* (Volume 63/Issue 1). Together with Klaudia Witte and Laura Chouinard-Thuly, I was one of the guest editors for this special issue. The special issue comprised a collection of eleven articles, of which I co-authored three articles (Chouinard-Thuly, Gierszewski, et al. 2017; Gierszewski et al. 2017; Müller et al. 2017) as well as the issue's editorial (Witte et al. 2017). Chapters 3 and 5 of my thesis have been published as part of this special issue. Moreover, I participated in the "Science-Schau-Fenster" in the City of Siegen, a public exhibition about current research projects at University of Siegen. In 2016, I organized the event "Molly knows best"[12], the fish oracle for the European Football Championship. We used this event to communicate our science to the broad public and to test the functionality of interactive stimuli by applying a 3D real-time tracking system developed within the Virtual Fish Project (Müller et al. 2014; Müller, Gierszewski, et al. 2016; Müller et al. 2018).

Overall, the Virtual Fish Project demonstrates a successful, interdisciplinary collaboration between biologists and computer scientists at University of Siegen. Over the course of the project, 5 publications in international peer-reviewed journals, 5 peer-reviewed conference proceedings, and 1 other article (not peer-reviewed) have, so far, been published. Overall, 12 scientific contributions in form of talks or posters to national and international conferences were made to communicate the science behind the Virtual Fish Project. Based on the new developments and scientific insights gained in the project, a follow-up research proposal was submitted to the DFG in 2017 (Wi 1531/12-2), which I was involved in writing. Hopefully, the achievements of the Virtual Fish Project will pave the way for more innovative and collaborative work in future studies on fish behavior (see also Chapter 10 for a synopsis and an outlook for future research).

[11] Symposium and workshop website: http://iec2015-symposium.wixsite.com/virtual-reality
[12] Website for „Molly knows best": https://virtualfishproject.wixsite.com/em2016-fisch-orakel

Chapter 5

The virtual lover: variable and easily guided 3D fish animations as an innovative tool in mate-choice experiments with sailfin mollies – II. Validation

Stefanie GIERSZEWSKI[a], Klaus MÜLLER[b], Ievgen SMIELIK[b], Jan-Marco HÜTWOHL[b], Klaus-Dieter KUHNERT[b] & Klaudia WITTE[a]

[a]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein-Straße 2, Siegen, 57076, Germany and [b]Institute of Real-Time Learning Systems, Department of Electrical Engineering & Computer Science, University of Siegen, Hölderlinstraße 3, Siegen, 57076, Germany

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5.1 Abstract

The use of computer animation in behavioral research is a state-of-the-art method for designing and presenting animated animals to live test animals. The major advantages of computer animations are: (1) the creation of animated animal stimuli with high variability of morphology and even behavior; (2) animated stimuli provide highly standardized, controlled and repeatable testing procedures; and (3) they allow a reduction in the number of live test animals regarding the 3Rs principle. But the use of animated animals should be attended by a thorough validation for each test species to verify that behavior measured with live animals toward virtual animals can also be expected with natural stimuli. Here we present results on the validation of a custom-made simulation for animated 3D sailfin mollies *Poecilia latipinna* and show that responses of live test females were as strong to an animated fish as to a video or a live male fish. Movement of an animated stimulus was important but female response was stronger toward a swimming 3D fish stimulus than to a "swimming" box. Moreover, male test fish were able to discriminate between animated male and female stimuli; hence, rendering the animated 3D fish a useful tool in mate-choice experiments with sailfin mollies.

5.2 Introduction

The use of artificial stimuli to study fish behavior has already a long history. Ter Pelkwijk and Tinbergen (1937; Tinbergen 1948) were two of the pioneers, using dummy fish to investigate courtship and agonistic behavior in three-spined sticklebacks *Gasterosteus aculeatus*. They showed that dead sticklebacks and schematic wooden models put on a stick and moved by hand with varying shape and belly redness could be used as visual releasers to evoke courtship and/or aggression in live male and female sticklebacks. Although they provide 3D cues, such dummy fish are very limited in their possible changes to morphology and behavior, but they are still used today (Kim and Velando 2014). Tinbergen and Perdeck (1950) used dummy heads of hering gulls *Larus argentatus argentatus* Pont. to investigate the begging response of chicks to the parents' beaks. This was the first field experiment in behavioral biology. Magnus (1954) used a rotating cylinder with stripes to investigate the visual releasing stimulus for males to follow females in the silver-watched fritillary *Argynnis paphia*. Other dummies were used to investigate the reaction of turkeys to birds of prey (Schleidt 1961) and the begging response of black bird *Turdus merula* and European song thrush *Turdus e. ericetorum* chicks (Tinbergen and Kuenen 1939). Thanks to rapid technical development over the last decades, we now have access to several elaborate methods to create highly realistic and varied artificial stimuli. Ward et al. (2008) used realistic stickleback replicas, made of colored resin plaster, to study decision-making strategies in shoaling fish. They automated and standardized movement by using a motorized guided line system that moved replicas through the test tank. This method was further developed leading to bio-inspired robot systems to study mate-choice, collective movement, and social networks directly within groups of live fish (Kopman et al. 2013; Landgraf et al. 2016). Screen-based techniques for stimulus presentation, including video playback, video editing, and computer animation, are valuable alternatives in test situations in which live fish can usually choose between two live stimulus fish presented in separate tanks or behind glass walls. Early screen-based methods used video playbacks of live animals (Rosenthal 1999; Oliveira et al. 2000; Rosenthal 2000). Manipulation of video playbacks was very limited in its early stages and restricted to variation in hue or color output defined by the monitors, as was done by Rowland (1995) to investigate female stickleback attentiveness toward different gray-toned and colored video sequences of male courtship. The development of video-editing software allowed more rigorous manipulations of shape and color, and limited variation in behavior of animated stimuli. (Rosenthal and Evans 1998) and Körner et al. (1999) used this technique to manipulate video playbacks of poeciliid fishes for presentation in mate-choice experiments. Not only did fish prove to be responsive toward video playback, but jumping spiders *Maevia inclemens* responded to prey insects, conspecifics, and heterospecifics (Clark and Uetz 1990). Clark et al. (1997) demonstrated the usability of this technique in the field and presented video-edited displaying male lizards *Anolis grahami* to conspecifics in the wild, who expressed natural behavior toward the video.

Advanced techniques are 2D and 3D computer animations (Woo and Rieucau 2011). Animations are more variable and the stimulus is detached from any basic, raw material. McKinnon and McPhail (1996) were one of the first using a computer generated 3D fish animation, based on morphological measurements of a male three-spined stickleback. They presented an animated rival male on a computer screen next to an aquarium containing a live test male. In presence of the animated rival, live male sticklebacks performed aggressive displays and bites to the rival. Following this new approach, it could be demonstrated that fish seemed to be similarly responsive to computer-animated

models as to natural stimuli and recognized them as “real” conspecifics (e.g., Baldauf et al. 2009). Furthermore, it was shown that results obtained with virtual stimuli were congruent and reproducible with live stimuli (Rosenthal et al. 2002; Egger et al. 2011; Amcoff et al. 2013). Zbinden et al. (2003; 2004) showed that three-spined sticklebacks could be successfully put into a feigned situation of sperm competition by showing them animations of courting or brood-caring virtual males. In this test situation, sticklebacks reacted by increasing their ejaculate size, indicating the high degree of realism the animation must have had for the observing fish.

The 2D computer animations often derive from digital photographs of live animals that are then edited using various image processing software. To gain a 3D fish animation, these photographs are transferred into digital wire mesh models that can be modulated and animated using software that is also used for developing computer games and animated movies (but see Künzler and Bakker 1998 for an alternative method). With the help of animation software, different motion patterns can be specified and virtual models perform simple to complex, realistic, species-specific behavioral patterns and visual displays. For example, Clotfelter et al. (2006) designed a complex 3D Siamese fighting fish *Betta splendens* that was able to perform species-specific opercula displays to study mate choice. Reasons for using animated stimuli instead of live stimulus fish, especially in mate-choice experiments, are obvious since the opportunities to manipulate virtual animals are nearly endless and do not require invasive techniques or surgery of live animals. A good example to illustrate the possibilities with virtual animals is the study of mate preferences in swordtail fish (*Xiphophorus* spp.). Basolo (1990) found a female preference for sword length in swordtails by surgically manipulating sword length in sedated live males. Rosenthal and Evans (1998) took advantage of video editing to partially dissolve the swordtail from the body to investigate the underlying mechanism of this preference. They used manipulated virtual stimulus males that had “normal” swords, only partial swords or no swords at all. They were even able to present sequences of single swords without the fish’s body, but moving as if connected to it, and found that female preference for swords reflects a bias for large apparent size. In a following study, Wong and Rosenthal (2006) used computer animated swordtails to investigate the evolution of mate preferences in swordtail fish. In this animation, the naturally swordless sheepshead swordtail *Xiphophorus birchmanni* was artificially equipped with a sword revealing a disdain for this trait by females of this species.

Variability of appearance is not the only advantage that virtual animals provide. Live stimulus fish used in experiments differ in their behavior and, hence, influence the test fish’s response. Live stimulus fish might not interact with the live test fish that can result in the rejection of test trials or the repetition of experiments with a new stimulus fish which is very time-consuming. Instead, behavior of virtual stimuli can be predefined and kept constant in every single trial. Recently, the need for such standardized and advanced methods gave rise to the development of free-to-use software for fish biologists, like the program *anyFish* 2.0 (Veen et al. 2013; Ingley et al. 2015). Müller et al. (2017) also developed user-friendly software to improve design and presentation of animated 3D fish for behavioral experiments. Their software is based on a robot operation system that enables users to steer 3D fish with a video game controller and makes it possible to implement a 3D tracking system (for details see Müller, Smielik, et al. 2016; Müller et al. 2017). Additionally, there are remarkable studies using non-fish animals that shall be mentioned here. The complex visual display repertoire of the Australian Jacky dragon *Amphibolurus muricatus* inspired researchers to design an animated 3D lizard opponent to be presented during experiments to get further knowledge on the display’s significance during interaction with conspecifics (Peters and

Evans 2003; Van Dyk and Evans 2008; Woo and Rieucau 2015). To investigate avian social perception, Watanabe and Troje (2006) used an animated 3D pigeon *Columba livia* and demonstrated its applicability in an operant conditioning paradigm. Parr et al. (2008) showed that chimpanzees *Pan troglodytes* responded to and discriminated between computer-animated facial expressions of a virtual chimpanzees. Virtual chimpanzees were even able to stimulate contagious yawning in live chimpanzees, indicating an empathic response to their virtual counterparts (Campbell et al. 2009). Neave et al. (2011) studied female preference for different dance moves in 3D animations of human males, and in a recent comparative study, Dolins et al. (2014) showed that humans and chimpanzees were equally able to navigate in a 3D, virtual environment. During experiments, virtual animals can be presented via all kinds of visual devices like tablets or smartphones, but most commonly via computer monitors (CRT or LCD). There are, however, restrictions and limitations concerning stimulus presentation because devices are specially designed for the visual system of humans (Oliveira et al. 2000; Baldauf et al. 2008; Chouinard-Thuly, Gierszewski, et al. 2017). A thorough validation should, therefore, be obligatory when using virtual stimuli (see e.g., Baldauf et al. 2009; Fischer et al. 2014).

Here, we validated the custom-made simulation for animated 3D sailfin mollies *Poecilia latipinna*, designed by Müller et al. (2017), for the use in mate-choice experiments with live sailfin mollies. First, to address common concerns whether to use CRT or LCD monitors for the presentation of visual stimuli, we tested which monitor type (CRT or LCD) was more suitable for stimulus presentation. Second, we tested whether different stimulus presentation types (animation, video, or live fish) were equally effective to attract live fish. Third, we disentangled movement from stimulus shape by presenting a static and/or swimming animated 3D box and 3D fish because movement can influence the attractiveness of virtual stimuli (Baldauf et al. 2009; Abaid et al. 2012; Nakayasu and Watanabe 2014; Woo and Rieucau 2015). And fourth, we investigated whether sailfin molly males were able to distinguish between animated 3D males and 3D females.

5.3 Materials and Methods

5.3.1 Study species

Male and female sailfin mollies used in experiments were mature descendants of three populations of wild mollies. Fish were caught from the Coleto Creek near Victoria (TX, USA) in 1998, from the Comal River in New Braunfels (TX, USA) in 2007 and from Mustang Island near Corpus Christi (TX, USA) in 2014. In the lab, the fish were kept in mixed-sex shoals and separated by populations as described in detail in Chapter 2.3. All experiments were performed under the German Animal Welfare Act (Deutsches Tierschutzgesetz) during 2014 and 2015, and no animals were harmed.

5.3.2 Video fish stimulus design

We recorded a male *P. latipinna* individual in a small tank (25 cm x 40 cm x 40 cm) filled with water, and the same tank without a male using a digital camera (Canon EOS 600D, full HD movie program, 50 fps, Canon Deutschland GmbH, Germany). Tank walls were covered with blue plastic sheets, except for the front, and the ground was covered with blue-colored sand. Illumination was provided by two LED strips (40 cm in length, 12 V,

6500 K) positioned above the longer sides of the tank. Short sequences were cut and combined to a video (Windows Movie Maker, Microsoft, v. 2012).

5.3.3 Animated 3D fish stimulus design

The 3D fish were designed with Blender (v. 2.70a, Blender Foundation, the Netherlands; Fig. 6 A-B) and then animated and presented during experiments using custom-made software (*FishCreator*, *FishSteering*, and *FishPlayer*) developed by Müller et al. (2017), as described in Chapter 4, "The Virtual Fish Project". Using *FishCreator*, different animated 3D fish stimuli and an artificial 3D box were created. To prevent pseudoreplication, as proposed by Rosenthal (2000), *FishCreator* enables generation of randomized models with textures taken from various live fish individuals. Stimulus sizes were adjusted to be within the natural range of this species (Supplementary Table S7, Appendix 3). Measurements were taken from live males (n = 13) resulting in 4.3 ± 0.7 cm (range 3–5.7 cm) for standard length and 5.4 ± 0.9cm (range 3.7–7 cm) for total length. Live females (n = 15) measured 3.9 ± 0.5 cm (range 3.3–5.3 cm) in standard length and 4.9 ± 0.7 cm (range 4.1–6.7 cm) in total length. In all treatments, animated 3D sailfin molly males were colorful with raised large dorsal fins. All animated 3D fish (and the 3D box) were also simulated swimming in a virtual tank when presented on screen. Color of the tank wall and the ground could be adjusted manually and then animated. Wall color was blue (105, 167, 205 RGB) in Treatment 2 and gray–white (240, 243, 218 RGB) in Treatments 3 and 4. The ground of the virtual tank was modulated to resemble the blue sand covering the experimental tank containing the live test fish. In contrast to previously used animation techniques (rotoscoping, key framing), 3D fish could be steered freely in space via gamepad using the application *FishSteering*. Swimming speed varied between 0 and 40 cm/s depending on the input given to the gamepad. Default swimming movements (e.g., undulatory movements, bending) were based on calculations from video analysis of live fish as described by Smielik et al. (2015). In the animation, a sailfin molly was steered to resemble a live fish swimming in a tank and interacting with another fish outside the tank (Gierszewski, personal observation). Behaviors included (I) swimming in varying heights and depths, (II) parallel swimming at the front wall and presentation the lateral side and raised dorsal fin (in males), and (III) swimming up and down in a position vertical to the front. A movie clip showing an exemplar animation of a 3D sailfin molly male can be found in the Supplementary Material Movie 1[13]. Movements were recorded with *FishSteering* and then loaded into *FishPlayer* for presentation during experiments. Additionally, an empty virtual tank was recorded for presentation between trials and during acclimatization periods. Resolution of the animation was optimized for the LCD monitors (1920 x 1200 pixels) and presented with a frame rate of 60 fps, which is well above the estimated threshold for motion perception in fish (Fleishman and Endler 2000; Oliveira et al. 2000).

5.3.4 General experimental procedure

All experiments were performed using the same experimental setup in the same experimental room (see experimental condition described in Chapter 2.3.2). The test tank (100 cm x 50 cm x 40 cm; see Fig. 17A) was divided into three compartments: two choice zones (20 cm in depth) at the outer sides of the tank and a neutral zone (60 cm)

[13] Supplementary Movie 1 available from: https://doi.org/10.1093/cz/zow108

in the middle. The bottom was covered with blue-colored sand and tank walls were covered with blue plastic sheets except for the front and two cut-outs (Treatments 1 and 2: 40 cm x 25 cm, Treatments 3 and 4: 40 cm x 34 cm) on either side providing a view of the presented stimuli. Six LED strips (12 V, 6500 K) were positioned at the longer sides of the tank, two above the rear wall and four above the front wall. Water temperature was 25 ± 1 °C and water level was 25 cm deep (34 cm for Treatments 3 and 4). Depending on the treatment, stimuli were either presented on 24'' LCD monitors (EIZO Foris FX2431, EIZO Nanao AG, Austria, 1920 x 1200 pixels resolution; see Fig. 17A), a 19'' CRT monitor (Samsung SyncMaster 997 MB, Samsung Electronics Display (M) (HSD), Malaysia, 85 Hz, 1280 x 960 pixels resolution; see Fig. 17B), or in small tanks (40 cm x 40 cm x 12 cm; see Fig. 17C). Monitors and tanks were positioned adjacent to the choice zones of the test tank at an approximate distance of 2 cm.

Test females were kept in small shoals separated from males several weeks prior to experiments. The day before testing, they were transferred to a 40 cm x 25 cm x 40 cm tank in the experimental room and kept under corresponding lighting and feeding conditions. These tanks featured blue-colored sand and blue plastic sheets on the walls. In Experiment 4, male test fish were used because they were expected to show a more distinct discrimination between the sexes because their reproductive motivation is not dependent on a reproductive cycle, as seen in females (Greven 2011). Males were not separated prior to experiments but directly taken from their home tank. This was done to prevent stress resulting from rivalry in separated male groups or isolation when kept alone. Males were assigned to a color group ("pale" or "colored"). "Pale" was defined as without or only slight black patterns and no orange patterns visible on the fins and on the body. "Colored" was defined as having distinct black and orange patterns. Assignment was done before males were taken from their home tank as colors may fade rapidly as a result of stress (Kawauchi 2006; Nilsson Sköld et al. 2013; Gierszewski, personal observation). Color is an indicator for social status in male sailfin mollies and dependent on group constellation, with dominant males being more colorful than subordinate male. Color and size are good predictors of mating tactics, with large colorful males mostly relying on courtship and small pale males mostly using a sneaker tactic with forced copulation (Snelson 1985; Fraser et al. 2014).

During the acclimatization period of 10 min, a single test fish could swim freely and explore the test tank for 5 min. Then the fish was positioned inside a Plexiglas cylinder (11 cm in diameter) in the middle of the test tank for 5 min. This procedure should guarantee an equal distance between the test fish and both stimuli, and increase the chance that test fish were aware of both stimuli before making a choice. Throughout this period, a tank (video or animation) containing no fish was shown on both sides so fish could get accustomed to the illumination emitting from the monitors. After acclimatization, both stimuli (depending on the treatment) were shown on opposite sides of the test tank. Test fish remained inside the cylinder for 1 min to watch the presented stimuli. Test fish were then released and given 5 min to choose between stimuli. We measured the time each test fish spent within choice zones with a stopwatch. After the first test trial, an intertrial interval (ITI) of 5 min was included during which test fish were gently put back into the cylinder and sides of the stimuli (and monitor types in Experiment 1) were switched to control for side bias. After the ITI, the procedure was repeated for a second test trial and time spent within the choice zones was recorded for another 5 min. Observations were done via camera (Prosilica GT1910c, Allied Vision Technologies GmbH, Germany) from a position not visible to the fish to prevent them from being stressed or influenced by the observer (Fig. 17A).

Stimuli were always presented simultaneously in a binary choice situation and their position (left or right) was alternated within experiments. For each test fish we measured the absolute association time (in seconds) the test fish spent with each stimulus within each choice zone. Association time is an indirect predictor for mate choice when no physical contact to the stimulus is possible and was used in different studies with this species (Witte and Noltemeier 2002; Witte and Klink 2005; Nöbel and Witte 2013). If test fish spent more than 90 % of the total time (first plus second trial) in the same choice zone, even though stimuli were switched, the choice was stated as side biased and fish were excluded from analysis in accordance to other studies (Schlupp and Ryan 1997; Dosen and Montgomerie 2004; Hoysak and Godin 2007; Williams and Mendelson 2010). Standard length of each test fish and live stimulus fish was measured in centimeters after testing. We noted the standard length for all presented stimuli. For animated 3D fish and video stimuli, total length was also measured. After experiments, all test fish were returned to their home tanks. For each experiment and treatment, we used new, live test fish. We performed the following experiments in the same sequence as presented below.

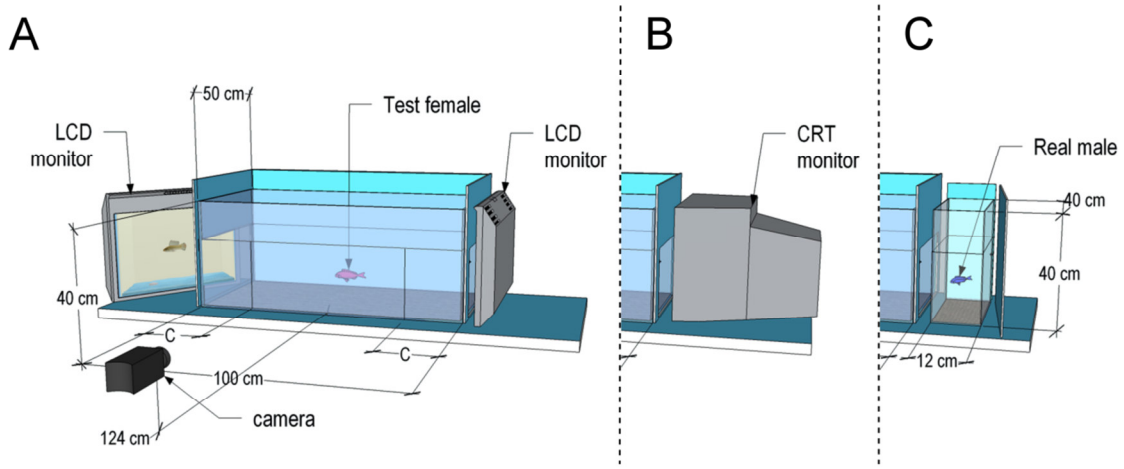

Figure 17. Overview of the experimental setup. (A) Test tank with the live test fish and two LCD monitors observed via camera as in Treatments 2.1, 2.2, and in Experiments 3 and 4. For illustration, the left LCD monitor is angled to show an animated scene. (B) Modified setup as used in Experiment 1. One LCD monitor was replaced by a CRT monitor. (C) Setup modified for the use in Treatment 2.3. Both LCD screens were replaced by small tanks filled with water and containing a live male or no fish. C = choice zone.

5.3.5 Experiment 1: video male on CRT versus video male on LCD monitor

Both monitor types were tested in a binary choice situation. Two identical videos of a male were presented on a LCD screen on one side and on a CRT screen on the other side of the tank (Fig. 17B). With reference to former studies (e.g., Witte and Ueding 2003; Witte and Klink 2005) it is known that sailfin molly females perceive and respond to video playbacks. As screens differed in overall size, the display area of the video was adjusted to be of the same size on both screens (35 cm x 19.8 cm) and resolution was set to 1280 x 960 pixels. For acclimatization and ITI, the video of an empty tank was shown so test fish could get accustomed the light emission from the monitors. White plastic sheets prevented females from viewing when position of monitor types was switched. We tested 18 females.

5.3.6 Experiment 2: comparison of different presentation types

In Experiment 2, we tested whether live test females differ in discrimination between a fish stimulus and a tank containing no fish when presenting these stimuli with different presentation types (animation, video, live). In each treatment, we used identical stimuli in every trial to keep stimuli as constant as possible to ensure comparability between presentation types. The side on which the fish stimulus was shown was alternated and distributed equally between left and right for all treatments.

Treatment 2.1: animated 3D male versus animated tank

In Treatment 2.1, live females were given a choice between a swimming 3D male animation on one side and a 3D animated tank (same tank but without an animated fish) on the other side, both stimuli presented on LCD screens. The tank was also shown on both sides during acclimatization and ITI. We tested 18 females.

Treatment 2.2: video male versus video tank

In Treatment 2.2, we presented live test females a video of a swimming male as one stimulus and a video of a tank as the alternative stimulus. Both stimuli were presented on LCD screens. During acclimatization and ITI, the tank was shown on both sides. We tested 18 females.

Treatment 2.3: live male versus real tank

In Treatment 2.3, live females could choose between a live male (presented in a real tank) and a real tank (filled with water but without a fish) as the alternative stimulus. During acclimatization and ITI, white plastic sheets prevented females from viewing the adjacent tanks. We used the same live male individual for the whole treatment for comparability between treatments with different stimulus presentation types. The live male was chosen to resemble the animated 3D male (Treatment 2.1) and the video male (Treatment 2.2). We tested 25 females.

5.3.7 Experiment 3: decoupling movement and shape of a stimulus

Here we tested whether live females distinguish between an animated 3D male and a 3D box (Fig. 18A) that were either static or swimming. By decoupling movement and shape of the stimuli we tested how these parameters affect association time.

Treatment 3.1: moving 3D male animation versus static 3D box animation

In this treatment, we presented live females an animation of a swimming 3D male and a static 3D box on LCD screens. The box represented dimensions (length, height, width) of the animated fish and was colored in the mean RGB value of the fish texture (207, 197, 149 RGB; see Fig. 18A). The animated male was moving around the animated tank, the box was positioned in the center of the animated tank, not moving. The dorsal fin of the

male was raised all the time to keep its lateral projection area constant for the duration of the experiment. We tested 23 females.

Treatment 3.2: static 3D male animation versus moving 3D box animation

In Treatment 3.2, we presented live females identical animations as used in Treatment 3.1, but now the male was static in the center of the animated tank and the box was "swimming" the identical path as the animated male did in Treatment 3.1. Lateral projection area of the fish was kept constant. We tested 22 females.

5.3.8 Experiment 4: animated 3D male versus animated 3D female

In Experiment 4, each live test male (pale and colored males) could choose between animations of a 3D male and a 3D female. Both animated fish were size matched and swam an identical path. *FishCreator* was used to generate three different sailfin mollies of each sex, so a total of nine combinations of male and female animations could be presented (see Fig. 18B and Supplementary Material Movie 2[14]). We tested 24 males.

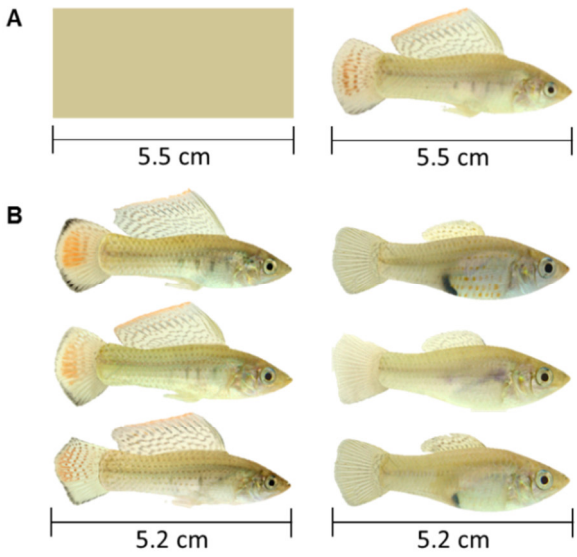

Figure 18. Overview of animated 3D fish stimuli used in Experiments 3 and 4. (A) Animated 3D box and animated 3D male used in Experiment 3. (B) Different animated 3D male and 3D female sailfin mollies presented in pairs of varying combinations in Experiment 4.

5.3.9 Data analysis

For data analysis, we used R 3.2.2 (R Development Core Team 2015). To test for differences between association times within Experiments 1 and 4, we used paired Wilcoxon signed-rank tests. To analyze association time in Experiments 2 and 3, we used linear mixed effect (LME) models with the *lme* function in the nlme package (Pinheiro et al. 2015) with association time as the outcome variable.

[14] Supplementary Movie 2 available from: https://doi.org/10.1093/cz/zow108

For Experiment 2, presentation type (animation, video, live), stimulus type (fish or tank), and size of the test females (SL) were fixed factors. Presentation type (PT) was equal to treatment, so treatment was not included as an additional factor. Following Crawley (2007) we used the function *contrasts* to define two orthogonal contrasts for PT: (PT1) live versus any virtual stimulus (sum of video plus animation), and (PT2) animation versus video. Identity of test fish (ID) was included as random factor. ID was nested in population. A plot of the standardized residuals against the fitted values revealed inhomogeneity of the residual variances. To account for this heteroskedasticity in the model, a weights function using the varIdent class of the *lme* function was included to allow different variances for each level of stimulus type and presentation type (Pinheiro and Bates 2000; Zuur et al. 2009). For Experiment 3, stimulus shape (fish or box), movement (moving or static), treatment, and test female size (SL) were set as fixed factors. Female identity (ID) was included as random factor and nested in population. We inspected model assumptions (Q/Q plots, normality of residuals, residuals against fitted values) visually. Given P values were considered significant if $p \leq 0.05$.

5.4 Results

Association times measured for each experiment, total number of test fish and those showing side biases as well as all size measurements of test fish and used stimuli can be found in the Supplementary Material[15] (Table S7, Appendix 3).

5.4.1 Experiment 1: video male on CRT versus video male on LCD monitor

Females (n = 16) spent significantly more time in front of the video male presented on the LCD screen than in front of the video male presented on the CRT screen (Wilcoxon signed-rank test: V = 26, p = 0.029; see Fig. 19). Thus, we used only LCD monitors in the following experiments.

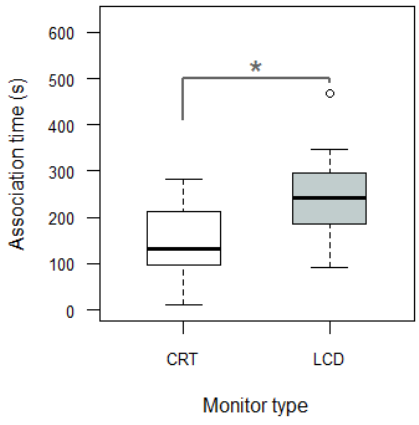

Figure 19. Results of Experiment 1: test of monitor type. Association times (s) for the video presentation of a swimming male on LCD and CRT screen are given. Boxplots of median, quartiles, and whiskers (1.5 interquartile range) are shown. Circles indicate outliers. N = 16; *p ≤ 0.05.

[15] Supplementary material available from: https://doi.org/10.1093/cz/zow108

5.4.2 Experiment 2: comparison of different stimulus presentation types

Results showed that association time was significantly affected by the stimulus type "fish" (LME: t = 11.500, p < 0.001), raising it on average by 258.4 ± 22.5 sec (see Estimate in Table 2) when compared with the empty tank. Presentation type (animation, video, live) and size of the test females did not affect association time (Table 2, Fig. 20A).

Table 2. LME estimates for effects on association time in Experiments 2 and 3. Absolute association time was the outcome variable throughout. Given are estimates with standard error, degrees of freedom, t-values and p-values for each fixed effect. Intercept estimates show the grand mean for each experiment. Intercept reference categories for factor estimates are 'live' (PT1), 'animation' (PT2), type 'tank' for Experiment 2, and treatment '3a', shape 'box' and movement 'static' for Experiment 3. Significant values ($p \leq 0.05$) are printed in bold. PT = presentation type; SL = standard length of test female.

Fixed effects	Estimate	Standard error	*df*	*t*	*p*
Experiment 2					
(Intercept)	20.708	57.554	55	0.360	0.720
PT1	13.697	11.883	55	1.153	0.254
PT2	0.677	11.009	55	0.062	0.951
type 'fish'	258.393	22.477	55	11.500	**<0.001**
SL	22.276	14.170	55	1.572	0.122
Experiment 3					
(Intercept)	124.516	50.157	30	2.483	**0.019**
Treatment '3.2'.	-12.223	30.290	28	-0.404	0.690
shape 'fish'	100.935	28.374	30	3.557	**0.001**
movement 'moving'	69.465	28.374	30	2.448	**0.020**
SL	0.567	3.947	28	0.144	0.887

5.4.3 Experiment 3: decoupling movement and shape of a stimulus

Association time was affected by movement (LME: t = 2.448, p = 0.02), raising it on average by about 69.5 ± 28.4 sec (see Estimate in Table 2) when the stimulus (fish or box) was moving. Stimulus shape also affected association time (LME: t = 3.557, p = 0.001), raising it by about 100.9 ± 28.4 sec (see Estimate in Table 2) when the animated stimulus was a fish (Fig. 20B).

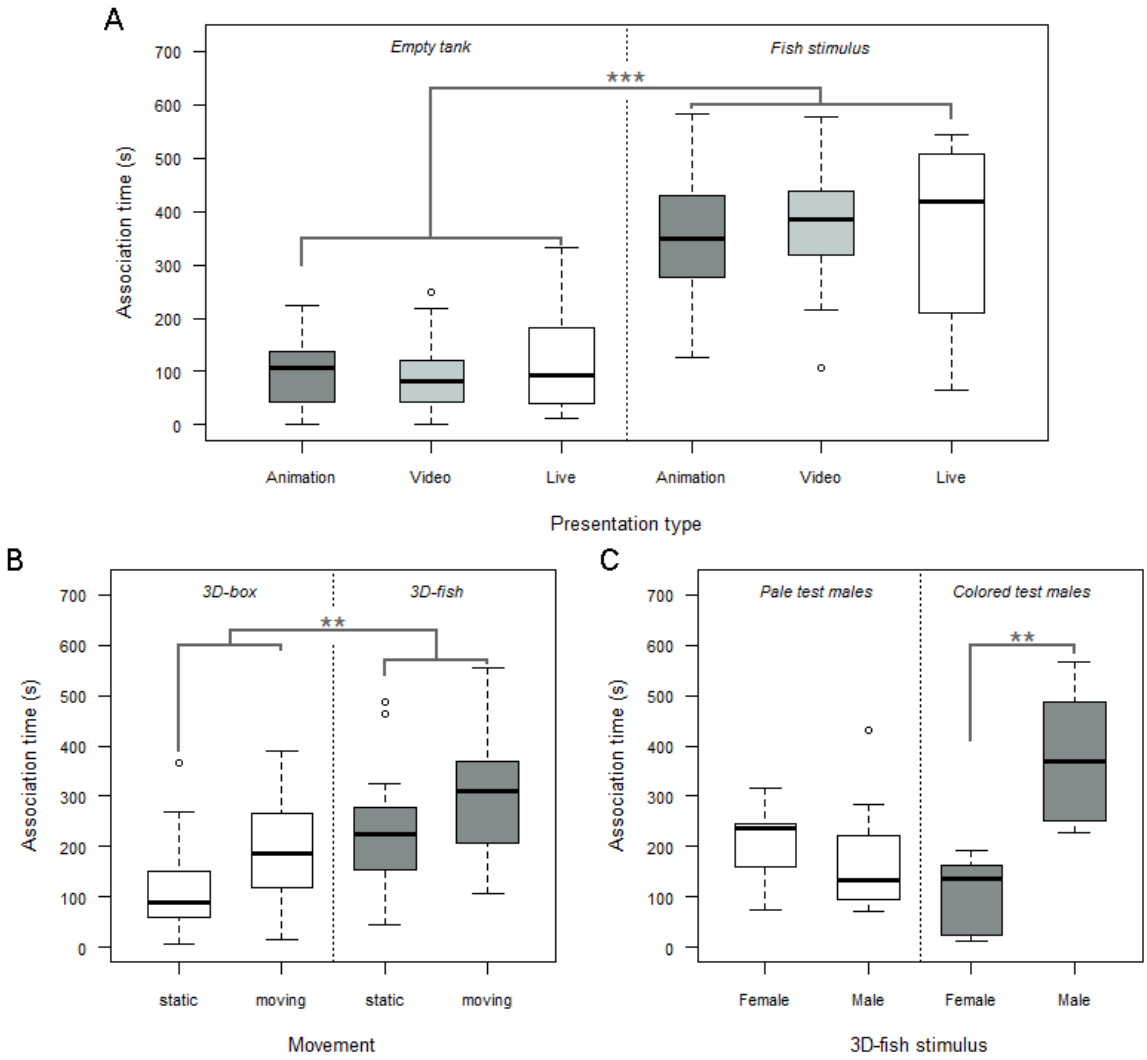

Figure 20. Results of Experiments 2, 3, and 4. (A) Association time obtained in Experiment 2 testing for stimulus presentation type (animation, video, live) when a tank or a fish was presented as either 3D animation, video, or live stimulus. $N_{animation}$ = 15, n_{video} = 16, n_{live} = 17. (B) Association time obtained in Experiment 3 for stimulus shape when static or moving. $N_{box/static}$ = 15, $n_{box/moving}$ = 17, $n_{fish/static}$ = 17, $n_{fish/moving}$ = 15. (C) Association time for the animated 3D male and 3D female stimuli in Experiment 4 for pale and colored live test males, n_{pale} = 7, $n_{colored}$ = 8. Boxplots showing median, quartiles, whiskers (1.5 interquartile range), and outliers (circles). **P < 0.01, ***p < 0.001.

5.4.4 Experiment 4: animated 3D male versus animated 3D female

Pale test males (n_{pale} = 7) showed no significant preference for either male or female 3D animation (Wilcoxon signed-rank test: n_{pale} = 7, V = 12, P = 0.813). Colored males ($n_{colored}$ = 8), however, spent significantly more time with animated male stimuli than with animated female stimuli (Wilcoxon signed-rank test: $n_{colored}$ = 8, V = 36, P = 0.008; see Fig. 20C). Thus, males could discriminate between animated 3D male and 3D female stimuli. Supplementary Material Movie 2[16] illustrates an exemplar response of a colored live male toward a 3D female animation, including following, displaying and gonopodial thrusting (at minute 00:10).

[16] Supplementary Movie 2 available from: https://doi.org/10.1093/cz/zow108

5.5 Discussion

Our results showed that animated 3D sailfin mollies can be a useful tool in mate-choice studies with live sailfin mollies. The response of live females to an animated 3D stimulus was as strong as to a video male or even a live male. Movement alone was important, but females responded stronger to a swimming fish stimulus than to a box “swimming” the same path. Colored test males were able to discriminate between animated 3D male and 3D female fish, hence, validating the 3D fish as biologically relevant stimuli in choice experiments. Additionally, test females spent significantly more time in front of a male video when presented on a LCD screen than on a CRT screen.

Live test females reacted attentively toward a 3D male animation when presented together with an animated tank as an alternative stimulus and spent significantly more time in front of the male. This experimental design also served as a control for the usage of animated stimuli in previous studies with fish, showing that a fish animation was preferred over an empty scene (Künzler and Bakker 1998; Clark and Stephenson 1999; Morris et al. 2003; Kuperberg et al. 2009; Culumber and Rosenthal 2013). In comparison to different stimulus presentation types (animation, video, live), that are commonly used in behavioral experiments, test fish significantly preferred the presented fish stimulus over a tank as an alternative stimulus, irrespective of the used presentation type. Stimulus presentation in all presentation types led to a similar response in sailfin mollies, thus, our animated male seemed to be as attractive as a live male, or a video male for sailfin molly females. Our results are in accordance with the results of Qin et al. (2014) in which zebrafish *Danio rerio* did not differentiate between live, video, and animated fish stimuli. Clark and Stephenson (1999) also found no difference in shoaling tendency toward animated, video, or live conspecifics in the tiger barb *Puntius tetrazona*.

By decoupling movement and shape of an animated stimulus, we showed that movement significantly increased attractiveness of a given stimulus (both box and fish), but that a swimming fish was more attractive to females than a swimming box. The shape of a moving stimulus matters. In terms of the freely steerable nature of our animated fish, this result underlines the usability of our new approach and presents a more flexible alternative to classic rotoscoping or key framing animation techniques. *FishPlayer* even allows the reuse of once created swimming paths with various fish models to gain consistency between experiments. Here, one should keep in mind, that freely steered stimuli might induce an individual experimenter effect. Therefore, we are currently developing an automatic swimming mode. Movement as a critical feature to evoke a response in live fish could also be shown in cichlids. Baldauf et al. (2009) showed that both male and female cichlids *Pelvicachromis taeniatus* preferred a moving 2D animation of the opposite sex over a stationary one. Sometimes the manner in which a stimulus is moving seems to be even more important than its appearance. Woo and Rieucau (2015) discovered the importance of syntax for recognition of visual displays in the jacky dragon *Amphibolurus muricatus*. They showed that jacky dragons paid same attention toward displays of animated 3D jacky dragons independent of whether animations were highly realistic or abnormal as long as the display’s syntax was correct. A study by Abaid et al. (2012) highlighted the importance of moving speed and coordination of animated 2D zebrafish shoals for a shoaling preference in live test fish when compared to a static image.

Our results indicate that live test males were able to distinguish between animated 3D males and 3D females. Despite the assumption that test males would generally spend more time with the female animation, pale test males showed no preference for either

stimulus. Colored males, however, spent significantly more time with the animated male. Animated males were large and colorful with large dorsal fins raised all the time, which might have elicited stronger agonistic responses in colorful live test males. In the given test situation, we assumed that colorful test males recognized the animated male as a rival of similar or lower quality and, hence, tried to chase him away, thus, spending more time in the choice zone in front of him. Colorful males are more dominant in their home tanks and mostly rely on courtship to attract females, but also spend lots of time chasing rival males to secure their own paternity. Pale males tend to stay close to females for copulation, but also close to dominant males as these constantly court fecund females. Here, pale males get their opportunities for sneak copulation. This might explain why pale males showed no distinct preference for either stimulus, nevertheless still discriminating between the two stimuli. It might be that pale and colorful males used different parameters to make their decision (e.g., quality of competitor compared to self). Discrimination between sexes of animated fish served as a control in other studies as well (Turnell et al. 2003; Baldauf et al. 2009).

5.5.1 Conclusion and future directions

The major advantages of 3D computer animations in behavior research are: (I) the creation of highly variable virtual animals which decreases pseudoreplication when compared to video playback. Stimuli are designed according to well-defined parameters providing high variability of morphology and appearance when compared to live test animals or videos. Parameters like shape, size, color, and behavior, can even be varied beyond natural extents. Moreover, 3D computer animations allow for specific manipulation and control of behavioral patterns, which is more difficult with 2D animation or video, and almost impossible with live animals. The 3D animations can be moved within the 3D environment as live fish do. (II) Computer-animated animals allow a high degree of standardization in test situations, and, thus, provide highly controlled, fast, and repeatable testing procedures. And (III) they allow reduction in the number of live test animals, which is in line with the three guiding principles (3R's) of replacement, reduction, and refinement (Richmond 2010) proclaimed in the guidelines for the treatment of animals in behavioral research and teaching (ASAB 2014).

Regarding the advantages of this promising method, one has to keep in mind that a thorough validation is obligatory before using animations in tests with live animals because its usability might be species specific. Prior to experiments, it should be investigated whether "behavior" of animated animals can elicit similar responses like live stimuli to test animals. Our presented results validated the usage of animated 3D fish as a powerful tool in mate-choice tests in sailfin mollies. The next step will be to implement a 3D fish animation that can interact with a live fish in real time to further study and discover underlying mechanisms in mate-choice decisions (Müller, Gierszewski, et al. 2016). This interactive approach will open new horizons for studying fish behavior.

5.6 Acknowledgements

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Chapter 6

Does body size of the model female affect mate-choice copying in sailfin molly females? A comparative study using live and virtual fish

Stefanie GIERSZEWSKI[a], Shumail B. AHMAD[a,b], Janine GÜRKE[a], Klaus MÜLLER[c], Jan-Marco HÜTWOHL[c], Klaus-Dieter KUHNERT[c] & Klaudia WITTE[a]

[a]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein-Straße 2, Siegen, 57076, Germany, [b]University of Manchester, Oxford Rd, Manchester, M13 9PL, UK, [c]Institute of Real-Time Learning Systems, Department of Electrical Engineering & Computer Science, University of Siegen, Hölderlinstraße 3, Siegen, 57076, Germany

This chapter is a manuscript draft intended for publication.

6.1 Abstract

Theoretical models on social learning suggest that individuals should adopt different rules that define from whom they should learn and under which circumstances. Mate-choice copying, which is a form of social learning, describes an alternative mate-choice strategy that relies on observing the mate choice of others. The observer gains information about the choice (or rejection) of a mate by a conspecific or heterospecific (the 'model') and copies his or her choice (or rejection) by mating with the same mate (or the same phenotype). Based on evidence related to age-dependent effects on mate-choice copying found in guppies (*Poecilia reticulata*), one social learning strategy postulates to "*copy older individuals*". In this study, we tested whether this strategy also applies for mate-choice copying in sailfin mollies (*Poecilia latipinna*). For this, we performed copying experiments using a comparative approach with (I) live fish stimuli, and (II) computer animated (i.e., virtual) fish stimuli created with *FishSim* Animation Toolchain. In two experiments, we tested live focal females within three different treatments each. We either presented a prior non-preferred male stimulus (live or virtual) with (I) a model female larger than the focal female (live or virtual); (II) a model female which was always smaller than the focal female (live or virtual); or (III) no model female. We assumed that larger females were older and smaller females were younger. Focal females copied the choice of both larger and smaller model females and there was no difference in copying behavior whether we used live or virtual fish stimuli. When there was no live model female present, focal females were consistent in their mate choice for a male. However, this was not the case in the experiment using virtual fish. We conclude that a strategy such as "*copy older individuals*" does not apply to sailfin molly mate-choice copying, at least not in our experimental setup. We suggest that the strategy of mate-choice copying is highly flexible across different animals and rather relies on the interplay of social and environmental parameters than on single universal rules.

6.2 Introduction

During decision making, e.g., which habitat, food patch or mating partner to choose, animals may benefit from using public information gathered by observing others (Danchin et al. 2004). Theoretical models of social learning presume that certain rules have evolved which specify when an individual will learn or copy from others, e.g., if the choice is difficult to make ("when-strategies"), and from whom it will copy ("who-strategies"; Laland 2004). Mate-choice copying (short: MCC) is a form of social learning that is defined as non-independent mate choice, in which an individual's probability of choosing a given mate increases if other individuals have chosen that mate previously (Pruett-Jones 1992). The study of MCC focuses on detecting differences in quality and quantity of public information that affect an observer's copying behavior (Witte and Nöbel 2011; Witte et al. 2015). When deciding whom to copy, individuals should evaluate the costs and benefits associated with their own choice and that of copying the choice of the observed individual, the model. It was assumed that the identity and specific characteristics of both the observer and the model critically affect the probability of public information use (Coussi-Korbel and Fragaszy 1995).

Previous studies on MCC have indeed identified the particularly important role of the model female for observing focal females and depicted certain characteristics, presumably associated with model female quality (reviewed by Witte et al. 2015). Dugatkin and Godin (1993) first demonstrated age-dependent effects related to MCC in guppies (*Poecilia reticulata*). Based on prior studies on fish growth (Ricker 1979), Dugatkin and Godin (1993) correlated age with body size, that is, younger females being smaller and older females being larger in body size. Larger and thus older females are presumably more experienced in mate choice and are, therefore, good models to copy from. They found that smaller and, hence, younger focal females copied the choice of larger and older model females but not vice versa. With regard to earlier studies (Dugatkin and Godin 1993; Kirkpatrick and Dugatkin 1994), Laland (2004) defined one who-strategy as "*copy older individuals*". The relevance of this strategy was later supported by Amlacher and Dugatkin (2005) and Vukomanovic and Rodd (2007) who performed additional experiments investigating age-related effects on MCC in guppies. A model female's age (as defined by her body size) was discussed to serve as a sign of model female quality with regard to experience. Experience in mate choice can be considered key when deciding which mate to choose, with older/larger models being presumably more experienced compared to younger and smaller models (Dugatkin and Godin 1993; Vukomanovic and Rodd 2007). One may further argue that a model's body size provides additional information to observing females aside from their experience in mate choice. For example offspring size, as well as many other life-history traits in poeciliids, is typically related to female body size (Reznik and Miles 1989; Johnson and Bagley 2011). Body size in relation to specific environmental factors and constraints may further characterize a model female's success (Johnson and Bagley 2011). For example regarding her ability to avoid predators or acquire good food resources, which may as well refer to the strategy "*copy successful individuals*" (Laland 2004). In the context of collective movement and leadership, female guppies were shown to follow biomimetic robots resembling larger females more closely compared to those resembling smaller female guppies (Bierbach, Mönck, et al. 2018).

Aside from model female size, additional aspects contributing to a model female's quality were found to affect MCC. Hill and Ryan (2006) showed that focal female sailfin mollies (*Poecilia latipinna*) copied the choice of conspecific (high-quality) model females but not that of heterospecific (low-quality) Amazon mollies (*Poecilia formosa*), despite them

living in sympatry and frequently mating with sailfin molly males. Similarly, female zebra finches (*Taeniopygia guttata castanotis*) payed attention to the phenotype of the model female and only copied the choice of wild type model females compared to models of a novel phenotype (Kniel et al. 2017). In humans, it was shown that women attend to the phenotype of the model resulting in a man being more attractive to observing women when paired with an attractive (Waynforth 2007) or more popular (Little et al. 2015) model partner. Here, a lack of mate-choice experience (defined as lifetime number of sex partners) was identified as an important driver for copying (Waynforth 2007) and younger women were more influenced by the choice of a popular model than older ones (Little et al. 2015).

In the current study, we aimed to test whether model female size affects MCC in female sailfin mollies, which are closely related to guppies. So far, the effect of age and/or size of the model female have never been tested in other poeciliids, despite guppies, or even other animals. Therefore, the question remains whether the strategy "*copy older individuals*" (Laland 2004) describes a universal rule in decision making related to MCC. If so, this relationship should be present in other animals as well. Female sailfin mollies copy the choice of conspecific females (see Chapter 2.2 in this thesis for more information) but whether their decision to copy may indeed be age or size-dependent is not known. In accordance with the documented findings in guppies, we assume that focal females will copy the choice of larger model females (i.e., model females larger than focal females), but not that of smaller model females (i.e., model females smaller than focal females). Further, we use this study to implement a new and innovative experimental approach for studying of MCC in sailfin mollies: the use of computer animation. We predict that our findings on MCC will not differ between the two methods (live stimulus fish versus virtual stimulus fish). When there is no model female present (i.e., no public information available), focal females will be consistent in their mate choice for a male.

6.3 Materials and Methods

6.3.1 Study species

In all experiments described in this chapter, we used mature focal female sailfin mollies from the Mustang Island population (TX, USA). A detailed description of the study species and its holding conditions can be found in Chapter 2. Prior to experiments, we kept focal females separated from males in small groups for several weeks to increase their choosing motivation. Live males, as used in Experiment 1, were taken directly from their home tank. All fish were returned to their home tanks after testing.

6.3.2 Experimental setups

All experiments were performed in the fish housing room using experimental setups E1 and E2 as described in Chapter 2.3.1. In this study, we used two different experimental setups: a classic setup for the use of live stimulus fish (Experiment 1) and a modified setup for the use of virtual stimulus fish presented via computer monitor screens (Experiment 2). Experiment 1 was performed in 2015 and Experiment 2 in 2017.

In Experiment 1, we used a large experimental tank (100 cm x 50 cm x 40 cm). On its shorter sides, two smaller stimulus tanks each (20 cm x 25 cm x 40 cm) were placed leaving a small space of approximately 5 mm between the glass walls to insert opaque plastic partitions (Fig. 21A). All tanks were filled with tap water, as used for fish housing, and had a thin layer of gravel. The hind wall of the large tank as well as the hind walls and outer sides of the smaller tanks were covered with blue plastic sheets to prevent the fish from being disturbed by their surroundings. Illumination was provided by two fluorescent tubes (Philips TL-D 90 De Luxe, 58 W) positioned approximately 138 cm above the middle of the test tank. We marked two mate-choice zones of 20 cm on the front wall of the large tank. Inside of the large tank, we used small sticks made of glass to mark choice zones in front of two orthogonal smaller tanks, resulting in two choice areas of 20 cm x 25 cm at each side of the tank (see also Figs. 21A and 22). Behavioral observations of focal fish were performed by a single experimenter, placed on a chair in front of the large tank at a distance of 100 cm.

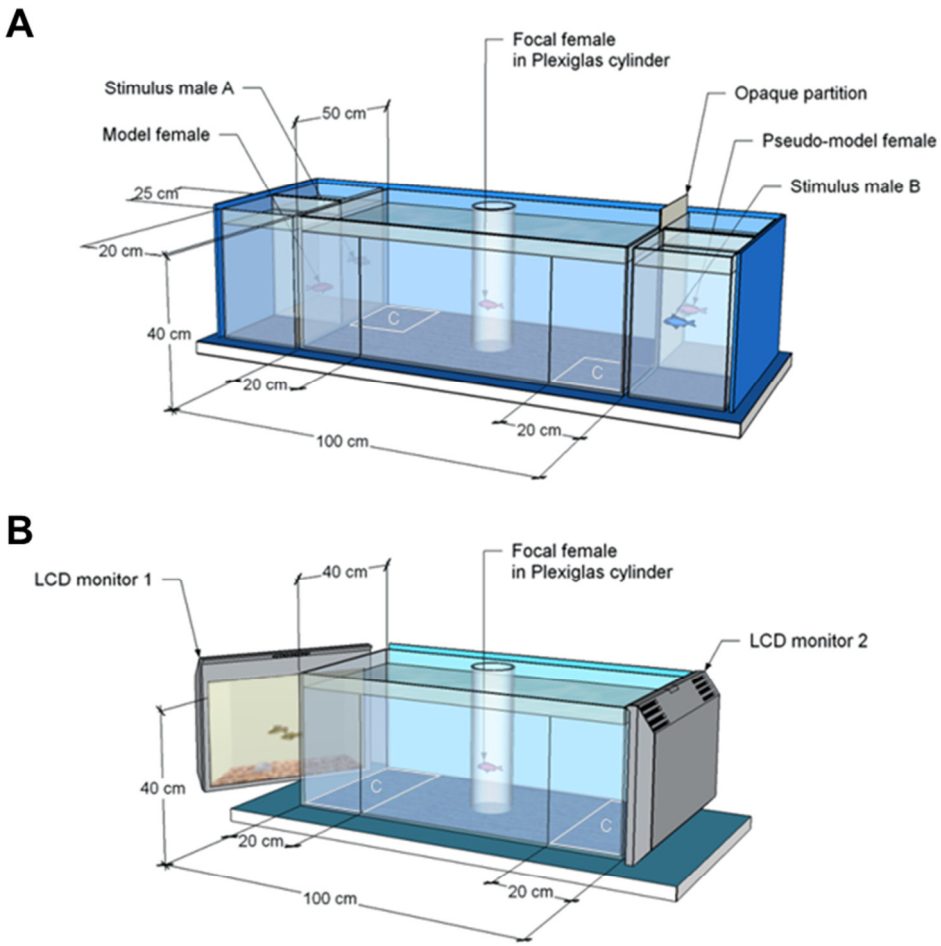

Figure 21. Overview of the experimental setups (situation of the observation period). (A) Classic setup for the study of MCC using live stimulus fish in Experiment 1. Test tank showing the live focal female inside a Plexiglas cylinder during the observation period. On either side of the test tank, adjacent to each mate-choice zone (C), two smaller tanks contain live stimulus fish. The focal female's view of the pseudo-model female is occluded by an opaque partition. **(B)** Modified setup for the study of MCC using virtual stimulus fish in Experiment 2. The smaller tanks are replaced by two LCD monitors that are positioned on either side of the test tank. Only one live fish is used, the focal female. For illustration, the left LCD monitor is angled to show an animated scene of a virtual male and together with the virtual model female.

In Experiment 2, we used a slightly smaller experimental tank (100 cm x 40 cm x 40 cm) to ensure that two 19" LCD monitors (1280 x 1024 pixels resolution; Belinea GmbH, Germany) covered the whole area of the shorter sides of the tank (Fig. 21B). These two monitors were used to replace the small stimulus tanks on either side of the test tank (see Experiment 1) and to present computer animations to focal females. Monitors were positioned at a distance of approximately 2 cm from the glass wall. We marked a mate-choice zone (20 cm) in front of each monitor as described above. Inside the test tank, zones covered the whole length of the tank's shorter glass walls (compared to almost half the size in Experiment 1) resulting in a choice area of 20 cm x 40 cm on either side (see Figs. 21B and 22). Again we used glass sticks on the gravel for additional markings of the choice zones inside the tank. Illumination as well as the general treatment of the experimental tank was identical to that of Experiment 1. To decrease disturbance of focal fish by their surroundings, the experimental tank as well as the control terminal of the experimenter were shielded from the rest of the room with a beige colored curtain.

6.3.3 General experimental procedure with live stimulus fish (Experiment 1)

The general experimental procedure was adopted from a classic study by Schlupp et al. (1994) and has previously been used in other studies on MCC in sailfin mollies as well (Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003). Prior to experiments, three live females were chosen according to predefined criteria as mentioned for the respective experimental treatment (see below). Additionally, two live stimulus males were chosen, being similar in size and overall appearance (color and fin size). The standard length of all fish was measured prior to testing using scale paper. Stimulus males were unknown to focal females and always presented in a binary choice situation. Each experimental trial started with an acclimatization period of 20 minutes in which the focal female was introduced to the test tank and allowed to swim freely and explore the tank. Two stimulus males were in one of the smaller tanks each, on opposite sides of the large tank placed in diagonally position from each other. Two opaque partitions were inserted between the test tank and the smaller stimulus tanks to prevent the fish from seeing each other (Fig. 22.1).

Following acclimatization, focal females were gently caught within a clear Plexiglas cylinder (11 cm diameter) and positioned in the middle of the test tank to ensure an equal distance to each stimulus male. We removed the partitions and allowed the focal female to observe both stimulus males from within the cylinder for 10 minutes (Fig. 22.2). The first mate-choice test (M1) commenced, in which we released the focal female and she was allowed to swim freely and choose to spend time with the males for 10 minutes (Fig. 22.3). We measured the time the focal female spent within each mate-choice zone (association time in seconds) using two stopwatches. Association time is an indirect predictor for mate choice when no physical contact is possible and was previously used as a good predictor for sexual preference in studies with fish (Bischoff et al. 1985; Forsgren 1992; Berglund 1993; Kodric-Brown 1993; Witte and Noltemeier 2002; Witte and Ueding 2003; Nöbel and Witte 2013; Gierszewski, Keil, et al. 2018; Gierszewski, Baker, et al. 2018). After 10 minutes, we inserted both partitions to block the view of the stimulus males and put the focal female back into the cylinder in the middle of the tank. To control for a possible side bias in focal females, we then switched the position of both male stimuli. We gently caught them with a net and transferred them to the other small tank with the help of a small plastic container filled with the same water as used for the experimental tanks. We again removed both partitions and the focal female was allowed

to observe both males and their new positions for 5 minutes. The focal female was then released from the cylinder and allowed to choose between both males for another 10 minutes. After M1, we returned the focal female to the cylinder and calculated which male was preferred by the focal female, i.e., the male with whom she had spent >50 % of the total time within both choice zones. We considered a focal female to be side biased if she had spent >90 % of the total time (both choice zones combined) in either only one zone (Experiment 1) or on only one side of the test tank (Experiment 2), even if male positions had been switched. If a female had a side bias, we terminated the trial and she was excluded from analysis.

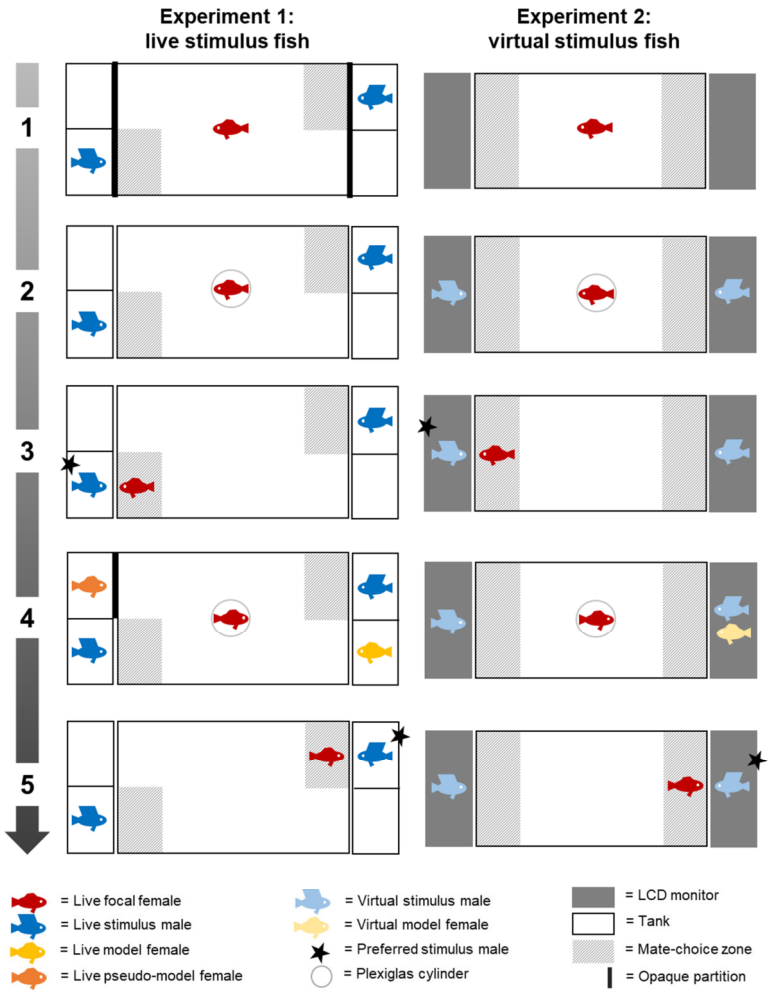

Figure 22. Comparing the experimental procedure of the MCC experiment using live stimulus fish (left) and using virtual stimulus fish created with *FishSim* (right). The figure describes the presence and position of fish used during an exemplar trial of the MCC experiment, depending on the respective experimental stage: (1) acclimatization period, (2) focal female watches stimulus males, (3) first mate-choice test (M1), (4) observation period, and (5) second mate-choice test (M2). The position of the preferred male is shown exemplarily for each treatment.

After calculating which male was preferred by the focal female, the observation period started. For this, we introduced a live model female into the empty small stimulus tank next to the prior non-preferred male. This model female served as a source of additional public information for that male to the observing focal female. We also introduced a live female into the small tank next to the prior preferred male. This so-called pseudo-model,

however, was not visible to the focal female but hidden by an additional partition (Fig. 21A and Fig. 22.4). We did this to ensure that both males were handled equally, concerning potential behavior expressed in reaction the presence of an additional female in close proximity. We removed partitions covering both males and the focal female was allowed to observe this new situation: the prior non-preferred male together with the model female, the prior preferred male apparently alone. After 10 minutes, we returned both partitions and removed the model female and pseudo-model female. We again removed the partitions and released the focal female from the cylinder to let her swim freely and spend time with both males in a second mate-choice test (M2; Fig. 22.5). The procedure of M2 was identical to that of M1 and male position was again switched after 10 minutes. The focal female was allowed to observe the new male position for 5 minutes from inside the cylinder and was afterwards given 10 minutes to spend time with both males. After M2, we terminated the experimental trial and calculated the time spent with both males and which male was preferred by the focal female. We returned all fish to their home tanks. Females were only used once as the focal female but were also reused once as the model female. Their role as focal female was always first.

6.3.4 Adopting the experimental procedure for the use with virtual fish stimuli (Experiment 2)

In Experiment 2, we performed the same MCC experiment but using computer-animated (i.e., virtual) fish stimuli instead of live fish. For this, we used the research tool *FishSim* Animation Toolchain (short: *FishSim*), developed by Müller et al. (2017) and validated by Gierszewski et al. (2017; see Chapter 5) for the use in mate-choice experiments with sailfin mollies.

We modified the experimental procedure for the use with *FishSim*, as described in detail by Gierszewski, Baker, et al. (2018) and varied the duration of some experimental stages as summarized in Table 3. The general succession of experimental stages, however, remained the same (Fig. 22 and Table 3). With regard to former studies on mate choice in poeciliids using computer animation (Nicoletto and Kodric-Brown 1999; Rosenthal et al. 2002; Kingston et al. 2003; Fisher et al. 2006; Fisher and Rosenthal 2007; Butkowski et al. 2011; Verzijden and Rosenthal 2011; Culumber and Rosenthal 2013; Gierszewski et al. 2017), we reduced the choosing time during mate-choice tests to 2 x 5 minutes (prior and after male position was switched) instead of 2 x 10 minutes as used in Experiment 1 (Table 3). This was done to decrease the possibility of focal females realizing that virtual fish were not real. However, we kept the duration of the observation period constant, since both Dugatkin (1998) and Witte and Noltemeier (2002) found variation in observation time significantly affecting public information use in both guppies and sailfin mollies.

We used the tool *FishPlayer* (Chapter 4.4) to present prior created animated sequences to live focal females. Sequences were organized as playlists that could be replayed automatically and simultaneously for each monitor separately during experiments (Table 3). Since *FishPlayer* continuously runs a playlist from top to bottom once started, we included a “pause”-sequence with a specific duration of 1:30 minutes whenever it was necessary to handle the focal female (e.g., while catching her with the cylinder; Table 3). During a pause, as well as during acclimatization, only an empty virtual tank was displayed on both monitors, with no virtual fish present (Table 3).

Prior to testing, we prepared animation playlists for M1 for each monitor separately as given in Table 3. We placed a focal female inside the test tank and started the presentation in *FishPlayer*, commencing the acclimatization of 20 minutes (Fig. 22.1 and Table 3). Before the timer in *FishPlayer* reached the end of acclimatization, the experimenter slowly walked to the experimental tank and positioned the focal female inside the cylinder in the middle of the tank. Animated sequences for the first part of M1 automatically started showing both virtual males on either side. Focal females were allowed to watch both males (Fig. 22.2 and Table 3). A few seconds before the timer in *FishPlayer* reached 2:30 min, focal females were gently released from the cylinder and allowed to swim freely and spend time with both males for 5 minutes (Fig. 22.3 and Table 3).

We measured association time with two stopwatches as done in Experiment 1. The second part of M1 was performed identical to the first part with male position switched automatically. Afterwards, we again positioned the focal female in the cylinder in the middle of the tank and calculated which virtual male was preferred during M1. We rearranged the playlist entries in *FishPlayer* accordingly. During the subsequent observation period, we presented an animated scene in which the prior non-preferred virtual male was shown swimming together with a virtual model female for 10 minutes (Figs. 21 and 22.4 and Table 3). Then, the first part of M2 started and we released the focal female from the cylinder to spend time with both virtual males for 5 minutes (Fig. 22.5 and Table 3). The experimental procedure for M2 was identical to that of M1 with male positions switched again in the second part (Table 3). After M2, we terminated the experimental trial and calculated which male was preferred by the focal female as done in Experiment 1. We returned focal females to their home tanks. Focal females were only used once.

6.3.5 Treatments within Experiment 1 and Experiment 2

To investigate whether model female size affects MCC in observing focal female sailfin mollies, we performed two different treatments. Here, we either presented model females which were larger or smaller than focal females during the observation period. Further, we performed a control for choice consistency in which no model female was visible to focal females to prevent public information use. Overall, the general procedure was identical for both treatments and the control in Experiments 1 and 2.

We classified whether focal females had copied the choice of a model female according to the definition by Pruett-Jones (1992), in which MCC is defined as a significant increase in time spent of a focal female with a previously non-preferred male after observing him together with a model female. In theory, this increase will consequently also increase the chance of mating with that same male.

Table 3. Overview of the different experimental stages in Experiments 1 and 2. Comparison of each stage of the MCC experiment using live fish stimuli (Experiment 1) and virtual fish stimuli (Experiment 2). The duration of each experimental stage is given as well as information on what stimuli were visible for focal fish at a given stage. For Experiment 2, the order of animated sequences for the left and right LCD monitor, as arranged in *FishPlayer*, are given. A short description of the used animated scenes is given for each stage. In this example, Male B was not preferred in M1 and is, hence, shown together with a (virtual) model female during the observation period. The thick black line marks the point at which it is calculated which male was preferred by the focal female in M1. At this point, animated sequences in *FishPlayer* (Experiment 2) are rearranged before starting M2. M1 = First mate-choice test, M2 = Second mate-choice test.

Experimental stage	**Experiment 1**			**Experiment 2**		
	Duration (minutes)	**Stimuli visible on left side of test tank**	**Stimuli visible on right side of test tank**	**Duration (minutes)**	**Animated scene left monitor**	**Animated scene right monitor**
Acclimatization	20:00	Two empty small tanks, no fish	Two empty small tanks, no fish	20:00	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli	10:00	Male A alone in 1st small tank; 2nd small tank empty	1st small tank empty; Male B alone in 2nd small tank	02:30	Male A alone	Male B alone
1st part of M1	10:00			05:00		
Pause	not specified	Inserted opaque partition	Inserted opaque partition	01:30	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	05:00	Male B alone in 1st small tank; 2nd small tank empty	1st small tank empty; Male A alone in 2nd small tank	02:30	Male B alone	Male A alone
2nd part of M1	10:00			05:00		
Pause	not specified	Inserted opaque partition	Inserted opaque partition	01:30	empty virtual tank, no fish	empty virtual tank, no fish
Observation period	10:00	Male B alone in 1st small tank; Model female in 2nd small tank	Opaque partition in front of 1st small tank (pseudo-model female inside but not visible); Male A alone in 2nd small tank	10:00	Male B with model female	Male A alone
1st part of M2	10:00	Male B alone in 1st small tank; 2nd small tank empty	1st small tank empty; Male A alone in 2nd small tank	05:00	Male B alone	Male A alone
Pause	not specified	Inserted opaque partition	Inserted opaque partition	01:30	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	05:00	Male A alone in 1st small tank; 2nd small tank empty	1st small tank empty; Male B alone in 2nd small tank	02:30	Male A alone	Male B alone
2nd part of M2	10:00			05:00		

Treatment 1: model female always larger than focal female

In Treatment 1 of Experiment 1 (T1.1), we presented a model female which was larger than the focal female next to the prior non-preferred male during the observation period. To ensure equal handling of both males, model female and pseudo-model female were chosen to be similar in size. Therefore, we used a large pseudo-model female in T1.1. In the corresponding Treatment 1 of Experiment 2 (T2.1), we presented an animated sequence showing a virtual model female which was larger than the focal female next to the prior non-preferred virtual male. The behavior of both virtual males was identical. We hypothesized that focal females would copy the choice of larger model females, irrespective of presentation type (live or virtual stimulus fish).

Treatment 2: model female always smaller than focal female

In Treatment 2 of Experiment 1 (T1.2), we presented a model female which was smaller than the focal female next to the prior non-preferred male during the observation period. Again, we used a similar sized pseudo-model female. In the corresponding Treatment 2 of Experiment 2 (T2.2), we presented an animated sequence showing a virtual model female smaller than the focal female next to the prior non-preferred virtual male. Her behavior, as well as that of both virtual males was identical to that shown in the animated sequence in T2.1. We hypothesized that focal females would not copy the choice of a smaller model females, irrespective of presentation type.

Control for choice consistency

In the control of Experiment 1 (C1), we prevented focal females from using public information for their mate choice during the observation period. We introduced a model female and pseudo-model female, taken into account that the presence of both females might have affected the behavior of both males during the observation period of T1.1 and T1.2. Both females were chosen to be similar in size to that of the focal female. During the control, however, sight of both the model female and pseudo-model female was blocked by two opaque partitions. Here, focal females observed both males as apparently being alone. In the corresponding control of Experiment 2 (C2), no virtual model female was shown. Both virtual males were presented alone, showing identical behavior as used in T2.1 and T2.2. We hypothesized that focal females would be consistent in their choice for a male when no public information was provided, irrespective of presentation type.

6.3.6 Computer animation design

We used the tool *FishCreator* to create virtual 3D sailfin molly males and females for Experiment 2, using different textures of male and female bodies and fins which derived from digital photographs of live fish, as described in Chapter 4.2 (Gierszewski et al. 2017; Müller et al. 2017). We created a set of 30 unique virtual stimulus males with a virtual SL ranging between 45 to 53 mm with a mean SL of 49.6 ± 2.6 mm (measured on screen using scale paper). Sailfin molly females have been shown to prefer large males (Witte and Ryan 1998), therefore, we intended virtual males to represent large and high quality individuals. During each treatment and the control, a pair of virtual males did not differ more than 5 mm in SL within each trial. If a virtual male was used in more than one

trial per treatment, he was always presented in combination with a different male in the next trial. Depending on the respective treatment, virtual stimulus males were shown together with either a larger (T2.1) or a smaller (T2.2) virtual model female during the observation period. For this, we created a set of 15 unique virtual model females with a constant virtual SL of 50 mm (larger model) and a second set of the identical model females having a constant virtual SL of 25 mm (smaller model). Model female sizes were chosen to approximately represent the upper and lower range of SL measured for mature females in the lab population.

We used the tool *FishSteering* to create animated sequences of the prior created virtual fish using a video game controller (SONY Playstation 3 Wireless Controller, Sony Computer Entertainment Inc., Japan), as described in Chapter 4.3. During animation, virtual fish were simulated to be swimming in a 3D virtual tank environment with brownish gravel at the bottom resembling the gravel inside the experimental tank and a light background (240, 243, 218 RGB). General swimming behavior of virtual fish was automatically generated according to a predefined algorithm based on video analysis of live swimming sailfin mollies (Smielik et al. 2015; see Chapter 4.3).

For the use during mate-choice tests, we created an animation sequence of a single virtual male with a total duration of 7:30 minutes (2:30 minutes time to observe + 5 minutes choosing time; see Table 3). Additionally, we created a second animation of a single male with duration of 5 minutes for use in the first part of M2, after the observation period (Table 3). Both sequences consisted of a swimming path of a single virtual male swimming in the virtual tank, simulated to court an invisible female “outside” of the monitor. Male courtship behavior consisted of raising the dorsal fin and displaying its lateral side while swimming from left to right along the screen. Occasionally, the virtual male would thrust his gonopodium. Thanks to a specific functionality of *FishSim*, we were able to use the identical swimming path to animate all 30 virtual male fish. By this, we kept the behavior of the virtual males constant but allowed for variation in their appearance.

For the use during the observation period, we prepared a single animation sequence with a total duration of 10 minutes. Here, we created two swimming paths: one for the virtual male and one for the virtual model female. The two fish were animated to resemble male and female engaged in courtship with the male following the female, occasionally swimming in front of her, thrusting his gonopodium and raining its dorsal fin in view of the model female. During the observation period, the identical animation was used for both virtual male stimuli to keep their behavior constant (similar to the use of a pseudo-model female in Experiment 1). However, only the virtual model female next to the prior non-preferred virtual male was visible (Table 3). During the control (C2), again, the identical animation was used during the observation period but the model female was set invisible on both sides (correspondent to covering the view towards both the model female and the pseudo-model female in Experiment 1). For each experimental trial of Treatments T2.1 and T2.2, as well as C2, we prepared a set of folders containing animation sequences (so-called “records”) together with the corresponding virtual fish individuals according to a pseudo-random table. Animation sequences could then be loaded into *FishPlayer* for the presentation to live focal females as shown in Table 3.

6.3.7 Data analysis

We used SPSS v. 24 (IBM Statistics) and R 3.3.1 (R Development Core Team 2015) for data analysis. For both Experiments 1 and 2, we compared the mean SL of the two stimulus males (live or virtual) using Mann-Whitney-U tests for each treatment and control. Additionally, we applied Kruskal-Wallis tests with Dunn-Bonferroni post-hoc tests to compare the mean SL of all three live females (focal female, model female, pseudo-model female) used in each treatment and the control of Experiment 1.

We used time spent within mate-choice zones as a measure of mate choice for a given live or virtual stimulus male. For both Experiments 1 and 2, we assessed whether the overall choosing motivation (total time spent in both mate-choice zones within a mate-choice test) differed from M1 to M2 for each treatment and control using Wilcoxon tests for paired samples. Further, we calculated a preference score for the preferred and non-preferred male (e.g., time spent with a live or virtual non-preferred male divided by the total time spent with both males) for M1 and M2 separately. We compared preference scores to analyze whether focal females showed a significant preference for one of the two stimulus males (preferred male: male they spent >50% of the total time of both choice zones within M1) using 1-sample t-tests. We used the same test to analyze whether the prior preferred male in M1 was still the preferred one in M2. To assess whether focal females had copied the choice of the model female, we analyzed whether preference scores for the prior non-preferred stimulus male differed between M1 and M2 using Wilcoxon tests for paired samples. To assess the strength of a possible change of preference for the prior non-preferred stimulus male, we calculated a copying score (preference score for prior non-preferred male in M2 – preference score for non-preferred male in M1) for each focal female. Despite from possible effects of model female size on MCC, we tested whether focal female SL would also have affected copying behavior. For this, we performed Spearman rank correlations between focal female SL and the respective copying scores.

To validate the effectiveness of live versus virtual stimuli over the course of all experiments, we analyzed whether focal females differed in their relative time spent within mate-choice zones. Since we had reduced choosing time per mate-choice test in the virtual condition (2 x 5 min) compared to the live condition (2 x 10 min), we calculated a motivation score for each focal female. Motivation scores were defined as the sum of the absolute time spent in both mate-choice zones in M1 and M2, divided by the total time to choose per trial (2400 sec for live stimuli; 1200 sec for virtual stimuli). We compared whether motivation scores differed among PT (live versus virtual) for each treatment and the controls using a LM. Motivation score was the dependent variable, presentation type (PT: live, virtual) and treatment (larger, smaller, control) were fixed factors. We included focal female standard length (SL) as a covariate. Since the factor "treatment" was comprised of three levels, we conducted two orthogonal comparisons using the function *contrasts* (Crawley 2007). We set the contrasts of the model (1) to compare the sum of the controls against the sum of treatments presenting a model female [control → (larger+smaller)], and (2) to compare treatments with a larger model female against those with a smaller model (larger → smaller).

To evaluate whether focal female's copying behavior was different between the two presentation types (PT) across treatments, we fit a linear model (LM) using the function *lm* of the "lme4" package in R (Bates et al. 2015). For this, we set the change of preference (copying score) as the dependent variable, presentation type (PT: live, virtual) and treatment (larger, smaller) as fixed factors. We included focal female SL as a

covariate. To analyze whether a possible effect of model female size was different among PT, we included an interaction between PT and treatment in our model.

We inspected model assumptions (Q/Q-plots, residuals, residuals against fitted values) visually and compared the distribution of the residuals against a normal distribution using a Shapiro-Wilk normality test according to (Korner-Nievergelt et al. 2015). We considered p-values significant if $p \leq 0.05$.

6.4 Results

Detailed information on the number of live focal females used in our study, their mean standard length, as well as the standard length of all model females and stimulus males (live and virtual) is given in Table 4. We only used preference scores for analyzing a potential change of focal female's preference for the prior non-preferred male, however, we provide a graphical overview of the absolute time spent with each stimulus male for both Experiments 1 and 2 in supplementary Figures S1 and S2 (Appendix 4).

Table 4. Overview of the number and size of all live and virtual fish used in this study. Given are absolute numbers of live focal females who were tested, excluded, and analyzed in all experimental treatments and controls. The standard length (SL) of all live and virtual fish used in this study is given as mean ± SD. T = tested; E = excluded (due to side bias); A = analyzed; FF = focal female; MF = model female; PMF = pseudo-model female; MA = stimulus male A; MB = stimulus male B.

	Number of live focal females			SL (mm)				
	T	**E**	**A**	**FF (analyzed)**	**MF**	**PMF**	**MA**	**MB**
Experiment 1: live stimulus fish								
T1.1[a]	20	5	15	34 ± 4	45 ± 4	41 ± 4	39 ± 3	40 ± 4
T1.2[b]	27	12	15	42 ± 5	29 ± 2	31 ± 3	41 ± 4	39 ± 2
C1[c]	12	2	10	34 ± 5	41 ± 5	41 ± 6	37 ± 2	37 ± 2
Experiment 2: virtual stimulus fish								
T2.1[a]	19	4	15	36 ± 3	50 ± 0	/	51 ± 2	50 ± 3
T2.2[b]	20	5	15	35 ± 4	25 ± 0	/	49 ± 2	49 ± 2
C2[c]	18	3	15	34 ± 5	/	/	50 ± 2	50 ± 3

[a]Larger model female; [b]Smaller model female; [c]No model female.

6.4.1 Experiment 1: Live stimulus fish

In Experiment 1, there was a significant difference in focal female SL across treatments and the control (Kruskal-Wallis test: $n = 40$, $\chi^2(2) = 15.424$, $p < 0.001$). Overall, focal female SL was significantly different between the control and the treatment with a smaller model female (Dunn-Bonferroni post-hoc test: $z = 3.273$, $p = 0.003$) and between both size treatments (larger versus smaller model female; Dunn-Bonferroni post-hoc test: $z = 3.405$, $p = 0.002$).

Treatment 1.1: Live model females larger than focal females

Standard length (SL) of the three different types of live females (focal female, model female, pseudo-model female) used in Treatment 1.1 was significantly different (Kruskal-Wallis test: n = 45, $\chi^2(2) = 26.352$, $p < 0.001$). Focal females were significantly smaller compared to the model females (Dunn-Bonferroni post-hoc test: z = -5.099, $p < 0.001$) and pseudo-model females (Dunn-Bonferroni post-hoc test: z = -3.062, p = 0.007). Model females and pseudo-model females did not differ in SL (Dunn-Bonferroni post-hoc test: z = 2.037, p = 0.125). Stimulus males used within trials in Treatment 1.1 were also not different in SL (Mann-Whitney-U test: n = 30, U = 77, p = 0.148).

Focal females (n = 15) spent on average 588 ± 227 sec (mean ± SD) in both choice zones in M1 and 536 ± 192 sec in M2. Choosing motivation did not differ across mate-choice tests (Wilcoxon test: n = 15, Z = -0.454, p = 0.65). In M1, the median preference score for the preferred stimulus male was 0.72 (1^{st} and 3^{rd} quartile: 0.63, 0.84) and 0.28 (0.16, 0.37) for the non-preferred stimulus male respectively. The stimulus male with whom focal females spent most of their time with (> 50 % of total time in choice zones) was significantly preferred (1-sample t test: n = 15, df = 14, t = 6.053, $p < 0.001$). From M1 to M2, preference scores for the prior non-preferred male significantly increased to 0.59 (0.47, 0.73) after the observation period (Wilcoxon test: z = -3.181, p = 0.001; Fig. 23). Copying scores for the prior non-preferred male [0.32 (0.26, 0.41)] were not correlated with focal female SL (Spearman rank correlation: $n_{cscore} = 15$, $n_{SL} = 15$, rho = -0.084, p = 0.765). In M2, the prior preferred male was not anymore preferred (1-sample t test: n = 15, df = 14, t = -1.093, p = 0.293). Out of 15 focal females, ten females (66.6 %) reversed their initial mate choice in favor for the prior non-preferred male in M2.

Treatment 1.2: Live model females smaller than focal females

Standard length of the three types of females used in Treatment 1.2 was significantly different (Kruskal-Wallis test: n = 45, $\chi^2(2) = 29.981$, $p < 0.001$). Focal females were significantly larger than the model females (Dunn-Bonferroni post-hoc test: z = 5.162, $p < 0.001$) and pseudo-model females (Dunn-Bonferroni post-hoc test: z = 4.163, $p < 0.001$). Model females and pseudo-model females were of similar SL (Dunn-Bonferroni post-hoc test: z = -0.999, p = 0.954). Stimulus males used within a trial in Treatment 1.2 did not differ in SL (Mann-Whitney-U test: n = 30, U = 86.5, p = 0.285).

Focal females (n = 15) spent on average 630 ± 156 sec in both choice zones in M1 and 608 ± 165 sec in M2. Choosing motivation was not different between M1 and M2 (Wilcoxon test: n = 15, Z = -0.031, p = 0.975). In M1, preference score for the preferred stimulus male was 0.86 (0.71, 0.90) and 0.14 (0.10, 0.29) for the non-preferred stimulus male. The stimulus male with whom focal females spent most of their time with was significantly preferred (1-sample t test: n = 15, df = 14, t = 7.719, $p < 0.001$). From M1 to M2, preference scores for the prior non-preferred male significantly increased to 0.51 (0.32, 0.69) after the observation period (Wilcoxon test: z = -2.954, p = 0.003; Fig. 23). Copying scores for the prior non-preferred male [0.29 (0.15, 0.47)] were not correlated with focal female SL (Spearman rank correlation: $n_{cscore} = 15$, $n_{SL} = 15$, rho = -0.323, p = 0.765). In contrast to M1, the prior preferred male was not anymore preferred in M2 (1-sample t test: n = 15, df = 14, t = 0.262, p = 0.797). Out of 15 focal females, seven females (46.6 %) reversed their initial mate choice in favor for the prior non-preferred male in M2.

Control for choice consistency (C1)

Standard length of the three types of females used in the control C1 was significantly different (Kruskal-Wallis test: n = 30, $\chi^2(2) = 8.495$, p = 0.014) with focal females being significantly smaller than the model females (Dunn-Bonferroni post-hoc test: z = -2.485, p = 0.039) and pseudo-model females (Dunn-Bonferroni post-hoc test: z = -2.562, p = 0.031). Model females and pseudo-models did not differ in SL (Dunn-Bonferroni post-hoc test: z = -0.076, p = 1). The presented live stimulus males within a trial were also not different in SL in C1 (Mann-Whitney-U: n = 20, U = 48.5, p = 0.912).

Focal females (n = 10) spent on average 732 ± 189 sec in both choice zones in M1 and 723 ± 131 sec in M2 and choosing motivation did not differ between M1 and M2 (Wilcoxon test: n = 10, Z = -0.102, p = 0.919). In M1, preference score for the preferred stimulus male was 0.66 (0.57, 0.70) and 0.34 (0.30, 0.44) for the non-preferred male. The stimulus male with whom focal females spent most of their time with was significantly preferred (1-sample t test: n = 10, df = 9, t = 5.139, p < 0.001). Here, focal females were consistent in their mate choice since preference scores for the prior non-preferred male in M2 [0.42 (0.35, 0.52)] were not different from those in M1 (Wilcoxon test: n = 10, z = -1.784, p = 0.074; Fig. 23). However, the prior preferred male was not anymore preferred in M2 (1-sample t test: n = 10, df = 9, t = 0.571, p = 0.582). The copying score for the prior non-preferred male was 0.11 (-0.02, 0.21). Out of 10 focal females, only two females (20 %) reversed their initial mate choice in favor for the prior non-preferred male in M2.

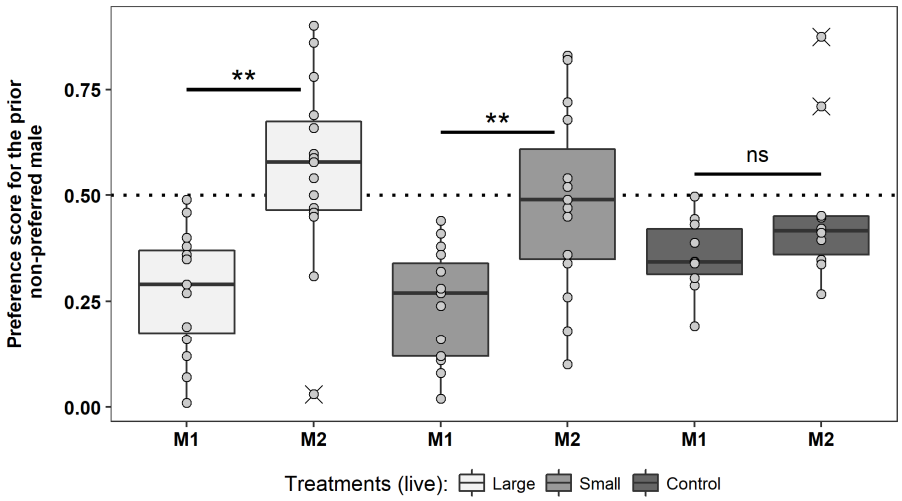

Figure 23. Results of Experiment 1 for both size treatments and the control for choice consistency using live stimulus fish. Preference scores for the (prior) non-preferred male stimulus in M1 and M2. The dotted line represents a preference score of 0.5. Dots represent raw data of individual focal females and outliers are depicted by a cross. M1 = 1st mate-choice test, M2 = 2nd mate-choice test. N_{large} = 15, N_{small} = 15, $N_{control}$ = 10, ** = p < 0.01, ns = not significant.

6.4.2 Experiment 2: virtual stimulus fish

In Experiment 2, focal female SL was not different across both treatments and the control (Kruskal-Wallis test: n = 45, $\chi^2(2) = 8.311$, p = 0.873).

Treatment 2.1: Virtual model females (50 mm) larger than focal females

Virtual stimulus males used within trials in Treatment 2.1 were not different in SL (Mann-Whitney-U test: n = 30, z = -0.817, p = 0.414). Focal females (n = 15) spent on average 297 ± 111 sec in both choice zones in M1 and 393 ± 142 sec in M2. Choosing motivation was different across mate-choice tests (Wilcoxon test: n = 15, Z = -2.131, p = 0.033) with focal females spending more time with the virtual stimulus males after observing them with a larger virtual model female. In M1, preference score for the preferred stimulus male was 0.71 (0.64, 0.89) and 0.29 (0.11, 0.36) for the non-preferred stimulus male. The stimulus male with whom focal females spent most of their time with was significantly preferred (1-sample t test: n = 15, df = 14, t = 7.043, p < 0.001). From M1 to M2, preference scores for the prior non-preferred male significantly increased to 0.50 (0.30, 0.66) after the observation period (Wilcoxon test: z = -3.01, p = 0.003; Fig. 24). The copying score for the prior non-preferred male [0.22 (0.02, 0.40)] and focal female SL were not correlated (Spearman rank correlation: n_{cscore} = 15, n_{SL} = 15, rho = 0.2, p = 0.474). In M2, the prior preferred male was not anymore preferred (1-sample t test: n = 15, df = 14, t = 0.44, p = 0.667). Out of 15 focal females, seven females (46.6 %) reversed their initial mate choice in favor for the prior non-preferred virtual male in M2.

Treatment 2.2: Virtual model females (25 mm) smaller than focal females

Virtual stimulus males within a trial did not differ in SL (Mann-Whitney-U test: n = 30, z = -0.817, p = 0.414). In Treatment 2.2, focal females (n = 15) spent 300 ± 109 sec in both choice zones in M1 and 340 ± 161 sec in M2, thus choosing motivation did not differ (Wilcoxon test: n = 15, Z = -0.909, p = 0.363). Preference score for the preferred stimulus male in M1 was 0.80 (0.71, 0.89) and 0.20 (0.11, 0.29) for the non-preferred stimulus male. The stimulus male with whom focal females spent most of their time with was significantly preferred (1-sample t test: n = 15, df = 14, t = 10.095, p < 0.001). From M1 to M2, preference scores for the prior non-preferred male significantly increased to 0.51 (0.21, 0.55) after the observation period (Wilcoxon test: z = -2.953, p = 0.003; Fig. 24). Median copying score for the prior non-preferred male was 0.29 (0.12, 0.41). Copying score was not correlated to focal female SL (Spearman rank correlation: n_{cscore} = 15, n_{SL} = 15, rho = -0.29, p = 0.295). In M2, the prior preferred male was not anymore preferred (1-sample t test: n = 15, df = 14, t = 0.882, p = 0.393). Out of 15 focal females, ten females (66.6 %) reversed their initial mate choice in favor for the prior non-preferred virtual male in M2.

Control for choice consistency (C2)

Standard length of virtual stimulus males used within a trial in C2 was not different between trials (Mann-Whitney-U test: n = 30, z = -0.481, p = 0.631). Focal females (n = 15) spent 371 ± 110 sec in both choice zones in M1 and 412 ± 116 sec in M2 and choosing motivation did not differ across mate-choice tests (Wilcoxon test: n = 15, Z = -1.25, p = 0.211). Preference score for the preferred stimulus male in M1 was 0.85 (0.77, 0.97) and 0.15 (0.03, 0.23) for the non-preferred stimulus male. The stimulus male with whom focal females spent most of their time with was significantly preferred (1-sample t test: n = 15, df = 14, t = 9.173, p < 0.001). Focal females were not consistent in their preference for a virtual stimulus male in the control, since preference scores for the prior non-preferred male significantly increased to 0.48 (0.11, 0.71) from M1 to M2 (Wilcoxon test: z = -2.669, p = 0.008; Fig. 24). Here, the copying score was 0.24 (0.04, 0.49). The

prior preferred male was not anymore preferred in M2 (1-sample t test: n = 15, df = 14, t = 0.803, p = 0.436). Out of 15 focal females, six females (40 %) reversed their initial mate choice in favor for the prior non-preferred virtual male in M2.

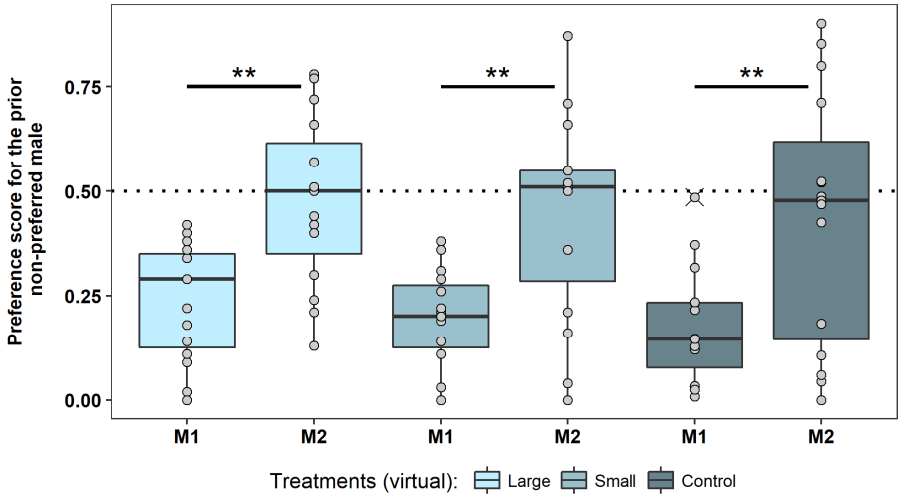

Figure 24. Results of Experiment 2 for both size treatments and the control for choice consistency using virtual stimulus fish. Preference scores for the (prior) non-preferred male stimulus in M1 and M2. The dotted line represents a preference score of 0.5. Dots represent raw data of individual focal females and outliers are depicted by a cross. M1 = 1st mate-choice test, M2 = 2nd mate-choice test. N_{large} = 15, N_{small} = 15, $N_{control}$ = 15, ** = p < 0.01.

6.4.3 Comparing choosing motivation and copying behavior across presentation types (live versus virtual)

There was no difference in relative choosing motivation (motivation score) among presentation types (live and virtual; Table 5). However, the relative choosing motivation was significantly higher in controls where no model female was present compared to treatments with any model female (larger or smaller; LM: df = 80, t = 2.738, p = 0.008; Table 5). Whereas there was no difference in motivation scores whether the presented model female was larger or smaller. Overall, focal female SL did not affect motivation scores.

Table 5. LM estimates for relative choosing motivation across presentation type and treatment. Motivation score was the dependent variable throughout. Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor. Intercept reference category for the factor estimate for PT "virtual" is PT "live". Significant p-values (p ≤ 0.05) are printed in bold. PT = presentation type, SL = standard length of focal females.

Fixed factors	Lower	Estimate	Upper	SE	*df*	*t*	*p*
(Intercept)	0.030	0.321	0.612	0.146	80	2.194	**0.031**
PT „virtual"	-0.022	0.051	0.125	0.037	80	1.399	0.166
Control → (larger + smaller)	0.010	0.038	0.065	0.014	80	2.738	**0.008**
Larger → smaller	-0.038	0.007	0.051	0.022	80	0.296	0.768
SL	-0.002	0.006	0.014	0.004	80	1.570	0.120

The comparison between focal female's copying scores for the prior non-preferred male revealed no significant difference across presentation types (live or virtual) or treatments (larger or smaller model female). Further, focal female SL did also not affect copying scores (Table 6).

Table 6. LM estimates for effects on the copying score for the prior non-preferred virtual male across presentation type and treatment. Change of preference for the prior non-preferred virtual male stimulus was the dependent variable throughout. Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor. Intercept reference categories for factor estimates are PT "live" (PT "virtual"), treatment "larger" and PT "live" x treatment "larger" (PT "virtual" x treatment "smaller"). Significant p-values ($p \leq 0.05$) are printed in bold. PT = presentation type, SL = standard length of focal females.

Fixed factors	Lower	Estimate	Upper	SE	*df*	*t*	*p*
(Intercept)	0.117	0.644	1.171	0.263	55	2.448	**0.018**
PT „virtual"	-0.197	-0.030	0.137	0.083	55	-0.358	0.722
Treatment „smaller"	-0.160	0.036	0.232	0.098	55	0.372	0.712
SL	-0.026	-0.012	0.004	0.008	55	-1.410	0.164
PT "virtual" x treatment "smaller"	-0.295	-0.033	0.230	0.131	55	-0.251	0.803

6.5 Discussion

In our current study, we investigated whether the size of the model female affects public information use in focal female sailfin mollies with regard to MCC. We hypothesized that our findings would be in accordance with previous findings in the closely related guppy, which showed that smaller focal females copied the choice of larger model females but not vice versa (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Vukomanovic and Rodd 2007). Results were, thereby, in accordance with the social learning strategy "*copy older individuals*" as proposed by Laland (2004).

In contrast, our study did not confirm this very same relationship for model female size and MCC in sailfin molly females. Our data shows that focal females copied the choice of a model female for a previously non-preferred male, irrespective of whether she was larger or smaller in size. Moreover, we found similar results using two different experimental approaches for studying MCC: (I) a classic experimental design with live stimulus fish; and (II) a modified procedure using virtual fish stimuli created with *FishSim*. We could already show that there was no difference in attractiveness of a virtual male stimulus (created with *FishSim*) presented to live focal females compared to the presentation of live or video recorded male stimuli (Gierszewski et al. 2017; see also Chapter 4). By this subsequent study, we were also able to demonstrate that *FishSim* is further perfectly capable of conducting MCC experiments in an easy-to-use and highly controlled way. We showed that live focal females copied the choice of a live model female for a prior non-preferred male and, likewise, that of a virtual model female. Overall, there was no difference in focal female's copying behavior across presentation types (live or virtual). Therefore, virtual model females were equally efficient in inducing copying behavior, showing that they too provide relevant information for observing live focal females. Over the course of the experiment, focal female's relative choosing motivation did not seem to be affected by whether live or virtual fish stimuli were presented during the different stages of the MCC experiment. Therefore, focal females

were equally attracted to virtual male stimuli compared to live males throughout the experiment. Our results serve as an important validation, justifying the use of *FishSim* to manipulate public information in MCC experiments with sailfin mollies. Furthermore, by using *FishSim* we had total control of the body size of a virtual stimulus and were therefore independent from available sizes of potential live fish stimuli available in the fish stock at the time of the experiment.

When no model female was present during the observation period (i.e., no public information), focal females were consistent in their mate-choice for a prior preferred male (Experiment 1). However, focal females did not choose consistently in Experiment 2, in which we used virtual fish. It is not unusual that, overall, individual mate choice may be subject to high degrees of inconsistency over time (Rosenthal 2017). It was found that mate choice can be highly flexible and, for the chooser, depends on both intrinsic (i.e., an individual's state) and extrinsic (i.e., environmental and/or social factors) factors (summarized in Tinghitella et al. 2013). However, prior studies on MCC in sailfin mollies have generally demonstrated consistency in mate choice when no public information was provided in various experimental setups (Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003; Witte and Ueding 2003; Gierszewski, Keil, et al. 2018; Gierszewski, Baker, et al. 2018). Still, focal females might have lost interest in the prior preferred male during the control when public information was missing. Since we always presented two virtual stimulus males that were very similar in quality, we assume that both males were perceived as equally attractive to focal females while relying only on personal information during mate choice. Notably, we do not conclude choice inconsistency being an artefact of using virtual stimuli during MCC experiments. In similar studies using the same experimental approach, we demonstrated that focal females indeed chose consistently with virtual fish stimuli (see Chapters 7 and 8 in this thesis and Gierszewski, Baker, et al. 2018).

There are some reasons which possibly might explain the contrary results of our study compared to those found in guppies (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Vukomanovic and Rodd 2007). Tinghitella et al. (2013) showed that the social environment and an individual's age interact to affect mate choice in three-spined sticklebacks (*Gasterosteus aculeatus*). Further, mate choice decisions were more relaxed (individuals being less choosy) in female-biased groups. Social environment and age also seem to interact in female variable field crickets (*Gryllus lineaticeps*; Atwell and Wagner 2014). Here, young female field crickets were found to be most choosy with high male density compared to older females (Atwell and Wagner 2014). We assume that the use of public information for mate choice may be highly variable across species and populations and may be similarly affected by personal and social factors as described above. An individual's tendency to copy one another's mate choice may, hence, represent the status quo of a social group and the respective environment it inhabits.

In the study by Dugatkin and Godin (1993), the experimental procedure differed from ours in a way that focal females' initial preference for a male was not assessed in a first mate-choice test. Instead, the experiment started with the observation period, for which the male being preferred by the model female (either smaller or larger than the focal female) was determined by coin flipping. Afterwards, focal females were given time to choose between the two males in one mate-choice test. It is possible, that these differences in procedure might have accounted for differences in copying behavior. Further, all individuals used by Dugatkin and Godin (1993) derived from a single stock tank. Therefore, it is likely that individuals had further knowledge of individual fish and knew each other (but see Vukomanovic and Rodd 2007). Bierbach, Girndt, et al. (2011)

showed that knowledge about the sexual behavior of conspecifics affected mating decisions in Atlantic molly males (*Poecilia mexicana*). In our study, focal females and model females used in Experiment 1 derived from different tanks and did not know each other. Moreover, both stimulus males were unknown to focal females. By this, we were able to exclude social experience to influence MCC decisions in our study. The same was true for Experiment 2, in which focal females could not have previous knowledge about the virtual males. Since rearing conditions in all tanks were kept as similar as possible (see Chapter 2.3), we are confident that differences in female body size reflected female age and not differences in growth rate, resulting from different environmental parameters. Furthermore, mixed-sex shoals in our rearing conditions were female-biased, a sex-ratio which is also commonly found among adult fish in the wild (Snelson and Wetherington 1980). Overall, sailfin mollies and guppies might simply not utilize the same social learning strategies even though they are closely related.

We conclude that age of the model female (as defined by her body size) seems not to play a role for MCC in sailfin molly females. At least not for the population used in our study and kept under the rearing conditions described in Chapter 2.3. Consequently, the social learning strategy "*copy older individuals*" (Laland 2004) seems not to be a universal rule regarding its relevance for MCC. We assume that MCC represents a highly flexible mate-choice strategy, which depends on an individual's status quo of living (social group composition plus environmental parameters). Plath et al. (2019) demonstrated changes in mating preferences in Western mosquitoefish (*Gambusia affinis)* as a response to the presence of different animated predators. It can be assumed that also the deployment of alternative strategies, such as MCC, may be subject to these changes and therefore need to be highly flexible. The occurrence of universal roles on when to deploy a certain strategy can therefore rather be considered a disadvantage. However, more research has to be done to increase evidence supporting this claim and future studies should take environmental aspects as well as social aspects (e.g., group composition and sex-ratio) into account.

6.6 Acknowledgements

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Chapter 7

In the “spot”light:

Do gravid spots predict model female quality in mate-choice copying of female sailfin mollies?

Stefanie GIERSZEWSKI[a], Derek BAKER[a,b], Klaus MÜLLER[c], Jan-Marco HÜTWOHL[c], Klaus-Dieter KUHNERT[c] & Klaudia WITTE[a]

[a]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein-Straße 2, Siegen, 57076, Germany, [b]University of Calgary, Calgary, AB, Canada, [c]Institute of Real-Time Learning Systems, Department of Electrical Engineering & Computer Science, University of Siegen, Hölderlinstraße 3, Siegen, 57076, Germany

Parts of this chapter have been published as a case study with specific focus on the technical background and the step-to-step usage of *FishSim* in the Journal of Visualized Experiments (Gierszewski S, Baker D, Müller K, Hütwohl JM, Kuhnert KD, Witte K. 2018. Using the *FishSim* Animation Toolchain to Investigate Fish Behavior: A Case Study on Mate-Choice Copying In Sailfin Mollies. J. Vis. Exp. 141: e58435).

7.1 Abstract

More and more studies identify mate-choice copying as a widespread and biologically relevant mate-choice strategy across the whole animal kingdom. The extent to which the quality of a model affects mate-choice copying in an observing individual, however, is still not well understood. In this study, we investigated the role of female gonopore pigmentation - the so-called gravid spot - as a means of a model female’s quality in sailfin mollies (*Poecilia latipinna*). We used *FishSim* to create virtual stimulus males, as well as virtual model females, which were animated and then presented on computer monitors to live focal females in a binary choice experiment. We performed two experimental treatments, in which we manipulated the absence or presence of a gravid spot in virtual model females, and a control for choice consistency without any virtual model female. Our results show that focal females copied the choice of the model female irrespective of the presence or absence of a gravid spot. In the control, in which no model female was visible, focal females were consistent in their choice for a virtual male. We conclude that the gravid spot seems not to be a sign of model female quality for observing focal female sailfin mollies when deciding to copy the choice of the model or not.

7.2 Introduction

Mate choice is one of the most important decisions animals make in their life history. Animals have evolved different strategies for finding the best mating partners. On the one hand, they may rely on personal information when evaluating potential mating partners independently. On the other hand, they may deploy the strategy of mate-choice copying (MCC) instead and, thereby, utilize public information gained from observing the mate choice of conspecifics (Danchin et al. 2004; also see Chapter 1). Mate-choice copying is a form of social learning and, hence, a non-independent mate-choice strategy (Witte and Nöbel 2011), which has been observed in both vertebrates (Galef and White 1998; Galef and White 2000; Waynforth 2007; Galef et al. 2008; Kniel, Dürler, et al. 2015) and invertebrates (Mery et al. 2009; Dagaeff et al. 2016; Nöbel, Allain, et al. 2018). So far, MCC was predominantly studied in fish and is found both under laboratory conditions (Dugatkin and Godin 1992; Munger et al. 2004; Widemo 2005; Heubel et al. 2008; Frommen et al. 2009; Bierbach, Girndt, et al. 2011) and in the wild (Witte and Ryan 2002; Goulet and Goulet 2006; Alonzo 2008; Godin and Hair 2009). Mate-choice copying is especially valuable for an individual if two or more potential mating partners are apparently similar in quality, and a “good” mate choice — in terms of maximizing fitness — is difficult to make (Nordell and Valone 1998). The quality of a model female herself can affect whether focal females copy her choice or not (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Hill and Ryan 2006; Vukomanovic and Rodd 2007). Respectively, “good” or “bad” model female quality has been attributed to her being more or less experienced in mate choice, for example with regard to size and age (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Vukomanovic and Rodd 2007; but see Chapter 6 in this thesis), or by her being conspecific or heterospecific (Hill and Ryan 2006). Since MCC is considered to play an important role in the evolution of phenotypic traits as well as speciation and hybridization (Danchin et al. 2004; Verzijden et al. 2012; Witte et al. 2015; Varela et al. 2018), the consequences of copying a “false” choice may be tremendous in reducing the fitness of the copier (Nöbel, Danchin, et al. 2018). If an individual decides to copy the choice of another individual, it is important to evaluate if the observed model is a reliable source of information, i.e., that the model itself is making a “good” choice due to him or her being well experienced in mate choice. Here the question arises: what visual features may characterize a reliable model to copy from in sailfin molly females?

A distinct visual feature in female sailfin mollies and other poeciliids is the gravid spot (also known as ‘anal spot’, ‘brood patch’ or ‘pregnancy spot’; Greven 2005). This darkly pigmented area in their anal region derives from melanization of the tissue lining the ovarian sac (Norazmi-Lokman et al. 2016). The size and presence of the gravid spot are variable across conspecific females and may further individually change during the progression of ovarian cycles (Constantz 1989; Norazmi-Lokman et al. 2016). For example, in guppies, the gravid spot was found to be largest prior to parturition (Constantz 1989). Since female guppies are most receptive immediately after giving birth (Constantz 1989), Dosen and Montgomerie (2004) argued that males should tend more to the most gravid females, that is, those having the largest gravid spot, during their mating efforts. In an early study by Peden (1973) using dummy fish, he could show that anal spots served to attract males in *Gambusia* and further facilitated gonopodial orientation for internal insemination. Benson (2007) manipulated the gravid spot in female green swordtails (*Xiphophorus helleri*) by injection of tattoo ink and found that significantly more males courted females whose gravid spot had been augmented. Similarly, female gravid spot size was also found to be a significant predictor for male mating attempts in western mosquitofish (*Gambusia affinis*; Deaton 2008).

During the assessment of important features of female sailfin mollies for 3D fish development for *FishSim* (see Chapter 4.2), we found that gravid spot size had the

highest coefficient of variation (113.8 %CV) between females (compare MP20 in Figs. 11 and 12 in Chapter 4.2, as well as Table S2 in Appendix 1). The smallest female sailfin molly ever measured in the lab bearing a gravid spot had a SL of only 20 mm (Gierszewski, personal observation). So far, the reported range of size at first reproduction in sailfin mollies is 27.8-37.1 mm SL (population means of wild caught individuals; Reznik and Miles 1989). Typically, sailfin molly females show an ovarian cycle of about 30 days, which is asynchronous among females within a group (Sumner et al. 1994; see Chapter 2.1.2 for more detailed information on sailfin molly reproductive biology). Similar to other poeciliids, the gravid spot was discussed to serve as a means of fertility advertisement in female sailfin mollies (Farr and Travis 1986; Sumner et al. 1994). Farr and Travis (1986) considered the gravid spot as a sign of maturity in sailfin mollies and further associated its development with the presence of partially or fully-yolked ova or embryos. Sumner et al. (1994) argued that the presence of a visible gravid spot mainly served as a sign for a non-receptive status in females. However, 33 % of the studied receptive females also had a spot.

Sailfin molly females were shown to copy the choice of conspecific females (see Chapter 2) but, so far, the potential role of the gravid spot for MCC has never been tested, nor experimentally manipulated. In contrast to the rather invasive study by Benson (2007), we used *FishSim* (Gierszewski et al. 2017; Müller et al. 2017; see Chapter 4 for more information) to perform a MCC experiment with virtual stimulus males and virtual model females, presented on computer monitors to live focal females. We tested whether absence or presence of a gravid spot in virtual model females affects the mate-choice of observing focal females. We performed two different experimental treatments in which we visually manipulated the quality of the virtual model female. During the observation period, we either presented the prior non-preferred virtual male (I) together with a virtual model female with a gravid spot ("spot" treatment); or (II) together with a virtual model female without a gravid spot ("no spot" treatment). Additionally, we tested in a control without any model female whether focal females chose consistently when no public information was provided.

Considering the link between the gravid spot and a female's reproductive status, we predicted that the gravid spot serves as a sign of model female quality by providing information on her current reproductive state to observing focal females. We investigated two alternate hypotheses. First, if the gravid spot is a general sign for maturity, as predicted by Farr and Travis (1986), it denotes a presumably reliable and experienced model compared to an immature model (without the spot). Here, focal females are more likely to copy the choice of a model with a spot but not that of a model without a spot. Second, if the gravid spot marks non-receptivity due to already developing broods, as predicted by Sumner et al. (1994), the model is presumably less reliable since non-receptive females would be considered less choosy. In this case, focal females will not copy their choice but that of models without spot. If no public information is provided during the control, we predicted that focal females would be consistent in their mate-choice.

7.3 Materials and Methods

7.3.1 Study species

We tested live focal females descendant from wild sailfin mollies caught on Mustang Island near Corpus Christi, TX, USA in 2014. All focal females were mature adults and were only tested once. Fish housing conditions were correspondent to that described in Chapter 2.3. Prior to experiments, we kept focal females separated from males in small

groups in small separation tanks (40 cm x 25 cm x 40 cm) for at least two weeks to increase their choosing motivation. After experiments, we returned all females to their original home tanks.

7.3.2 Experimental setup

We used the identical experimental setup as described in Chapter 6.2.3 (experimental setup E2, Chapter 2.3.1; see Fig. 25). Computer animations were presented in a two-choice paradigm on two 19'' LCD screens (Belinea, Modell 1970 S1-P; 1280 x 1024 pixels resolution). Monitors were positioned adjacent to the experimental tank (100 cm x 40 cm x 40 cm) at an approximate distance of 2 cm. The bottom of the experimental tank was layered with brownish gravel and its back wall was covered with blue plastic sheets. The experimental tank as well as the terminal featuring the computer running *FishSim*, were shielded from the rest of the room by a beige colored curtain, to decrease disturbance of focal fish by their surroundings. The experimental tank was illuminated by two neon tubes (ORSAM L58W/965). The experimenter was sitting at a computer terminal, about 100 cm away from the experimental tank, to reduce disturbance of the fish and to manage the presentation of computer animations in *FishSim*. Experiments were performed from June to August in 2017.

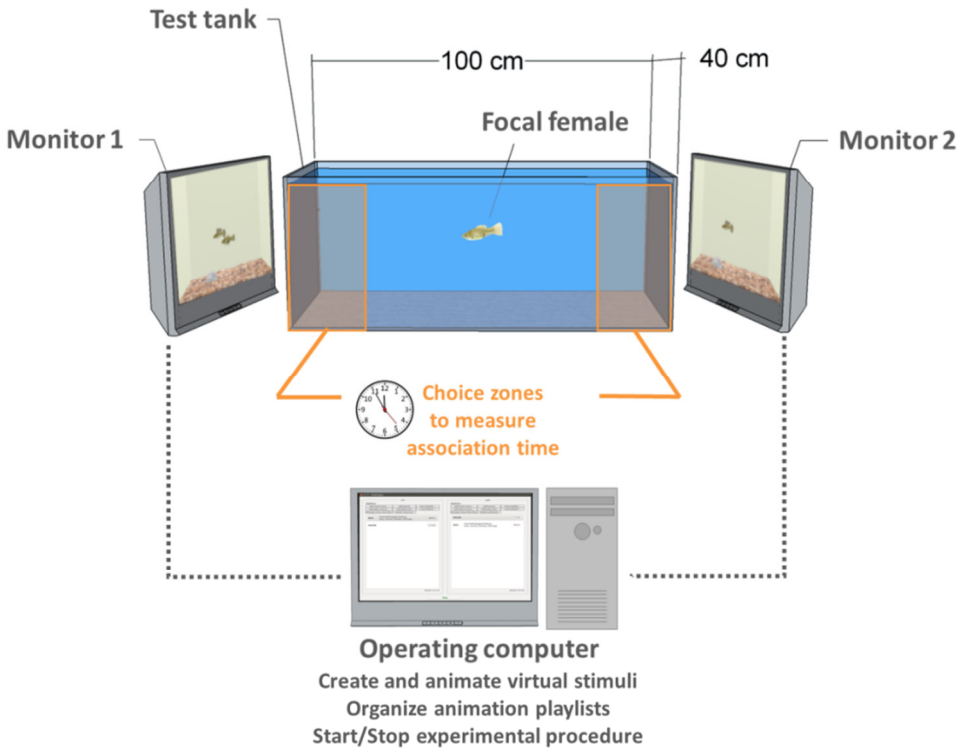

Figure 25. Experimental setup for the MCC experiment with computer animation. The operating computer connects to two presentation monitors (Monitors 1 and 2) which replay animations to live focal females inside the test tank. For illustration, both LCD monitors are angled to show an animated scene.

7.3.3 General experimental procedure

We followed the classic experimental approach for testing MCC in sailfin mollies (Schlupp et al. 1994; Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003) and adopted its procedure for the use of computer animations created with *FishSim* (Gierszewski et al. 2017; Müller et al. 2017; see Chapter 4 for more information on *FishSim*; Fig. 26). A detailed step-by-step protocol on how we used the *FishSim* software in this study was published in Gierszewski, Baker, et al. (2018) and can be found in Appendix 5.

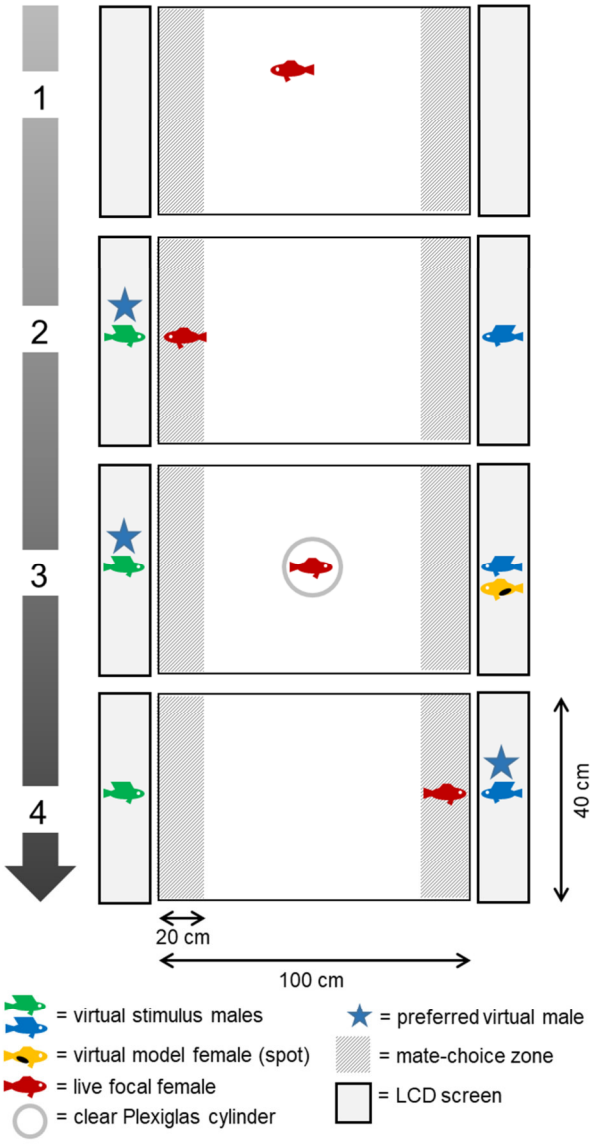

Figure 26. General overview of the most important experimental steps for a MCC experiment using virtual fish stimuli. (1) Acclimatization period. (2) First mate-choice test: live focal female chooses between virtual stimulus males. (3) Observation period: focal female watches the prior non-preferred male together with a virtual model female with gravid spot. (4) Second mate-choice test: the focal female again chooses between virtual stimulus males. In this example, she copies the choice of the virtual model.

Prior to testing, we loaded all animated sequences needed for the first mate-choice test (M1) into *FishPlayer* (Table 7). Per experimental trial, we used a set of virtual fish stimuli comprised of a pair of virtual stimulus males and one virtual model female (except for the control; see below). Within experiments, each focal female was presented with a unique set of virtual fish stimuli. Each pair of virtual males was used only once within a treatment but was re-used across treatments in different order. Virtual male stimuli were always presented simultaneously in a binary choice situation according to a pseudo-random table.

First, we introduced a focal female into the test tank and let her acclimate for 30 minutes, to get accustomed to the tank and the illumination emitting from the two monitors. During acclimatization, both monitors depicted the scene of a virtual empty tank without any virtual fish (Fig. 26.1; Table 7). After acclimatization, we gently positioned the focal female inside a Plexiglas cylinder in the middle of the test tank, to ensure an equal distance between the two male stimuli. We started the presentation of animated sequences in *FishPlayer* and the first mate-choice test (M1) commenced (Fig. 26.2; Table 7). After having observed both virtual male stimuli for 2:30 minutes, we released the focal female from the cylinder and let her choose between the two virtual stimulus males for a period of 5 minutes (Table 7). Afterwards, we placed the focal female back inside the cylinder in the middle of the tank and the position of the virtual males was automatically switched to control for a possible side bias in focal females (1st part of M1; Table 7). After focal females had observed the switch of male positions for 2:30 minutes, we again released her from the cylinder and let her choose between the two males for another 5 minutes (2nd part of M1; Table 7). We then placed the focal female back into the cylinder and calculated which virtual male had been preferred by the focal female in M1. Additionally, we calculated whether females showed side biases or not. If a focal female spent more than 90 % of the total time in only one mate-choice zone, even if virtual males were switched, her choice was judged to be side biased and the experiment was terminated. All females with a side bias were retested once after at least two days with different stimulus males and, if their side bias persisted, were further excluded from the final analysis. Focal females that were too stressed during mate-choice trials and only stayed in one corner of the pool were excluded from analysis due to stress.

According to which male had been preferred in M1, we stopped *FishPlayer* and rearranged the playlist entries for the subsequent observation period. For focal females, changes made to the entries in *FishPlayer* were not visible on screen. Instead, an empty virtual tank was displayed throughout. We again started *FishPlayer*, commencing an observation period of 10 minutes (Fig. 26.3; Table 7). During the observation period, the prior non-preferred virtual male was presented to be swimming together with a virtual model female. The prior preferred virtual male, however, was presented to be alone. After 10 minutes, we released the focal female from the cylinder and she was allowed to choose between the two virtual males for 5 minutes, starting the second mate-choice test (M2; Fig. 26.4; Table 7). The procedure of M2 was identical to that of M1. After the first part of M2 we switched the position of the two virtual males and let the focal female observe the new position of both males for 2:30 minutes. She was then again allowed to choose between the two virtual males for 5 minutes during the second part of M2 (Table 7). After M2, we terminated the experimental trial.

Table 7. Experimental stages of the MCC experiment using *FishSim*. The duration of each experimental stage as well as the order of animated sequences for the left and right LCD monitor, as arranged in *FishPlayer*, are given. A short description of the used animated scenes is given for each stage. In this example, Male B was not preferred in the first mate-choice test and was, hence, shown together with a virtual model female during the observation period. The thick black line marks the point when it is calculated which male was preferred by the live focal female in M1. At this point, animated sequences in *FishPlayer* need to be rearranged before commencing M2. M1 = First mate-choice test, M2 = Second mate-choice test.

Experimental stage	Duration	Animated scene left monitor	Animated scene right monitor
Acclimatization	30 min	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli	2:30 min	Male A alone	Male B alone
1st part of M1	5 min		
Pause	1:30 min	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	2:30 min	Male B alone	Male A alone
2nd part of M1	5 min		
Pause	1:30 min	empty virtual tank, no fish	empty virtual tank, no fish
Observation period	10 min	Male B with model female	Male A alone
1st part of M2	5 min	Male B alone	Male A alone
Pause	1:30 min	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	2:30 min	Male A alone	Male B alone
2nd part of M2	5 min		

For each virtual male stimulus, we measured the absolute association time (in seconds) a focal female spent within a mate-choice zone with a stopwatch. Association time is a well-established measure to determine mate choice in sailfin mollies when no direct contact is provided (Witte and Noltemeier 2002; Witte and Klink 2005; Nöbel and Witte 2013; Gierszewski et al. 2017). Association time is an indirect measure of female mate preference and several studies showed that the time females spent with a male was positively correlated with the probability of copulation with that same male in different species of fish (Bischoff et al. 1985; Forsgren 1992; Berglund 1993; Kodric-Brown 1993). After Pruett-Jones (1992), focal females were considered to copy the choice of the model female if they showed a significant increase in time spent with a prior non-preferred virtual male after having observed this same male together with a virtual model female. An increase in association time directly results in a higher probability of mating with that same male.

After testing, we measured the standard length (SL) of each focal female to the nearest millimeter using scale paper and took digital pictures (Pentax Optio W80, RICOH IMAGING, Germany) of each female in a small tank (26 cm x 16 cm x 17.5 cm) with a uniform light background. Following a similar approach as described by Dosen and Montgomerie (2004) for color quantification in guppies, we used the free image processing and analysis tool ImageJ (version 1.51j8; https://imagej.nih.gov/ij/) to quantify a female's gravid spot area relative to her total body surface area (excluding fins; Fig. 27). By this, we were able to test whether the extent of a focal female's own gravid spot, at the time of the experiment, might have affected her copying behavior.

We observed that the laterally visible spot area may differ between the left and right side of a female's body. Therefore, we took pictures of both the left and right side of each female. Further, since females were able to swim freely in the small tank, we took each picture twice and calculated mean values for each measurement. For this, we assessed the average visible gravid spot area (visible gravid spot area = average spot area measured from two pictures of the left side + average spot area measured from two pictures of the right side) in relation to the overall visible average body surface area (visible body surface area = average surface area measured from two pictures of the left side + average surface area measured from two pictures of the left side) for each focal female. All fish were later returned to their home tanks.

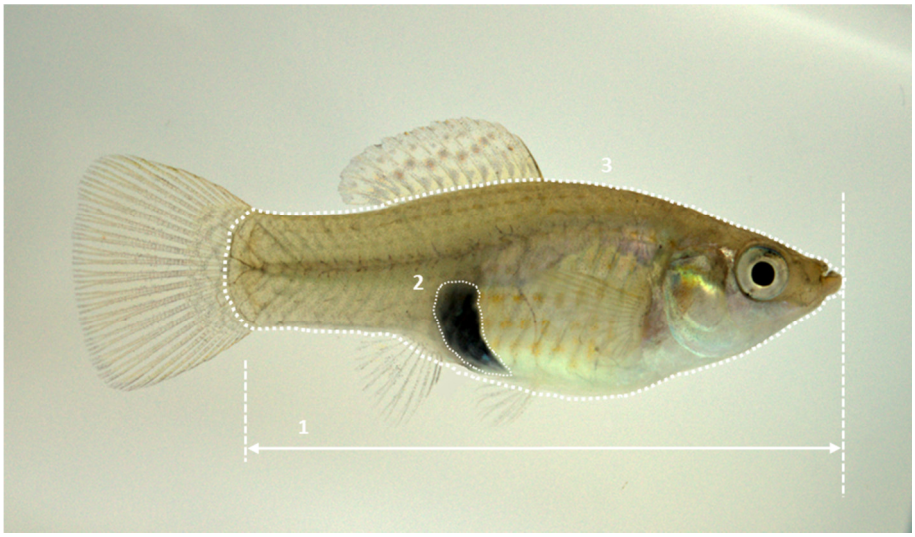

Figure 27. Scheme for gravid spot measurement in focal females. Gravid spot measurement was done based on lateral photographs of focal females taken after experiments. Given are measuring points calculated for each focal female sailfin molly: (1) Standard length, (2) gravid spot area, (3) total body surface area. Markings only serve as a means of illustration and may not be perfectly accurate.

7.3.4 Treatments

To evaluate the effect of the presence or absence of the gravid spot in virtual model females on MCC in live focal females, we performed two different treatments and a control as described below. Both treatments and the control differed in what was presented during the observation period but the general experimental procedure was the same throughout. Focal females were randomly assigned to either the "spot" or "no spot" treatment (see below). The control was performed after the two treatments had been finished.

Treatment 1: virtual model female with a gravid spot ("Spot")

During the observation period in Treatment 1, the prior non-preferred virtual male was presented together with a virtual model female with a gravid spot.

Treatment 2: virtual model female without a gravid spot ("No spot")

In Treatment 2, we presented the identical virtual model females but this time without a gravid spot together with the prior non-preferred virtual male. Behavior of the two virtual fish was identical to that shown in Treatment 1.

Control for choice consistency

During the control, the procedure was identical to that in both treatments. No virtual model female was visible during the observation period; however, the behavior expressed by the virtual stimulus males was identical to that shown in the two treatments.

7.3.5 Computer animation design

We created and animated 3D virtual fish stimuli using *FishSim* (Gierszewski et al. 2017; Müller et al. 2017; see Chapter 4). We created all virtual fish stimuli on the basis of high-quality lateral photographs of male and female sailfin mollies of the Mustang Island population in the lab. To prevent pseudo-replication (Hurlbert 1984; Chouinard-Thuly et al. 2017) and to present a natural variation of virtual stimuli, we mixed body and fin textures of the same sex and manipulated body size to create unique virtual fish stimuli. Thereby, we created 30 different virtual stimulus males for the presentation during mate-choice tests, resulting in 15 unique pairs of male stimuli. Males were of similar quality and of large body size with a virtual standard length (measured on screen) of 43 ± 1.4 mm (range: 41-45 mm). It was shown that females prefer larger over smaller males with a detectable size difference of > 5 mm (Witte and Ryan 1998). Therefore, size difference between virtual males presented to focal females was less than 5 mm.

We further created a set of n_S = 15 virtual model females with a prominent gravid spot, and n_{NS} = 15 identical virtual model females without a gravid spot. For this, we manipulated the textures used by *FishSim* with the free picture editing software GIMP. We chose a reference picture of a female having a very large gravid spot; relative gravid spot area of the reference picture was 4.7 % of the total body surface (excluding fins; measured with ImageJ as described above). We cut the female's dark gravid spot area of the picture and inserted the same gravid spot onto each model female's body texture as a new picture layer (Fig. 28; Manipulated texture for "Spot" treatment). This procedure ensured that, during experiments, the area of the gravid spot had the same size and identical position on each virtual model female. To create model females without a gravid spot, we used the same textures but removed already existing gravid spots by replacing them with body texture from the surrounding abdominal area of each female picture using the stamp tool in GIMP (Fig. 28; Manipulated texture for "No spot" treatment). All virtual model females had a virtual SL of 50 mm.

During animation, virtual fish stimuli were simulated to be swimming in a virtual tank environment. The virtual tank had brownish gravel at the bottom resembling the gravel inside the experimental tank, a grey stone object, and a light background (240, 243, 218 RGB; Fig. 29). Three different computer animations were created using *FishSteering* (Chapter 1.4.3): (I) a virtual male fish swimming alone inside the virtual tank and seemingly courting the live focal female "through the screen" for 7:30 minutes; (II) a similar scene of single courting male for 5 minutes; and (III) a virtual male and a virtual model female swimming together in the virtual tank and mutually engaged in courtship behavior for 10 minutes. To standardize the behavior of the virtual stimuli

throughout the experiment, the identical swimming paths were used in every trial and every experiment but the virtual stimuli could be replaced by different, prior created virtual males and model females. Animated sequences were then loaded into *FishPlayer* (Chapter 1.4.4) for the presentation during experiments as described above. During acclimatization and during pause sequences for fish handling, only the empty virtual tank was shown.

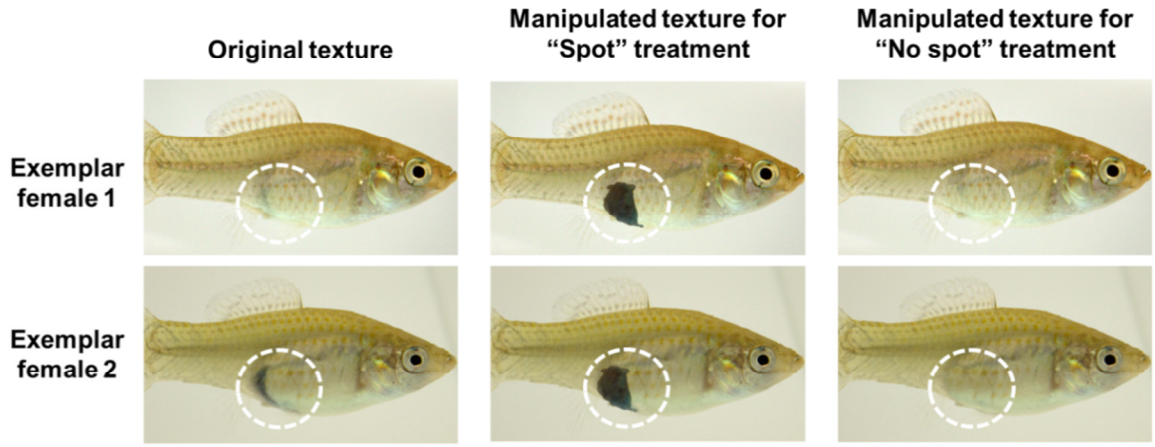

Figure 28. Exemplar pictures of two different female body textures prior to (original) and after manipulation for the "spot" and "no spot" treatments. The dotted circle marks the area that was digitally manipulated.

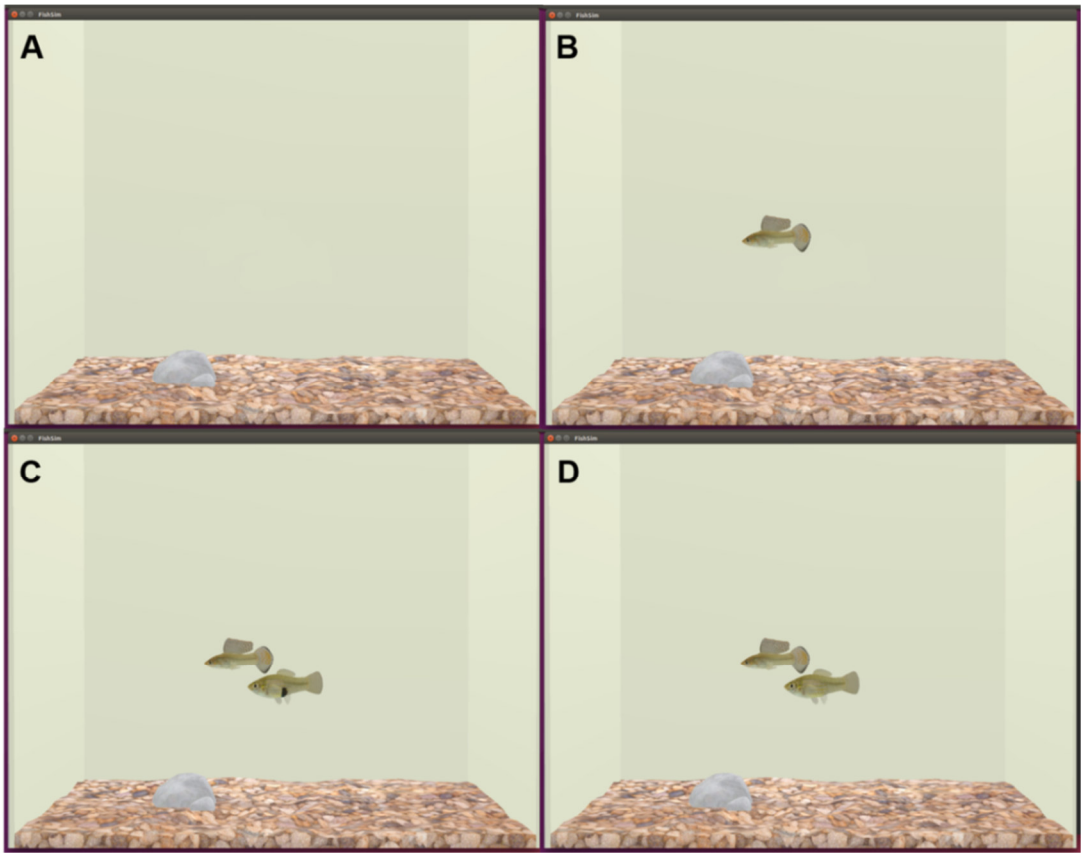

Figure 29. Screenshots of a scene in *FishSim*. (A) The empty default scene without a fish, (B) a scene showing a male alone, (C) a scene showing that same male together with a model female with a spot, and (D) a scene showing the identical male and the identical model female without a spot.

7.3.6 Data analysis

For data analysis, we used R 3.2.2 (R Development Core Team 2015). We uploaded the raw data we obtained in our MCC experiment as well as the R-code we used for our analysis to Figshare (doi: 10.6084/m9.figshare.6792347). We compared the standard length (SL) of focal females across treatments using a Kruskal Wallis rank sum test for independent data.

For each treatment and the control, we used association time to analyze whether the choosing motivation differed between the first and second mate-choice test (M1 and M2) using a paired Wilcoxon signed-rank test. Choosing motivation was defined as the total time a focal female spent in both choice zones within a mate-choice test. A change in choosing motivation does not necessarily reflect a change in preference for either male. However, if choosing motivation differed between M1 and M2, we used preference scores instead of absolute association time for further analysis to ensure comparability within and between treatments.

For each mate-choice test (M1 and M2), we calculated a preference score for the preferred and non-preferred male as the time a focal female spent with a male divided by the time she spent with both males in the mate-choice zones. Preference scores for the prior non-preferred stimulus male were further used to test whether these scores changed from M1 to M2 when public information was provided, compared to the control treatment in which public information was absent. For this, we fit a linear mixed effect (LME) model with the *lme* function from the 'nlme' package (Pinheiro et al. 2015) with preference score for the prior non-preferred male (pref_NP) as the dependent variable. We included mate-choice test (Mtest: M1, M2) and treatment (treatment: spot, no spot, control) as fixed factors as well as focal female's standard length (SL) as a covariate. To account for the repeated measures design, focal female identity (ID) was included as a random factor. We were especially interested in whether the effect of mate-choice test was different among treatments; therefore, we included an interaction between mate-choice test and the treatment in our model. We conducted two orthogonal comparisons for "treatment" using the function *contrasts* (Crawley 2007). We set the contrasts of the model (I) to compare the control against the mean of all treatments in which any virtual model female was presented during observation [control → (spot, no spot)], and (II) to compare the treatment showing a virtual model female with spot against that without a spot (spot → no spot). A plot of the standardized residuals of a factor against the fitted values revealed heteroscedasticity of the residual variances for "Mtest". Therefore, we included a weights function using the varIdent class of the *lme* function to allow for different variances for each level of "Mtest" (Pinheiro and Bates 2000; Zuur et al. 2009). We used the R package 'phia' (De Rosario-Martinez 2015) for a post hoc analysis with Holm-Bonferroni correction of significant interaction terms.

We further analyzed whether copying scores for the prior non-preferred male were different across treatments. The copying score for a male describes the change in female preference for a male from the first to the second mate-choice test. The copying score is defined by the preference score of a male in the second mate-choice test minus the score of that same male in the first mate-choice test. Copying scores range between -1 and +1 and can either be positive or negative values in which negative values describe a decrease in preference and positive values an increase in preference for that male. Here, we fit an LME with copying score (copy_NP) as the dependent variable, treatment as a fixed factor, focal female SL as a covariate and focal female's spot area (spot_area) as a random factor. Here, we conducted the same two orthogonal comparisons for "treatment" as described above. To test whether copying scores were correlated with a focal female's relative gravid spot area, we performed a Spearman rank correlation for each treatment separately.

Additionally, we tested whether the number of focal females that reversed their initial mate preference in M2 differed across treatments. Mate-choice reversal is defined as whether there is a change in the preference for a male (from less than 50% to more than 50% of the time in both choice zones) from the first to the second mate-choice test. Mate-choice reversal is counted as a "Yes" (preference for a male has changed) or a "No" (preference for a male did not change). Here, we performed a post hoc pairwise G-test using the R package 'RVAideMemoire' (Hervé 2017) with correction for multiple testing.

We inspected model assumptions (Q/Q-plots, residuals, residuals against fitted values) for all models visually (Korner-Nievergelt et al. 2015). We further compared the distribution of the residuals against a normal distribution using Shapiro-Wilk normality tests. The given p-values were considered significant if $p \leq 0.05$.

7.4 Results

We tested a total number of n = 55 focal females. Two females had to be excluded due to technical problems during testing. One female was excluded due to stress since she did not acclimate to the test situation and was too afraid to enter either choice zone. We further excluded seven females from the final analysis due to their side bias in the first mate-choice test. Altogether, we analyzed a total of n = 15 focal females for each treatment and the control. Focal females had a mean SL of 32 ± 5 mm in Treatment 1, 33 ± 5 mm in Treatment 2 and 33 ± 3 mm in the control for choice consistency. SL did not differ between treatments and the control (Kruskal Wallis rank sum test: n = 45, χ^2 (2) = 0.329, p = 0.848). Due to their asynchronous reproductive cycle (Sumner et al. 1994), there was high variability in the relative gravid spot area among focal females. Of 45 focal females that were analyzed, 27 focal females had no visible gravid spot. Of the remaining 18 focal females, who had a visible gravid spot at time of the experiment, the average gravid spot area was 0.97 ± 0.75 % (range: 0.11 % - 2.85 %), in relation to their total body surface area (excluding fins). Overall, the gravid spot area in focal females was considerably smaller than in the virtual model female (4.7 %).

In our study, choosing motivation of focal females before and after observation of a virtual model female sexually interacting with a male did not differ in Treatment 1 (Wilcoxon signed rank test: n = 15, V = 44, p = 0.379) and in the control for choice consistency (Wilcoxon signed rank test: n = 15, V = 42, p = 0.33). However, choosing motivation was significantly higher after observation of a virtual model female without gravid spot, sexually interacting with a male in Treatment 2 (Wilcoxon signed rank test: n = 15, V = 22, p = 0.03). We, therefore, analyzed preference scores instead of absolute association time.

For preference scores of the prior non-preferred male, we found a significant interaction between M2 and the contrast "[control → (spot, no spot)]" for preference scores of the prior non-preferred male (LME: df = 42, t = -2.74, p = 0.009). However, preference scores were not affected by focal female SL. Further post hoc analysis of the interaction term revealed a significant difference of preference scores of the prior non-preferred male in M2 in Treatment 1 (spot: df = 1, χ^2 = 30.986, p < 0.001) and Treatment 2 (no spot: df = 1, χ^2 = 19.957, p < 0.001) but not for the control (control: df = 1, χ^2 = 2.747, p = 0.097). The results of this analysis are given in Figure 30A and Tables 8 and 9.

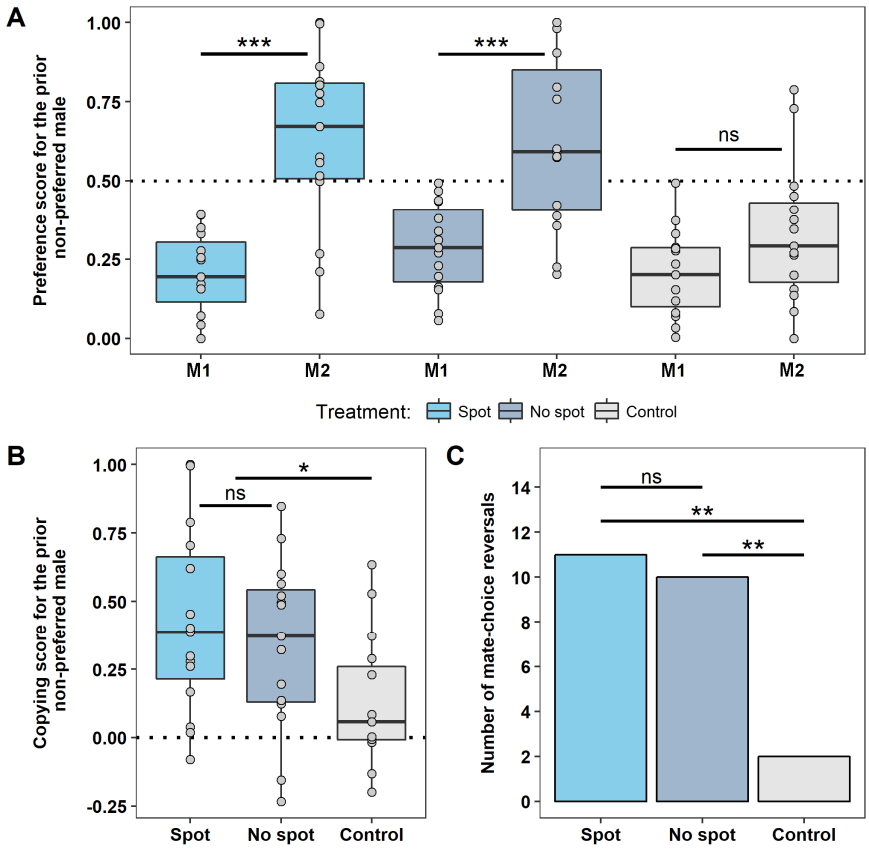

Figure 30. Results of the virtual MCC experiment manipulating model female quality by the visual absence or presence of a gravid spot. (A) Preference scores for the (prior) non-preferred virtual stimulus male in M1 and M2 for both treatments and the control. (B) Change of preference from M1 to M2 (copying score) for the prior non-preferred virtual male in the treatments and in the control. The dotted line depicts no change in preference, positive values show an increase in preference and negative values show a decrease in preference. Grey dots in A and B depict raw data of each focal female. (C) Number of mate-choice reversals in M2 for each treatment and the control. M1 = first mate-choice test, M2 = second mate-choice test, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. N = 15 for both treatments and the control.

Table 8. LME estimates for effects on preference scores for the prior non-preferred virtual male. Preference score for the prior non-preferred virtual male stimulus was the dependent variable throughout. Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor. Intercept estimates represent the grand mean of all treatments. Orthogonal comparisons of treatments are given. If treatments are combined in parentheses, mean values of these treatments are used in the comparisons. The intercept reference category for factor "M2" is "M1". Significant p-values ($p < 0.05$) are printed in bold. M1 = first mate-choice test, M2 = second mate-choice test, SL = standard length of focal females. 90 observations with n = 15 focal females per treatment.

Factors	Lower	Estimate	Upper	SE	*df*	*t*	*p*
(Intercept)	0.046	0.339	0.632	0.145	42	2.336	**0.024**
M2	0.207	0.296	0.384	0.044	42	6.750	**<0.001**
Control → (spot, no spot)	-0.041	-0.012	0.017	0.014	41	-0.852	0.4
Spot → no spot	-0.093	-0.043	0.008	0.025	41	-1.715	0.094
SL	-0.012	-0.003	0.006	0.004	41	-0.747	0.459
M2 x [control → (spot, no spot)]	-0.148	-0.085	-0.023	0.031	42	-2.743	**0.009**
M2 x (spot → no spot)	-0.067	0.042	0.15	0.054	42	0.777	0.441

Table 9. LME variance components for focal female ID. Variance and standard deviation for the random effect "focal female ID" and the residuals are given.

Random factor	Variance	SD
ID ((Intercept))	1.464×10^{-10}	1.21×10^{-5}
Residual	1.859×10^{-2}	0.1364

As we show in Figure 30B and Tables 10 and 11, we found a significantly higher copying score for the prior non-preferred male in treatments with a virtual model female compared to the control (LME: df = 20, t = -2.833, p = 0.01) but no significant difference between treatments (LME: df = 20, t = 0.618, p = 0.544). Copying scores were not affected by focal female SL. Copying scores were also not correlated with focal female's relative gravid spot area in Treatment 1 (Spearman rank correlation: n_{cscore} = 15, n_{spot_area} = 15, rho = 0.198, p = 0.48) or Treatment 2 (Spearman rank correlation: n_{cscore} = 15, n_{spot_area} = 15, rho = -0.365, p = 0.181).

Table 10. LME estimates for effects on copying scores for the prior non-preferred virtual male. Copying score for the prior non-preferred virtual male stimulus was the dependent variable throughout. Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor. Intercept estimates represent the grand mean of all treatments. Orthogonal comparisons of treatments are given. If treatments are combined in parentheses, mean values of these treatments are used in the comparisons. Significant p-values ($p \leq 0.05$) are printed in bold. SL = standard length of focal females. 45 observations with n = 15 focal females per treatment.

Fixed factors	Lower	Estimate	Upper	SE	*df*	*t*	*p*
(Intercept)	-0.889	-0.081	0.727	0.389	21	-0.208	0.837
Control → (spot, no spot)	-0.153	-0.088	-0.023	0.031	20	-2.833	**0.01**
Spot → no spot	-0.079	0.033	0.146	0.054	20	0.618	0.544
SL	-0.013	0.011	0.035	0.011	20	0.991	0.333

Table 11. LME variance components for focal female spot area. Variance and standard deviation for the random effect "spot_area" and the residuals are given.

Random factor	Variance	SD
spot_area ((Intercept))	0.028	0.166
Residual	0.075	0.275

The number of focal females that reversed their initial mate choice in favor of the prior non-preferred male in M2 was significantly larger in both treatments compared to the control (post hoc pairwise G-test: Spot vs. control, p = 0.002; no spot vs. control, p = 0.003) but not significantly different between Treatments 1 and 2 (Post-hoc pairwise G-test: spot vs. no spot, p = 0.69); see Fig. 30C).

7.5 Discussion

The gravid spot in sailfin molly females was previously described to serve as a means of fertility advertisement towards conspecific males (Farr and Travis 1986; Sumner et al. 1994). Whether a gravid spot may also provide information to conspecific females in the context of mate choice had not been tested so far. In the present case study, we investigated the potential role of a gravid spot as a source of public information for

observing conspecific females in the context of MCC. Our study shows that the gravid spot seems not to be a sign of model female quality for live focal females when deciding to copy the mate choice of a virtual model female for a virtual male. Focal females copied the choice of a virtual model female for a prior non-preferred virtual male regardless of whether the model female had a gravid spot or not. We found no difference in copying scores nor the number of mate-choice reversals between the two treatments, indicating that the copying effect was also equally strong whether the model female had a gravid spot or not. When no public information was provided in the control (no model female present), focal females were consistent in their mate choice. This supports that the observed change of preference within treatments can be explained by the presence of the virtual model female only, providing sufficient public information for copying the mate choice of others.

Even though the general presence and extent of the gravid spot are considered to be linked to a female's reproductive cycle, with the spot being largest prior to parturition and smallest or absent after giving birth (Sumner et al. 1994), systematic visual observations of the development of gravid spots in individual females are still missing. Even though sailfin molly females are most receptive short after parturition (Farr and Travis 1986; Travis 1989), they are able to store sperm for several months (Constantz 1989; Gierszewski, personal observation). Since insemination is possible at any time, females should always be choosy for the best quality mate. Although, Gabor and Page (2003) found female sailfin mollies were most choosy during their most receptive state (postpartum, i.e., most likely without spot as per definition by Sumner et al. 1994), even under risk of predation. Indeed, focal females in our study were significantly more motivated to choose between males after the observation period in the "no spot" treatment. Therefore, a model female without spot might have been recognized as being choosier, which would consequently render her choice for a male more valuable. Another viewing angle might include aspects of female competition to affect MCC (see also Chapter 8.5 for ideas on this viewpoint). If a model female in the "no spot" treatment was identified as immature (Farr and Travis 1986), observing focal females might have treated her as a low competitor (compared to the model with gravid spot) and were, consequently, more motivated to choose. Nonetheless, focal females copied the choice of a model, irrespective of the presence or absence of a gravid spot.

As postulated by Farr and Travis (1986), the gravid spot indicates sexual maturity in general, which would consequently also include females that just reached maturity but are still not experienced in mate choice yet. Variation in gravid spot size can also be high among individual females, with spots also being completely absent in otherwise mature and gravid females, as found by Sumner et al. (1994; also compare MP20 in Table S2, Appendix 1). Information on the general nature of gravid spots in sailfin mollies found in the literature is, therefore, quite mixed. With regards to our study on MCC and the tested hypotheses, we conclude that a gravid spot may not be a reliable visual indicator of model female quality to observing sailfin molly females. Here, we need to point out that we solely presented visual stimuli throughout. Olfactory cues were, therefore, completely absent. Sumner et al. (1994) showed that male sailfin mollies were only able to distinguish between receptive and non-receptive females if they were able to access olfactory cues as well. Future studies testing the effect of a model female's reproductive status on MCC might, therefore, benefit from a multimodal approach combining visual stimuli with olfactory cues in experiments (see e.g., Mehlis et al. 2008 and Thünken et al. 2014 for multimodal approaches using computer animation in fish).

Notably, our study demonstrates a highly standardized procedure for visual manipulation of public information provided in MCC experiments by using computer animated fish. We show that virtual model females provided valuable visual information to observing live focal females which led to copying behavior. In contrast, copying behavior was absent in the control when no virtual model female was present which serves as an additional

validation of our method. In contrast to an earlier study by Benson (2007), who injected live fish with tattoo ink to manipulate gravid spots, our method provides a completely non-invasive alternative for visual manipulation in animal behavior research.

7.6 Acknowledgements

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Chapter 8

Differences in courtship of an interacting pair affect mate-choice copying in observing sailfin molly females

Stefanie GIERSZEWSKI[a], Klaus MÜLLER[b], Jan-Marco HÜTWOHL[b], Klaus-Dieter KUHNERT[b] & Klaudia WITTE[a]

[a]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein-Straße 2, Siegen, 57076, Germany and [b]Institute of Real-Time Learning Systems, Department of Electrical Engineering & Computer Science, University of Siegen, Hölderlinstraße 3, Siegen, 57076, Germany

This chapter is a manuscript draft intended for publication.

8.1 Abstract

Social learning enables individuals to gather public information from observing the mate choice of others and then use this information for their own mate-choice decisions, e.g., by copying the same choice. Mate-choice copying is defined as non-independent mate-choice strategy, in which an individual's probability of choosing (or rejecting) a mate increases if other individuals have chosen (or rejected) that mate previously. Differences in both quality and quantity of public information may affect whether or not copying occurs. Sailfin molly females (*Poecilia latipinna*) often copy the mate choice of other females when observing them sexually interacting with males. To decipher which partner, the observed male or female, provides most valuable information for copying, we manipulated information provided by an interacting pair to observing focal females in a mate-choice copying experiment. Using computer animation, we varied the extent and direction of courtship behavior by either simulating (I) mutual, (II) female driven or (III) male driven courtship during the observation period. We further tested whether (IV) presence or (V) absence of male courtship per se affected female mate choice when no model female was present. Focal females copied the mate choice whenever a model female was present; however, the copying effect was strongest in the male driven courtship scenario, and weaker in the mutual and female driven courtship scenarios. When no model female was present (no public information), focal females chose consistently when males were actively courting them. In contrast, a lack of male courtship decreased focal female's preference for a previously preferred male. Our results provide first evidence that the strength of copying might be negatively affected when observing high sexual activity of the interacting pair, as well as highly sexually engaged model females. We suggest that sexually active model females might be recognized as strong competitors for focal females, leading to a weaker copying effect. This relationship has so far not been tested before and offers new lines of research on mate-choice copying in sailfin mollies.

8.2 Introduction

The study of mate-choice copying (hereafter abbreviated as MCC) has fascinated scientists for decades since Lee A. Dugatkin demonstrated that female guppies (*Poecilia reticulata*) did not choose a mating partner independently but instead utilized information gained from observing the mate choice of conspecific females for their own mate-choice decisions (Dugatkin 1992). MCC is an alternative mate-choice strategy that animals may use while evaluating potential mating partners. An individual may observe a conspecific or heterospecific – the "model" – in his or her mate choice and thereby gather so-called public information (Nordell and Valone 1998). If the observing animal then decides to use this information and mates with (or rejects) the same individual or the same phenotype (Kniel, Dürler, et al. 2015), as the model previously did, we speak of copying (Witte et al. 2015). MCC is a form of social learning (Witte and Nöbel 2011) and occurs in both vertebrates (Höglund et al. 1995; Galef et al. 2008; Frommen et al. 2009; Kniel, Dürler, et al. 2015; Gouda-Vossos et al. 2018) and invertebrates (Mery et al. 2009; Dagaeff et al. 2016; Danchin, Nöbel, et al., 2019). MCC is not limited to laboratory experiments but was also found in the wild (Witte and Ryan 2002; Goulet and Goulet 2006; Alonzo 2008; Godin and Hair 2009) which validates it as a biologically relevant mate-choice strategy. Even though MCC was predominantly studied in females, it is also deployed by males (Schlupp and Ryan 1997; Nöbel, Allain, et al. 2018); however, sexes may differ in whether or not they show copying behavior (Widemo 2005; Kniel, Dürler, et al. 2015). MCC is considered to contribute to the evolution of (novel) phenotypical traits and may play an important role in speciation and hybridization (Danchin et al. 2004; Verzijden et al. 2012; Kniel, Dürler, et al. 2015; Varela et al. 2018). Especially, since not only the choice for a particular individual was copied but new socially learned preferences (e.g., for a novel trait) were even generalized to other individuals (Witte and Noltemeier 2002; Godin et al. 2005; Kniel, Dürler, et al. 2015).

MCC is assumed most valuable when the costs involved alongside mate choice (e.g., time spent for mate searching or predation risk) are high or if two potential mates are very similar in quality (Pomiankowski 1987; Nordell and Valone 1998; Wong and Jennions 2003; Frommen et al. 2009). However, MCC also bears potential costs for the observer, which consequently may lead to maladaptive decisions and reduced fitness (Dubois et al. 2011; Nöbel, Danchin, et al. 2018; Witte et al. 2018). The observer therefore needs to assess different aspects about quantity and quality of public information to evaluate whether or not a source is reliable. For example, an increase in information quantity (i.e., increasing the number of model females or observation time) may favor copying behavior and even result in mate-choice decisions that are in contrast to predisposed heritable preferences (Dugatkin 1998; Witte and Noltemeier 2002). The model itself also plays an important role for the observer in deciding whether to copy its mate choice or not. Certain characteristics can define the quality of a model as being "good" or "poor" regarding how reliable his or her choice is in finding the best mating partner (e.g., body size and age: Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Vukomanovic and Rodd 2007; phenotype: Hill and Ryan 2006; Kniel et al. 2017). Further, different aspects about the behavioral interaction between the model and a potential mate may also affect whether or not copying occurs (e.g., differences in sexual interest: Wideomo 2005; physical contact or distance: Bierbach, Kronmarck, et al. 2011; Gierszewski, Keil, et al. 2018; or communication mode, i.e., visual and/or auditory: Kniel, Schmitz, et al. 2015). So far, it is still not known whose behavior of the interacting pair is most important for an observing individual. Does an observing female mostly tend to the behavior of the male or to that of the model female?

To further examine this question, we investigated how differences in courtship of an observed pair of sailfin mollies might affect mate-choice decisions in observing focal females. Restricting the expression of behavior by physical and/or visual barriers is an effective way to alter the interaction of a pair of live animals during experiments. However, it does not allow for the controlled manipulation of behavioral patterns expressed by single individuals, in particular that of courtship behavior. In our study, we performed, for the first time, a systematic manipulation of information quality (i.e., behavioral differences) provided during the observation period of a MCC experiment with the help of computer animation. Computer animation is a promising and non-invasive alternative method for behavioral manipulation and earlier studies demonstrated its usability for creating virtual animals whose behavior is under total control of the experimenter (Chouinard-Thuly, Gierszewski, et al. 2017; Chapter 3 in this thesis). In the context of mate choice, courtship vigor and intensity, as well as different levels of sexual activity, could be effectively altered using computer animation in studies with three-spined sticklebacks (*Gasterosteus aculeatus*; Künzler and Bakker 2001), Siamese fighting fish (*Betta splendens*; Clotfelter et al. 2006), Atlantic mollies (*Poecilia mexicana*; Bierbach, Jung, et al. 2013) and mosquitofish (*Gambusia holbrooki*; Sommer-Trembo et al. 2017).

In this study, we used the research tool *FishSim* Animation Toolchain (Gierszewski et al. 2017; Müller et al. 2017; see Chapter 1.4). We previously validated FishSim for the study of mate choice in sailfin mollies (Gierszewski et al. 2017; see Chapter 5) and showed that live females are able to assess public information provided by computer-animated (i.e., virtual) model females and copy their choice for a virtual male (Gierszewski, Baker, et al. 2018; Chapter 7). With the help of FishSim, we varied the extent and direction of courtship behavior expressed by the male and/or the model female partner by either presenting courtship that is (I) mutual, (II) only female driven, or (III) only male driven. Thereby, we wanted to decipher which information (courtship vs. no courtship), and from whom (male vs. model female), mostly influences the strength of copying behavior in focal females. From an earlier study by Witte and Ueding (2003) we know that focal females copy the rejection of a male when they have the opportunity to observe the model fleeing from that male. We hypothesize that the engagement (i.e., sexual interest) of the model female is most important for observing focal females in their decision whether or not to copy. We predict that, focal females will strongly express copying behavior if the model female is actively engaged in courtship: that is, when courtship is mutual or female driven. Additionally, we predict that focal females will only weakly express copying behavior when courtship is only male driven. When focal females have no opportunity to copy (i.e., when no model female is present and hence no public information available), we hypothesize that focal females will rely on the expression of male courtship per se (courting vs not courting) and alter their mate choice when virtual males do not show courtship.

8.3 Materials and Methods

8.3.1 Study species

In this study, we used mature female sailfin mollies descendant from wild mollies caught on Mustang Island (TX, USA) in 2014. In the lab, we kept fish in large mixed-sex housing tanks as described in detail in Chapter 2.3 in this thesis. Prior to experiments, we

separated groups of females in smaller tanks (40 cm x 25 cm x 40 cm) and kept them separated from males for two weeks to increase their choosing motivation.

8.3.2 Computer animation design

Using *FishCreator* (Gierszewski, Baker, et al. 2018; see Chapter 4.2), we created 30 different virtual males (virtual SL between 41-45 mm) resulting in 15 unique male stimulus pairings per treatment. Stimulus pairings were used only once within a treatment, but were reused between treatments in different order. Two different mating tactics exist among sailfin molly males, in which large colorful males tend to rely more on courtship prior to copulation, whereas small and inconspicuous males tend to rely more on sneaky matings (i.e., forced-copulations; Baird 1974; Luckner 1979; Snelson 1985; Seda et al. 2012; Fraser et al. 2014; Chapter 2.1.2). Therefore, we only presented males that were presumably dominant courting males, as indicated by their large body size and well developed and colorful caudal and dorsal fins. Further, we created 15 different virtual model females (virtual SL = 50 mm). We always presented large and therefore presumably high quality virtual model females (see Dugatkin and Godin 1993 and Vukomanovic and Rodd 2007 for guppies), even though body size has currently not been verified to serve as an indicator for model female quality for MCC in sailfin mollies (see Chapter 6).

We animated virtual fish using the tool *FishSteering* (see Chapter 4.3; Gierszewski, Baker, et al. 2018) and a video game controller (SONY Playstation 3 Wireless Controller; Sony Computer Entertainment Inc., Japan). Swimming behavior of virtual fish (e.g., undulatory movements, bending and speed) was generated automatically according to an algorithm based on videos of swimming live sailfin mollies (Smielik et al. 2015). Swimming speed of virtual fish varied between 0-40 cm/s, depending on the input given to the controller. Animations were rendered at a frame rate of 60 fps, which is considered above the estimated threshold for motion perception in fish (Fleishman and Endler 2000; Oliveira et al. 2000). Stimuli were always simulated to be swimming in a virtual tank environment with blue colored sand at the bottom and a grey-white background (240, 243, 218 RGB) to resemble the conditions found in the test tank (Figs. 31 and 32). Each animated sequence consisted of one or two swimming paths that were recorded for each virtual fish separately (male and/or female). A once recorded swimming path could be replayed with all prior created fish stimuli, thereby keeping the behavior constant across treatments but allowing for phenotypic variation.

For the use in all mate-choice tests (M1 and M2), we created one animated sequence of 7:30 minutes (2:30 min for focal females to observe and 5:00 min choosing time; compare Table 13 in Chapter 8.3.3 below) showing a single courting male. This sequence was identical across all treatments. Animated behavior of the virtual male resembled that of a live male swimming in a tank and interacting with a live female outside the tank as closely as possible: (I) swimming in varying heights and depths of the virtual tank; (II) parallel swimming at the front wall of the monitor screen; (III) lateral displays (showing the male's lateral side with raised dorsal fin); (IV) gonopodial thrusting or swinging; and (V) swimming up and down in a position vertical to the front, i.e., directed towards the prospective live focal female.

For the observation period, we created three different animated sequences of 10 minutes each, showing a male and a model female interacting. During experiments, visibility of the model female was only active together with the male that was not preferred by the

focal female in M1. Depending on the respective treatment, we varied the extent and direction of courtship by either presenting: a male and a model female mutually engaged in courtship (Treatment 1); a model female engaged in courtship and a male not courting/not interested (Treatment 2); a male engaged in courtship and a model female not interested (Treatment 3). Further, we used the identical sequences created for Treatments 1 and 2 to present variation in male courtship without presence of a model female during the observation. For this, we either presented a single male courting (Treatment 4) or not courting (Treatment 5) while automatically hiding the swimming path of the model female throughout. Specifics on the behavioral features presented in each treatment are given below (see Fig. 31 and Table 12).

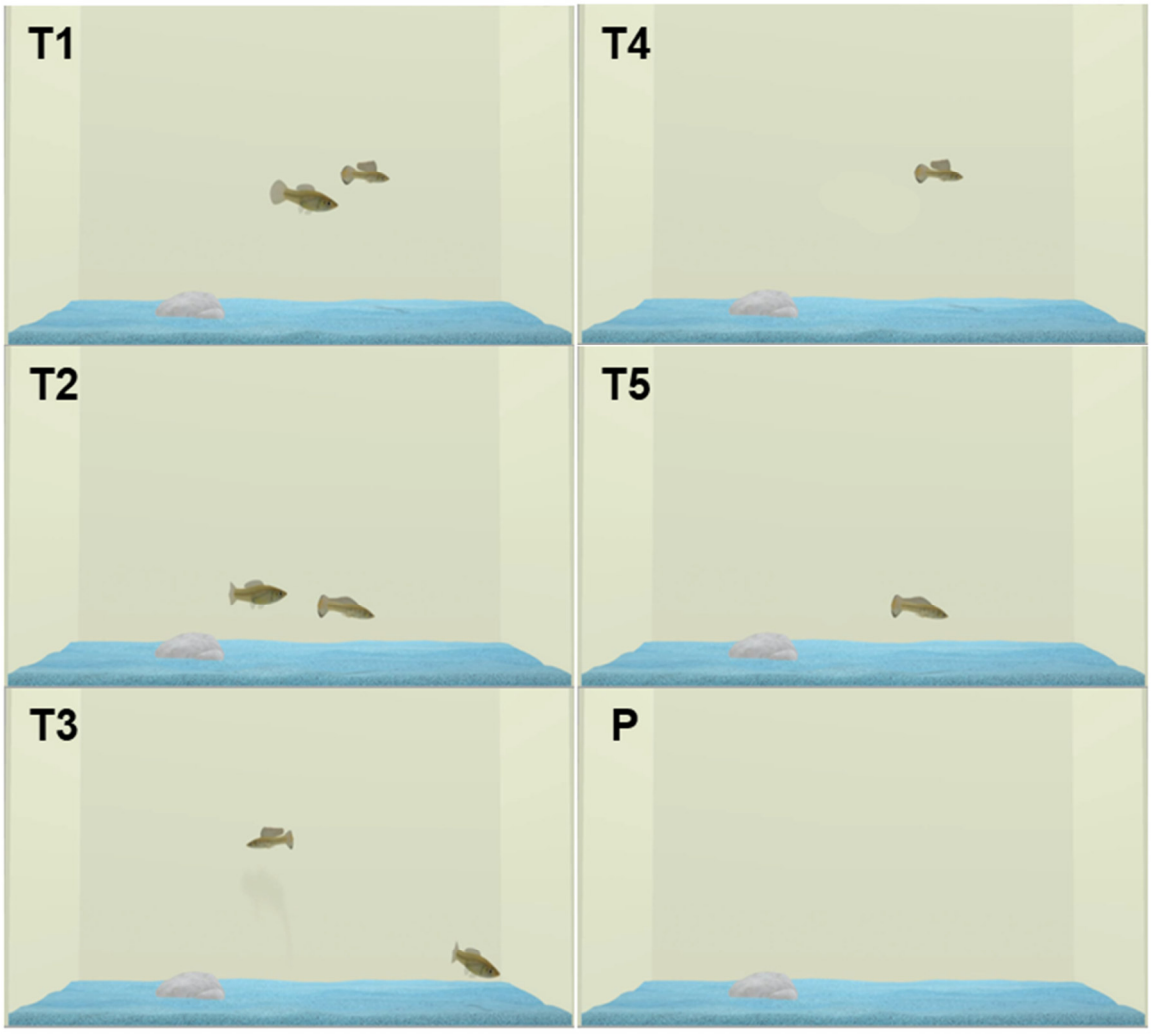

Figure 31. Screenshots of animated sequences used in the different stages of the MCC experiment. Screenshots show a virtual male and female swimming in a virtual tank environment. (T1) Mutual courtship. (T2) Female driven courtship, male not interested. (T3) Male driven courtship, female not interested. (T4) Actively courting male without model female. Similar to animations used during mate-choice tests. (T5) Male alone and not courting. (P) Empty virtual tank for acclimatization or handling time (pause).

Treatment 1 (T1): mutual courtship

For T1, we simulated the virtual male and virtual model female to be both mutually engaged in courtship and tried to reproduce natural courtship behavior of sailfin mollies as closely as possible (see Parzefall 1969; Baird 1974; Luckner 1979; Chapter 2.1.2 in this thesis). The male always followed the model female and they were actively swimming alongside each other in close proximity most of the time. The male frequently performed lateral displays (i.e., showing his lateral side with raised dorsal fin) next to and in front of the female (Fig. 31, T1). We tried to indicate gonopore nipping by the male as well as gonopodial thrusts, of which most were in close proximity to the virtual model female and may have been perceived as attempted copulations by observing focal females. In contrast to males, females do not actively court but may deliberately approach and follow them (Greven 2005). Overall, female behavior determines to a large extent whether copulation occurs or not. A responsive female frequently swims slowly away from a courting male without showing fleeing or escape behavior. She may engage in copulation by stopping and moving her body sideways presenting her urogenital area towards the male to facilitate a successful insertion of the gonopodium (Baird 1974; Luckner 1979; Greven 2005). Accordingly, the animated virtual female was gently swimming away from the virtual male and occasionally stopping in front of him to engage copulation (see Table 12 for an overview of male and female behavioral features). Mutual courtship as presented in this treatment was very similar to that presented in a previous study on MCC in sailfin mollies using the same method (Gierszewski, Baker, et al. 2018).

Treatment 2 (T2): female driven courtship

For T2, we simulated only the virtual model female to be actively engaged in courtship. Usually, males are observed being more engaged in courtship (see T1 above), but also females may deliberately approach them when they are eager to mate, e.g., virgins, post-partum females and females that have been separated from males (Baird 1974; Bisazza 1993 and references within). The virtual female actively followed and frequently approached the male, swimming in front of him and stopping to engage copulation. The male mostly swam away from her, never raised his dorsal fin, and never thrusted his gonopodium (see Fig. 31, T2 and Table 12).

Treatment 3 (T3): male driven courtship

For T3, we simulated only the virtual male to be actively engaged in courtship. Male courtship behavior was identical to that presented in T1, except that neither gonopore nipping nor copulations occurred (Table 12). The virtual model female frequently changed directions and swam slowly away from the male. She spent more time nipping on the ground or swimming alongside the tank walls. The female never stopped in front of the male to engage copulation and both fish were generally more often swimming apart from each other (Fig. 31, T3). In contrast to a study by Witte and Ueding (2003), in which video recordings of females showed escape behavior (e.g., thrashing away very fast with their fins clenched tightly to their body) we tried to simulate lack of sexual interest without fleeing.

Treatment 4 (T4): male actively courting

For T4, male courtship behavior was identical to that presented in T1 (mutual courtship) and T3 (male driven courtship) except that the model female was not visible (Fig. 31, T4 and Table 12). Focal females therefore would not have access to additional public information to evaluate the two males.

Treatment 5 (T5): male passive and not courting

For T5, male courtship behavior was identical to that presented in T2 (female driven courtship) in which he was very passive and never raised his dorsal fin or thrusted his gonopodium (Fig. 31, T5; see Table 12). The model female was not visible.

Table 12. Overview of all behavioral features of virtual fish visible during the observation period in Treatments 1-5. Given is the amount of animated behavioral features of male and model female visible to observing live focal females during the 10 minutes observation period. The general activity level of each virtual fish is given with regard to swimming speed and number of actions performed (behavioral features). A = activity level, DF = dorsal fin raising, GN = gonopore nipping, GT = gonopodial thrusts, C = copulation, S = stops to engage copulation.

	Virtual male					Virtual model female	
Treatment	**A**	**DF**	**GN**	**GT**	**C**	**A**	**S**
T1[1]	High	120	10	45	32	High	5
T2[2]	Low	0	0	0	0	High	12
T3[3]	High	120	0	45	0	Low	0
T4[4]	High	120	0	45	0	/	/
T5[5]	Low	0	0	0	0	/	/

[1]Mutual courtship; [2]Female driven courtship; [3]Male driven courtship; [4]Male alone, courting; [5]Male alone, not courting.

8.3.3 General experimental procedure

We followed the experimental protocol for testing MCC in fish using *FishSim* as described in detail by Gierszewski, Baker et al. (2018; see Appendix 5). All experiments were performed from June 2017 to May 2018 in the same experimental room (The virtual fish lab; see Chapter 2.3.2), using the identical experimental setup throughout (Fig. 32). The test tank (100 cm x 50 cm x 40 cm) was divided into three different zones, two mate-choice zones (20 cm in depth; C in Fig. 32) at the outer sides of the tank and a neutral zone in the middle (60 cm). We covered the bottom of the tank with a thin layer of blue colored sand and the rear tank wall was covered with a blue plastic sheet. We positioned a 24″ LCD monitor (EIZO Foris FX2431, EIZO Nanao AG, Austria, 1920 x 1200 pixel resolution) on either side of the test tank (adjacent to each mate-choice zone) for the presentation of computer animations. Monitors had an approximate distance of 2 cm from the glass wall. We illuminated the test tank with eight LED strips (12V, 6500K; combined in pairs of two) positioned above the longer sides of the tank. We installed two cameras (Prosilica GT1910c, Allied Vision Technologies GmbH, Germany) in front of and above the test tank to provide visual tracking and video recording of live focal fish with a custom-made real-time 3D tracking system (Müller et al. 2014; Müller,

Gierszewski, et al. 2016; see Fig. 32). We used two personal computers during experiments: one to run the stimulus presentation on both monitors with *FishSim* and a second to simultaneously record and track focal fish. Computer terminals were operated from a position not visible to the focal fish to prevent them from being stressed or influenced by the observer.

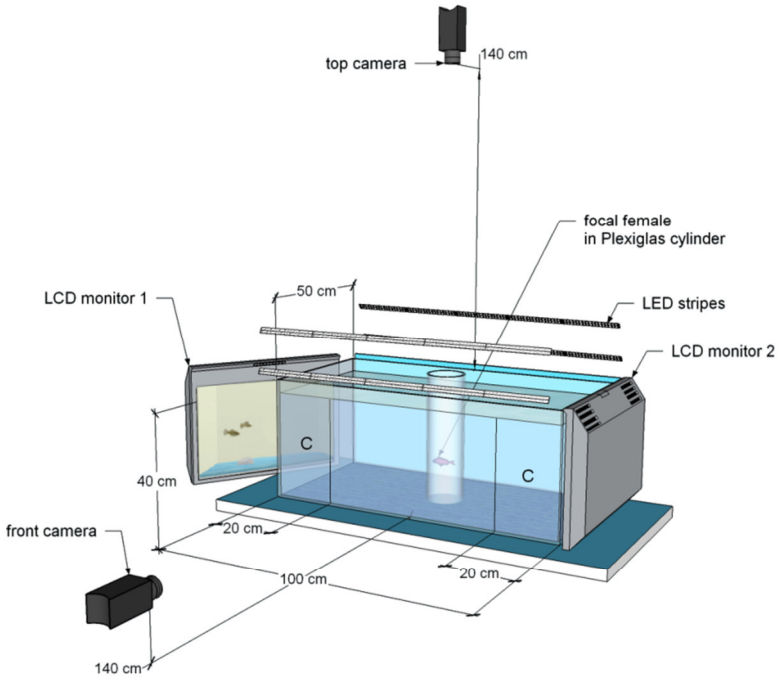

Figure 32. Overview of the experimental setup (situation of the observation period). Test tank showing a live focal female inside a Plexiglas cylinder (11 cm in diameter) in the middle. Two LCD monitors are positioned on either side of the tank as well as two cameras at its top and front. For illustration, the left monitor is angled to show an animated scene. C = mate-choice zone.

We used the tool *FishPlayer* (Chapter 4.4; Gierszewski, Baker et al. 2018) for automatic stimulus presentation. Here, prior created animated sequences were organized into playlists for each monitor separately and presented to live focal females during all experimental trials (Table 13). Stimuli were always presented simultaneously in a binary choice situation according to a pseudo-random table.

We started each trial with an acclimatization period of 30 minutes, in which a single focal female was allowed to swim freely and explore the test tank. An empty virtual tank was visible on both monitors throughout acclimatization (Table 13, Fig. 31P) so the female could get accustomed to the illumination emitting from the monitors. After acclimatization, we gently captured the focal female inside a Plexiglas cylinder in the middle of the tank (Fig. 32), ensuring an equal distance to both monitors. We commenced the first part of the first mate-choice test (M1) by activating the stimulus presentation in *FishPlayer*. The focal female was first able to watch both males for 2:30 minutes until we released her to swim freely and choose between both males for 5 minutes. Association time (i.e., time spent by focal females within the mate-choice zone for each virtual male) was measured automatically by the tracking system for each mate-choice test. Although association time is an indirect measure for mate choice when no physical contact is possible, it is a good predictor for sexual preference and is commonly used in studies with fish (Bischoff et al. 1985; Forsgren 1992; Berglund 1993; Kodric-Brown 1993; Witte and Noltemeier 2002; Witte and Ueding 2003; Nöbel and Witte

2013; Gierszewski, Keil, et al. 2018; Gierszewski, Baker, et al. 2018). Afterwards, we gently put the focal female back into the cylinder in the middle of the tank (Pause; see Table 13).

Table 13. Overview of all experimental stages and corresponding animated sequences. The duration of each experimental stage as well as the order of animated sequences for the left and right LCD monitor, as arranged in *FishPlayer*, are given. For each experimental stage, we state whether the focal female was free swimming or inside the cylinder and only able to watch the stimulus presentation. A short description of the used animated scenes is given for each stage. The thick black line marks the point at which it is calculated which male was preferred by the focal female in M1. At this point, animated sequences in *FishPlayer* are rearranged before starting M2. In this example, Male B was not preferred in M1 and was, hence, shown together with a virtual model female during the observation period. M1 = 1st mate-choice test, M2 = 2nd mate-choice test.

Experimental stage	Duration (minutes)	Focal female position	Animated scene left monitor	Animated scene right monitor
Acclimatization	30:00	Free swimming	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli	02:30	In cylinder	Male A alone	Male B alone
1st part of M1	05:00	Free swimming		
Pause (handling time)	01:30	Back to cylinder	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	02:30	In cylinder	Male B alone	Male A alone
2nd part of M1	05:00	Free swimming		
Pause (handling time)	01:30	Back to cylinder	empty virtual tank, no fish	empty virtual tank, no fish
Observation period	10:00	In cylinder	Male B with model female	Male A alone
Pause	00:30	In cylinder	empty virtual tank, no fish	empty virtual tank, no fish
1st part of M2	05:00	Free swimming	Male B alone	Male A alone
Pause (handling time)	01:30	Back to cylinder	empty virtual tank, no fish	empty virtual tank, no fish
Observing male stimuli (position switched)	02:30	In cylinder	Male A alone	Male B alone
2nd part of M2	05:00	Free swimming		

For the second part of M1, we repeated the procedure, however, the sides of both male stimuli were switched to control for a possible side bias in focal females (see Table 13). After M1, we put the focal female back into the cylinder and calculated which virtual male was preferred (i.e., with whom the female had spent more time) and whether she showed a side bias. We stated a focal female's choice as side biased if she had spent more than 90 % of the total time in both choice zones (first plus second part of M1) in the same choice zone, even though stimuli were switched. If females showed a side bias, we terminated the trial and retested them once after two days. If their side bias was persistent, they were excluded from analysis.

In the following observation period, the focal female was able to observe a sequence of the prior non-preferred virtual male interacting with a virtual model female for 10 minutes (see Table 13 and Fig. 31). The prior preferred male was swimming alone. After a short

pause, the focal female was released from the cylinder to choose between the same two virtual males for 5 minutes, starting the first part of the second mate-choice test (M2; Table 13). The procedure of M2 was identical to that of M1. After the first part of M2 we used the same animations but switched the position of the two virtual males. We let the focal female observe the new position of both males for 2:30 minutes from inside the cylinder. She was then again released from the cylinder and allowed to choose between the two males for 5 minutes during the second part of M2 (Table 13). We then terminated the trial. We calculated the time the female had spent with each stimulus male, measured her standard length (SL; body size from snout to end of caudal peduncle) and released her back into her home tank. All focal females were only used once (except in case of a side bias; see above). We classified whether or not focal females copied the choice of a virtual model female according to the definition for MCC by Pruett-Jones (1992). Therein, MCC is defined as an increase in time spent of a focal female with a previously non-preferred male after observing him together with a model female. In theory, this increase will consequently also increase the chance of mating with that same male or a male of that same phenotype.

To study whether differences in the observed behavioral interaction between the virtual male and the virtual model female affect MCC in focal females, we performed three different treatments (T1-T3) in which we varied the extent and direction of courtship behavior of the stimulus pair to be either mutual (T1), female driven (T2) or male driven (T3). The experimental procedure was identical for every treatment except for the animated sequence presented during the observation period. Additionally, we used the same procedure to assess whether differences in male courtship affected a focal female's mate choice when no model female was present. For this, we performed two treatments (T4-T5) in which we either presented both males courting (T4) or not courting (T5) during the observation period. This time, there was no virtual model female present and hence focal females had no access to another source of information. A detailed description of the used animated sequences for each treatment is given below.

8.3.4 Data analysis

We used R 3.3.1 (R Development Core Team 2015) for data analysis. We compared focal female SL across treatments using a Kruskal-Wallis rank sum test. For each treatment, we used time spent of a focal female within mate-choice zones as a measure of mate choice for a given virtual stimulus male. We compared whether the overall choosing motivation (total time spent in both mate-choice zones within a mate-choice test) differed from M1 to M2 using a paired Wilcoxon signed rank test. For each mate-choice test (M1 and M2), we calculated a preference score for the preferred and non-preferred male (e.g., time spent with a virtual non-preferred male divided by the total time spent with both males). For Treatments T1-T3, we used Wilcoxon signed rank tests to analyze whether (I) focal females showed a significant preference for one of the two virtual males (preferred male: male they spent > 50% of the total time with in M1); (II) preference scores for the prior non-preferred stimulus male differed between M1 and M2; and whether (III) the prior preferred male in M1 was still preferred in M2. For Treatments T4-T5, we performed the same analysis using preference scores for the prior preferred stimulus male respectively. We applied a Bonferroni correction to account for multiple testing and calculated adjusted p-values using the *p.adjust* function in R.

To analyze whether a possible change of preference for the prior non-preferred stimulus male (preference score for prior non-preferred male in M2 – preference score for non-preferred male in M1) was different between Treatments T1-T3, we fit a linear model (LM) using the *lm* function from the R-package 'lme4' (Bates et al. 2015). For this, we set preference score for the prior non-preferred male as the dependent variable and included treatment (treatment: mutual, female driven, male driven) as a fixed factor as well as focal female's standard length (SL) as a covariate. To meet the model assumptions, we performed a z-transformation to the covariate (Korner-Nievergelt et al. 2015). We conducted two orthogonal comparisons for "treatment" using the function *contrasts* (Crawley 2007). We set the contrasts of the model (I) to compare the treatment presenting mutual courtship against that showing only female driven courtship (mutual → female driven), and (II) to compare mutual courtship against only male driven courtship (mutual → male driven).

We did the same analysis to compare the change of preference for the prior non-preferred male across T4 and T5. Here, however, the fixed factor treatment had only two levels (courting, not courting) which were directly compared in the model. We inspected model assumptions (Q/Q-plots, residuals, residuals against fitted values) for all models visually (Korner-Nievergelt et al. 2015). We further compared the distribution of the residuals against a normal distribution using a Shapiro-Wilk normality test.

Additionally, we analyzed whether the number of focal females who reversed their initial mate choice in M2 (mate-choice reversals) differed between treatments. For T1-T3, we performed a post-hoc pairwise G-test using the R-package 'RVAideMemoire' (Hervé 2017) with Bonferroni correction. For T4 and T5, we calculated Fisher's exact test.

As a control, we tested whether the mere presence or position of the virtual model female alone could explain a possible change in preference in M2, independent from the respective male stimulus. We used a Binomial test to analyze whether the number of trials in which focal females first entered the mate-choice zone in M2 where they had previously seen the model female differed from chance. Using the same test, we compared whether the number of trials, in which focal females had spent most of their time on the same side of the test tank where they had seen the model, deviated from chance. P-values were considered significant if $p \leq 0.05$. All p-values are two-tailed.

8.4 Results

We provide a detailed overview of the number of tested focal females, as well as their standard length in Table 14. Absolute and relative time spent (preference scores) with virtual males for all treatments can be found in Appendix 6, Table S9. Several females showed a side bias during the course of the experiment. When analyzing all trials in which side biases occurred (1^{st} and 2^{nd} try of T1-T5 combined; n = 106), proportions of focal females preferring the right side (n_r = 47; 44.3 %) or left side (n_l = 59; 55.7 %) of the test tank did not differ from chance (Binomial test: p = 0.285). Therefore, we conclude that side biases were not due to problems related to the experimental setup. Overall, focal females analyzed in this study did not differ in SL across treatments (Kruskal-Wallis test: n = 75, $\chi^2(4) = 2.839$, p = 0.585).

Table 14. Number of focal females used during Treatments 1-5. Given are absolute numbers of live focal females who were tested, excluded, and analyzed in all experimental treatments. The standard length (SL) of all focal females analysed in this study is given as mean ± SD. T = total tested; TI = trial terminated due to technical issues; ES = excluded due to stress; SB = females with side bias; RT = successfully retested; E = total excluded; A = analysed.

	T	TI	ES	SB	RT	E	A	SL (mm)
T1[1]	24	2	/	16	9	9	15	35 ± 4
T2[2]	19	/	/	9	5	4	15	36 ± 3
T3[3]	30	/	/	16	1	15	15	35 ± 3
T4[4]	29	/	/	19	5	9	15	36 ± 3
T5[5]	22	/	1	12	6	6	15	36 ± 3

[1]Mutual courtship; [2]Female driven courtship; [3]Male driven courtship; [4]Male alone, courting; [5]Male alone, not courting

8.4.1 Public information with varying extent and direction of courtship

Treatment 1 (T1): mutual courtship

In T1, choosing motivation did not differ between M1 and M2 (Wilcoxon signed rank test: $V = 56$, $p = 0.847$). Focal females ($n = 15$) showed a significant preference for one of the two virtual stimulus males (Wilcoxon signed rank test: $V = 120$, $p < 0.001$) in M1. Preference scores for the prior non-preferred male significantly increased from M1 to M2 (Wilcoxon signed rank test: $V = 8$, $p = 0.005$; Fig. 33A). Preference for the prior preferred male was no longer significant in M2 (Wilcoxon signed rank test: $V = 84$, $p = 0.155$). Four out of 15 focal females (26.6 %) reversed their initial mate choice for a virtual male in M2.

Treatment 2 (T2): female driven courtship

In M1, choosing motivation differed between M1 and M2 with females spending significantly more time in both choice zones in M2 (Wilcoxon signed rank test: $V = 18$, $p = 0.015$). Focal females ($n = 15$) showed a significant preference for a virtual male in M1 (Wilcoxon signed rank test: $V = 120$, $p < 0.001$). From M1 to M2, preference scores for the prior non-preferred male significantly increased (Wilcoxon signed rank test: $V = 14$, $p = 0.02$; Fig. 33A). Preference for the prior preferred male was not significant in M2 (Wilcoxon signed rank test: $V = 82$, $p = 0.689$). Five out of 15 focal females (33.3 %) reversed their initial mate choice for a virtual male.

Treatment 3 (T3): male driven courtship

Focal females' ($n = 15$) motivation to choose between virtual stimulus males was significantly different between M1 and M2 with females spending more time with both males in M2 (Wilcoxon signed rank test: $V = 18$, $p = 0.015$). Focal females significantly preferred a virtual stimulus male in M1 (Wilcoxon signed rank test: $V = 120$, $p < 0.001$). Preference scores for the prior non-preferred male significantly increased from M1 to M2 (Wilcoxon signed rank test: $V = 0$, $p < 0.001$; Fig. 33A) and the preference for the prior preferred male did not persist since there was no difference in preference scores in M2

(Wilcoxon signed rank test: V = 44, p = 1). Eight out of 15 focal females (53.3 %) reversed their initial mate choice for a virtual male in M2.

Comparison between T1 to T3 when public information was provided

We found a significantly stronger change of preference for the prior non-preferred male when courtship was only male driven compared to the mutual courtship scenario (LM: df = 41, t = -2.119, p = 0.04; Fig. 33B and Table 16). However, there was no difference when comparing mutual courtship and only female driven courtship. Further, focal female SL did not affect a change of preference (Table 16). The number of focal female's mate-choice reversals did not differ across treatments (G-test: G = 2.446, df = 2, p = 0.294). In all treatments (T1-T3), focal females first entered the mate-choice zone where they had previously seen the model female in six out of 15 trials, which did not differ from chance (Binomial test: p = 0.607). In seven out of 15 trials, focal females spent most of their time on the side of the test tank where they had seen the model female during observation. This distribution was identical for all treatments (T1-T3) and did not different from chance (Binomial test: p = 1). Therefore, presence and position of the virtual model female alone did not explain the observed increase of preference for the prior non-preferred male in M2 across treatments.

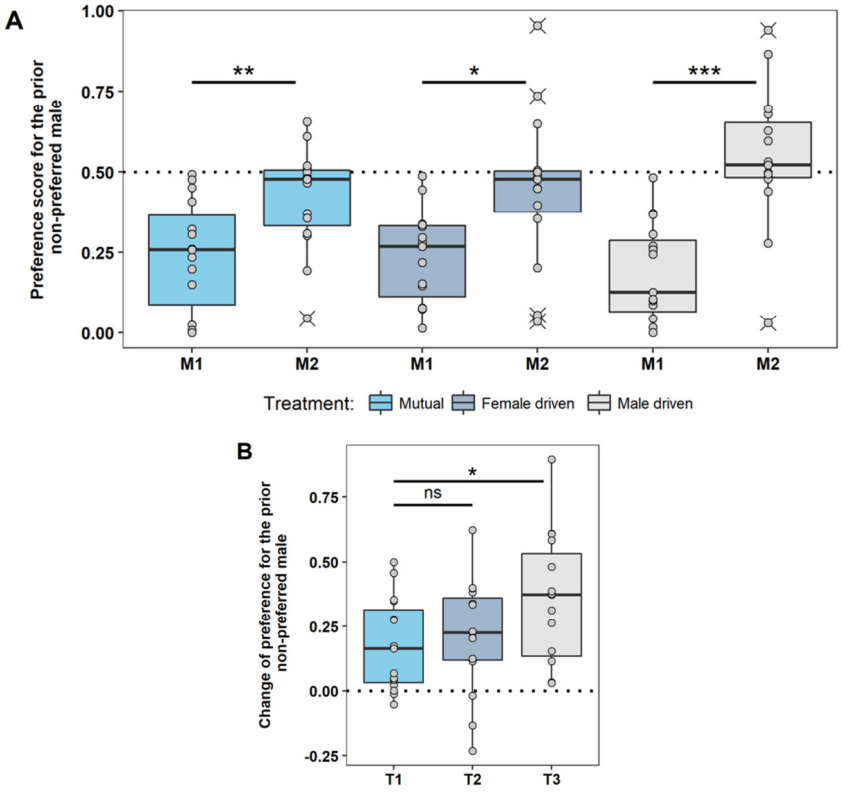

Figure 31. Results of Treatments T1-T3 providing public information but varying extent and direction of courtship behavior. (A) Preference scores for the (prior) non-preferred virtual stimulus male in M1 and M2. (B) Change of preference from M1 to M2 for the prior non-preferred virtual male. The dotted line depicts no change in preference, positive values show an increase in preference and negative values show a decrease in preference. Grey dots in A and B depict raw data of each focal female. Boxplots show median, quartiles, whiskers (1.5 interquartile range), and outliers (dots with cross). M1 = first mate-choice test, M2 = second mate-choice test, T1 = Treatment 1, T2 = Treatment 2, T3 = Treatment 3, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. N = 15 for each treatment.

Table 16. LM estimates for effects on the change of preference for the prior non-preferred virtual male comparing T1-T3. Change of preference for the prior non-preferred virtual male stimulus was the dependent variable throughout. Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor. Intercept estimates represent the grand mean of all treatments. Orthogonal comparisons of treatments are given. Significant p-values ($p \leq 0.05$) are printed in bold. SL = standard length of focal females. N = 15 focal females per treatment.

Fixed factors	Lower	Estimate	Upper	SE	*df*	*t*	*p*
(Intercept)	0.185	0.249	0.313	0.032	41	7.814	**<0.001**
Mutual → female driven	-0.068	0.024	0.116	0.046	41	0.529	0.599
Mutual → male driven	-0.188	-0.096	-0.005	0.046	41	-2.119	**0.04**
SL	-0.122	-0.056	0.01	0.033	41	-1.706	0.096

8.4.2 No public information but varying extent of courtship

Treatment 4 (T4): male actively courting

Focal females' (n = 15) choosing motivation did not differ (Wilcoxon signed rank test: V = 36.5, p = 0.191). During M1, focal females showed a significant preference for one stimulus male (Wilcoxon signed rank test: V = 120, p < 0.001). There was no significant difference in preference scores for the prior non-preferred male from M1 to M2 (Wilcoxon signed rank test: V = 22, p = 0.091; Fig. 34). However, preference for the prior preferred male was not anymore significant in M2 (Wilcoxon signed rank test: V = 78, p = 0.991). Six out of 15 focal females (40 %) reversed their initial mate choice for a virtual male.

Treatment 5 (T5): male passive and not courting

Choosing motivation was not different between M1 and M2 (Wilcoxon signed rank test: V = 44, p = 0.379). Focal females (n = 15) showed a significant preference for a stimulus male in M1 (Wilcoxon signed rank test: V = 120, p < 0.001). Preference scores for the prior non-preferred male significantly increased from M1 to M2 (Wilcoxon signed rank test: V = 16, p = 0.031; Fig. 34). In M2, focal female's preference for the prior preferred virtual male did not persist and they showed no preference for either stimulus male (Wilcoxon signed rank test: V = 70, p = 1). Six out of 15 focal females (40 %) reversed their initial mate choice for a virtual male.

Comparison between T4 and T5 when no public information was provided

Neither presence (T4) nor absence (T5) of male courtship (LM: t = 0.735, p = 0.469) or focal female SL (LM: t = -0.881, p = 0.386) did affect a change of preference for the prior non-preferred virtual male. We found an equal number of mate-choice reversals in T4 and T5 (Fisher's exact test: p = 1).

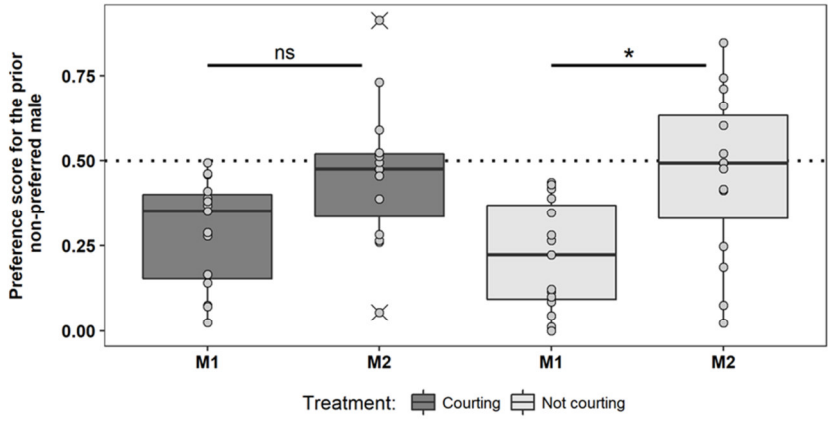

Figure 32. Results of Treatments T4 and T5 showing varying male courtship behavior without model female (no public information). Preference scores for the (prior) non-preferred virtual stimulus male in M1 and M2. Grey dots depict raw data of each focal female. Boxplots show median, quartiles, whiskers (1.5 interquartile range), and outliers (dots with cross). M1 = first mate-choice test, M2 = second mate-choice test, * = 0.05 > p > 0.01, ns = not significant. N = 15 for each treatment.

8.5 Discussion

In the present study, we investigated the role of public information quality and quantity for the expression of MCC in female sailfin mollies. By applying computer animations, we varied the extent and direction of courtship of a virtual male and a virtual model female during the observation period. Additionally, we investigated the role of male courtship in female mate choice in the absence of a model female (no public information). Here, we tried to decipher, whose behavior of the interacting pair presented during the observation period of a MCC experiment, provides most relevant information for copying to observing focal females.

8.5.1 Public information with varying extent and direction of courtship (T1-T3)

When public information was provided during the observation period (virtual model female present), focal females copied the choice of the virtual model for the prior non-preferred male and showed significantly higher preference scores for that male in the subsequent second mate-choice test (M2) in all three treatments. Copying behavior as found in our study is in accordance to previous studies on MCC in sailfin mollies using live fish stimuli (e.g., Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003; Witte and Ueding 2003; Gierszewski, Keil, et al. 2018), which validates our method.

Here, we wanted to decipher which information (courtship vs. no courtship), and from whom (male vs. model female), mostly influences the strength of copying behavior in focal females. Surprisingly, we found the strongest copying effect in T3 with male driven courtship. This result was in contrast to our prediction. We predicted the strongest copying scores in the situation with mutual courtship and/or female driven courtship based on previous studies emphasizing the role of the model female in MCC (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Godin et al. 2005; Hill and Ryan 2006; Kniel et al. 2017; Gierszewski, Baker, et al. 2018). These previous studies showed that the quality of a model female is key information for focal females to copy or not to copy.

In most cases, so far, model females were not explicitly presented as females highly interested in the stimulus male during an observation period. In our study, we could manipulate the behavior of model females in a way that they show behavior to be highly responsive to a male by means of computer animations, which were used to present sexually active females in T1 and T2. On one hand, a highly interested model female could be a good indicator for reliable information about a male for an observing female. Conversely, a highly interested and sexually active model female could be viewed as a strong competitor to the focal female for that male in focus. Female-female aggression in sailfin mollies is frequently observed and suggested to serve during competition for food and males and to establish dominance hierarchies (Makowicz and Schlupp 2015; Makowicz et al. 2020). Foran and Ryan (1994) showed that sailfin molly females actively compete for males, e.g., by blocking another female's access to a mate. This was also observed when sailfin mollies were associated with heterospecific females of the gynogenetic Amazon molly (*Poecilia formosa*), who compete for access to the same males (Foran and Ryan 1994). This ambiguous information (i.e., sexual behavior of a model female indicates a "high quality" male but simultaneously presents herself as a strong competitor for the focal female) may lead to a lower copying score in T1 and T2. In T3, the model female was present but was not sexually engaged in male courtship. This could be an explanation for the higher copying score in T3. To confirm this hypothesis, one should assume that the copying score of focal females decreases with increasing number of copulations between stimulus male and model female. Indeed, simulated copulation events only occurred in T1 (see Table 12) in which we found the weakest copying effect. Instead, copying behavior was significantly stronger when no copulations occurred and the model female was also not actively approaching or otherwise sexually interacting with the male (T3). Additionally, focal females' choosing motivation was significantly higher after observing female driven and male driven courtship without copulations. Similarly, Bierbach, Kronmarck, et al. (2011) found that Atlantic molly males vary in the strength of copying behavior depending on what sexual interaction they had previously observed. Male MCC was much weaker when the observed pair was able to interact physically and actually copulate, which consequently increased the risk for sperm competition perceived by the observer. It is, therefore, not unlikely that sailfin molly females showed similar fine-tuned changes in the strength of copying behavior based on fine-tuned differences in the sexual behavior of virtual model females in our study. Although more in light of sperm availability by males that recently copulated rather than sperm competition. Even though sailfin molly females are able to store sperm for several months (Constantz 1989) and insemination is possible at any time, it can still be assumed that being the first female to mate with a high quality male is more beneficial. Females showing more courtship vigor may be better in gaining access to males and may benefit from more or higher quality sperm (Makowicz and Schlupp 2015 and references within).

An alternative explanation for a weaker copying effect in T2 might be that female driven courtship might have been perceived as the female chasing away the male. This however would be a rejection of that male and we would therefore expect copying to not occur at all. The virtual male was also not fleeing from the model or showing any other signs of escape behavior. Witte and Ueding (2003) showed that the distinct rejection of a male by a model female clearly affects an observing female's mate choice. In their study, focal females significantly decreased time spent with a prior preferred male after that same male was rejected by a model female during the observation period. They presented video recordings of male driven courtship, in which model females were actively fleeing from courting males during the observation period. In the present study,

we never simulated a strong rejection of a male since the virtual model female was never actively fleeing or showing any other clearly negative dismissive behavior in response to the presence of the virtual male, even when courtship was only male driven (T3). Instead, the model female was behaving “neutral” to the male — she did not “stimulate“ him to copulate with her, but she also did not reject him. Quite the contrary, the calm mood of the virtual model female in response to the vigorously courting male may indeed have provided relevant information about the male being a “good” choice to exhibit copying behavior in focal females.

In all experimental treatments, the distance between virtual males and females on the monitors was not very large, though we simulated them to swim in opposite directions from one another. Due to the display dimensions of the monitors used in our experiment, the largest possible distance between virtual stimuli was around 30 cm. From an earlier study with sailfin mollies, we know that focal females still copied the choice of a model female even if she was interacting with a prior non-preferred male at a distance of 40 cm (Gierszewski, Keil, et al. 2018). Even though sexual interactions at distance were also limited in their extent since fish were restrained in Plexiglas cylinders. Close proximity (about 1 cm) of male and model female, as typically described in observations on natural courtship in sailfin mollies (Parzefall 1969; Baird 1974), is therefore not a prerequisite for MCC to occur.

8.5.2 No public information but varying extent of courtship (T4 and T5)

When public information was absent (no model female) during the observation period, focal females chose consistently and there was no significant increase in focal female’s preference scores for the prior non-preferred male However, this was only the case when virtual males were presented to be actively courting during the observation period (T4). When virtual males were passive and not courting (T5), preference scores for the prior non-preferred male significantly increased in M2. We assume that the absence of male courtship behavior caused focal females to lose interest in the prior preferred male, consequently spending more time with the prior non-preferred male in the second mate-choice test. This underlines our previous assumption that the general expression of sexual interest per se (of either male or model female or both), seems to be important for observing females for MCC in general.

8.5.3 Conclusions and future directions

Overall, MCC remains a complex and fascinating mate-choice strategy that, if utilized, depends on the specific interplay between quality and quantity of public information. Our results support previous findings that responsive behavior of a model female towards a male is an important driver for observing focal females to copy her choice. However, we also provide first evidence that the strength of copying might be negatively affected when observing high sexual activity of the interacting pair, with actual copulations, as well as highly sexually engaged model females. We suggest focusing future research on behavioral aspects influencing sailfin molly MCC, as well as studying a possible competitive effect in female-female relationships.

We presented a first attempt in artificial manipulation of extent and direction of courtship between the interacting pair presented during the observation period of a MCC

experiment with sailfin mollies. Our results indicate that focal females were able to assess differences in animated courtship behavior (i.e., copulation events, presence or absence of male and female sexual behavior) and altered their mate-choice decisions accordingly. This validates the use of *FishSim* to study future questions related to behavioral manipulation in mate choice. Our approach offers solutions for the systematic study of varying degrees of courtship, such as courtship direction, vigor, intensity or rate, and even number of copulations in relation to its effect on MCC in sailfin mollies and other poeciliids (*FishSim* can be applied for other fish species as well; see Gierszewski, Baker, et al. 2018). Possibilities for controlled testing of one parameter while keeping others constant, as well as non-invasive manipulation of behavioral and phenotypic traits, are powerful advantages of computer animation compared to studies using live animals. Implementation of such methods in research serves the 3R's principle – *reduce, replace, refine* – in research and may improve a study's validity (Russell and Burch 1959; ASAB 2018).

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Chapter 9

Mate-choice copying in sailfin molly females: public information use from long-distance interactions

Stefanie GIERSZEWSKI[a], Melissa KEIL[a] & Klaudia WITTE[a]

[a]Research Group of Ecology and Behavioral Biology, Institute of Biology, University of Siegen, Adolf-Reichwein-Straße 2, Siegen, 57068, Germany

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9.1 Abstract

Animals may use public information gained by observing sexual interactions between conspecifics and use this information for their own mate choice. This strategy, called mate-choice copying, is considered to play an important role for the evolution of mate preferences. Mate-choice copying is defined as non-independent mate choice, in which a female's probability of choosing a given male increases if other females have chosen that male previously. Using the livebearing sailfin molly (*Poecilia latipinna*), we asked if increasing the distance between a model female and a male would affect copying behavior of focal females. We tested focal females in two different treatments: (I) model female and male in close proximity and able to interact at close range; (II) model female and male positioned apart from each other and restricted from close-range interactions. We could show that focal females copied the choice of a model at short distance to the prior non-preferred male as predicted from previous experiments. Surprisingly, focal females also copied the choice of a model when positioned 40 cm apart from the male. When no model female and, hence, no public information was provided (choice consistency control), focal females were consistent in their mate choice, indicating that changes in mate preference observed in the two treatments were due to the simulated mate choice of the model female. Our results demonstrate that females gain and use public information and copy the mate choice of other females even when heterosexual conspecifics interact from a distance.

9.2 Introduction

Animals can use public information to receive information about the environment like resources, predators, parasites, and conspecifics as competitors or mates. Public information can be acquired by observing the performance or decisions of others and used to assess the quality of resources (Nordell and Valone 1998; Danchin et al. 2004; Valone 2007). In a feeding context, a forager can for example assess the behavior of its group mates to get information about food availability without personally sampling the whole food patch (Clark and Mangel 1986; Valone 1989; Giraldeau et al. 1994; Valone and Templeton 2002). Public information gained by observing interactions between other individuals or the environment is also frequently described as inadvertent socially acquired information, since the observed interactions are not deliberately directed to the observer (Dall et al. 2005). Here, the observer eavesdrops on its surrounding individuals. Therefore, the opportunity to acquire and use public information is especially high in group-living animals (Valone and Templeton 2002; Danchin et al. 2004; Dall et al. 2005; Valone 2007; Ioannou et al. 2011).

One form for the use of public information is mate-choice copying (MCC; Westneat et al. 2000; Witte and Nöbel 2011; Witte et al. 2015). Here, individuals observe heterosexual conspecifics during sexual interaction and choose the same individual as a mate as the so-called “model” (of same sex as the observer) did before (Dugatkin 1992) or an individual of the same phenotype as the chosen one (Kniel, Dürler, et al. 2015). MCC is an alternative non-independent mate-choice strategy that shows that not only genetically-based mate preferences for a certain trait determine mate choice, but that mate choice can also be influenced by non-genetic factors like social learning (Nordell and Valone 1998; Danchin et al. 2011). MCC is considered to have wide implications on the evolution of phenotypic traits (Agrawal 2001; Danchin et al. 2004; Verzijden et al. 2012; Witte et al. 2015), as it could not only be shown to favor the potential spread of preferences for novel phenotypes (Kniel, Dürler, et al. 2015; Kniel, Schmitz, et al. 2015) but also the avoidance of certain phenotypic traits by copying the rejection of a mate (Witte and Ueding 2003). Moreover, it has been shown that MCC can even override genetic preferences under certain conditions (Dugatkin 1996; Dugatkin 1998; Witte and Noltemeier 2002; Godin et al. 2005).

So far, MCC has been demonstrated in mammals (humans: Waynforth 2007; Place et al. 2010; norway rats *Rattus norvegicus:* Galef et al. 2008) birds (Japanese quail *Coturnix coturnix japonica;* Galef and White 1998; White and Galef 2000; zebra finches *Taeniopygia guttata castanotis:* Kniel, Dürler, et al. 2015; Kniel, Schmitz, et al. 2015; Kniel et al. 2017) and invertebrates (fruit fly, *Drosophila melanogaster:* Mery et al. 2009; Dagaeff et al. 2016). An extensive amount of work was also done in fish where MCC seems to be a widespread alternative mate-choice strategy. Since Dugatkin (1992) first experimentally demonstrated that female guppies (*Poecilia reticulata*) copy the mate choice of conspecific females, MCC has been detected in several other species of fish: in three-spined sticklebacks *Gasterosteus aculeatus* (Frommen et al. 2009), in ocellated wrasses *Symphodus ocellatus* (Alonzo 2008), in the white belly damselfish *Amblyglyphidodon leucogaster* (Goulet and Goulet 2006) in the pipefish *Syngnathus typhle* (Widemo 2008), in Atlantic mollies *Poecilia mexicana* (Heubel et al. 2008; Bierbach, Kronmarck, et al. 2011), in sailfin mollies *Poecilia latipinna* (Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003; Witte and Ueding 2003), in Amazon mollies *Poecilia formosa* (Heubel et al. 2008) and in the humpback limia *Limia nigrofasciata* (Munger et al. 2004). MCC can be considered as a biologically meaningful strategy, since it was found to occur in the wild as well (Witte and Ryan 2002;

Goulet and Goulet 2006; Alonzo 2008; Godin and Hair 2009). Further, it was demonstrated that not only females use MCC but that, despite the risk of sperm competition, also males copy the mate choice of conspecific males (Schlupp and Ryan 1997; Witte and Ryan 2002; Bierbach, Kronmarck, et al. 2011; reviewed by Plath and Bierbach 2011).

Until now, not much is known about what exact aspects of quantity and quality of public information affects MCC (Witte et al. 2015). Initial findings have shown that the quality of the model, whose choice is observed and potentially copied, seems to play an important role (age: Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; species: Hill and Ryan 2006; size: Vukomanovic and Rodd 2007; phenotype: Kniel et al. 2017), as well as the quantity of public information available for the observer (number of models and duration of observation: Witte and Noltemeier 2002). It is defined that a sexual interaction between the model and the male/female has to be observed to count as MCC and not the resulting consequences, as for example the number of eggs in a nest (Pruett-Jones 1992). But numerous previous studies in fish following an experimental approach similar to Schlupp et al. (1994) also proved it is sufficient when sexual interactions are restricted (e.g., by a transparent partition), meaning that no actual copulation needs to be observed to trigger copying behavior in the observer. So far, in all experiments regarding MCC the model individual was always presented directly next to a heterosexual conspecific – a potential mate (but see also Bierbach, Jung, et al. 2013) for homosexual copying behavior and Schlupp et al. 1994 for copying of a heterospecific model). In nature, however, individuals may also interact with each other at distance. Long-distance communication might be more prominent in cases of acoustic signals (reviewed by Naguib and Haven-Wiley 2001; see Ladich 2004 for fishes) or chemical cues, e.g., pheromones for mate attraction (see Shorey 2013). Nevertheless, visual signals can also be used to communicate from a distance, as seen by the long-distance jumping display in the lekking Jackson's widowbird (*Euplectes jacksoni*) used to attract females (Andersson 1989).

Here, the question arises whether females would still copy the mate choice when two conspecifics sexually interact at distance. In our current study, we used female sailfin mollies to test whether females use public information in mate choice even though conspecifics are interacting at distance. In the wild, sailfin mollies form loose shoals of around 20 individuals that change quickly through time and space (Travis 1994b). Therefore, longer distances between individuals can be expected. Natural sailfin molly courtship displays of males, however, involve individuals in direct or very close contact of about 1 cm (Parzefall 1969; Baird 1974). Male courtship displays typically include the following behavioral patterns: (I) approaching and (II) following of a female, (III) lateral displays (dorsal and caudal fin raised) and so-called sigmoid displays (IV) in front of or next to the female, (V) gonopore nipping, (VI) gonopodial thrusting and (VII) copulation (Parzefall 1969; Baird 1974; see also Chapter 2.1.2 in this thesis). In commonly used experimental designs to study MCC in fish, model and stimulus males are restricted in direct contact, but are still able to communicate visually through glass at a distance of around 1 cm at the nearest. Standard designs were previously used to demonstrate copying behavior in sailfin mollies (Schlupp et al. 1994; Schlupp and Ryan 1997; Witte and Ryan 1998, 2002; Witte and Noltemeier 2002; Witte and Massmann 2003) and, therein, model female and stimulus male were frequently observed to interact visually at the closest possible distance.

Marler and Ryan (1997) showed a genetically predefined preference for larger over smaller males in sailfin molly females. Later, MacLaren (2006) showed that female sailfin

mollies were able to perceive and distinguish between a large and a small dummy male positioned at a distance up to 68 cm by showing a preference for the larger male. Therefore, sailfin molly females are generally able to assess visual information necessary for mate choice at a distance up to 68 cm.

But the questions remain whether (I) sailfin molly conspecifics do interact sexually over larger distances and if so, (II) is this distant interaction sufficient for an observing female to extract public information for her own mate choice decision? Therefore, we investigated whether sailfin molly females would copy a choice of a model female when this model female is either close to a stimulus male (1 cm) or 40 cm away from that male. We predicted that females will copy the choice when the model female is next to the stimulus male as found in previous experiments. However, we further predicted that females will not copy the choice of a model female positioned farther away from the stimulus male (40 cm), since close sexual interactions (courtship) between the model female and the male are not possible.

9.3 Materials and Methods

9.3.1 Study organism

Male and female sailfin mollies used in experiments were mature descendants of wild mollies caught from a shallow freshwater drainage ditch at Mustang Island near Corpus Christi (Texas, USA) in 2014. In the lab, fish were kept in mixed-sex shoals in large housing tanks (see Chapter 2.3 for detailed information on the species and the housing conditions). Prior to experiments, focal females were kept separated from males in small groups for several weeks to increase their choosing motivation. All experiments were performed in 2016. After experiments, all fish were returned to their home tanks.

9.3.2 General experimental procedure

For testing the effect of distance on public information use in MCC, we used a square paddling pool for children to provide a large area (Intex Kinderpool Frame Pool Mini, blue, 122 cm x 122 cm x 30 cm; see Experimental setup E3 in Chapter 2.3.1). We filled the pool with tap water as used for fish housing. Water was aerated and filtered between experiments and partly changed once a week. Water temperature was 26 ± 1°C and the water level was 19.5 ± 1 cm in height, resulting in a total volume of approximately 280 L. Illumination during experiments was provided by two fluorescent tubes positioned approximately 138 cm above the middle of the pool (Philips TL-D 90 De Luxe, 58 Watt).

The following treatments and the control were modified after the classic copying experiment described by Schlupp et al. (1994), Schlupp and Ryan (1997), Witte and Ryan (1998) and Witte and Noltemeier (2002). Prior to the start of a trial, four clear Plexiglas cylinders (11 cm diameter) were positioned in the test pool (Fig. 35). Cylinders were used to keep each fish at a specific place inside the pool and to ensure a constant distance between them during experiments. All cylinders were large enough for fish to move around and behave normally. To mark a circular mate-choice zone (34 cm diameter) around each cylinder containing a stimulus male, we used flexible tubes (8 mm in diameter) filled with sand that lay on the ground of the pool. Both choice zones comprised around 12 % of the total pool area.

First, two stimulus males were positioned in two cylinders on opposite sides of the pool for acclimatization (Fig. 35). Two opaque frames made of white plastic were put around them to prevent the test female from seeing them, which was released into the pool beforehand. Each trial started with an acclimatization period of 25 minutes, in which the focal female was allowed to swim freely and explore the pool (Fig. 35, Stage 1). The focal female was then placed in a clear Plexiglas cylinder (11 cm diameter) in the middle of the pool and the frames were lifted to give view to the stimulus males. The focal female was allowed to watch both stimulus males for 10 minutes (Fig. 35, Stage 2). After we lifted and removed her cylinder, the first mate-choice test (M1) started and the focal female could swim freely for 10 min to spend time with both males. We measured the time the focal female spent within the mate-choice zone around the cylinder containing a stimulus male with stopwatches (Fig. 35, Stage 3).

After 10 minutes, the focal female was gently put back into the cylinder in the middle of the pool and a white and opaque plastic frame was put around her cylinder to block her view. The position of the stimulus males was switched to control for a possible side bias in the focal female. Males could be easily switched as their cylinders were closed at the bottom and they could be lifted out of the water to change position. This was done to decrease handling time for the experimenter and to reduce stress from handling the fish with a net. We lifted the plastic frame around the focal female and the focal female was given 5 minutes to get accustomed to the new situation before she was released again. She was given 10 minutes time to spend with the males. After M1, the focal female was placed back inside the Plexiglas cylinder in the middle of the pool and the plastic frame was put around her cylinder to block her view to the other cylinders. Time spent for both halves of M1 was summed up and it was calculated which stimulus male was preferred by the female during M1. The preferred male was defined as the male the focal female spent more than 50 % of the time she spent in both mate-choice zones within the two mate-choice trials à 10 min. After this first mate-choice test we put the focal female back into her clear cylinder in the middle of the pool and put an opaque white frame around her cylinder. To provide public information to the focal female during the observation period, a model female was introduced to the cylinder next to the male that was not preferred. To handle both males equally and to prevent the case that one male was more active than the other, a so-called “pseudo-model” female was placed next to the preferred male in a clear Plexiglas cylinder (Fig. 36). The pseudo-model female was obscured by a white plastic barrier in front of the cylinder and, hence, not visible to the focal female. The pseudo-model female, however, was still visible to the stimulus male next to her. The frame surrounding the focal female was lifted and an observation period of 10 minutes started in which the focal female could watch the simulated mate choice of the model female for the non-preferred male (Fig. 35, Stage 4).

After the observation period, the view of the focal female was again obscured; the model females were removed from their cylinders and the second mate-choice test (M2) started. Here, the procedure was identical to M1 in which the focal female could again spend 10 minutes with both males (Fig. 35, Stage 5). Then the position of the stimulus males was switched and the female was given 5 minutes to observe the new situation before she was again given 10 minutes time to spend with both males. Time spent was again summed up for both halves of M2 and it was calculated which stimulus male was preferred by the female during M2.

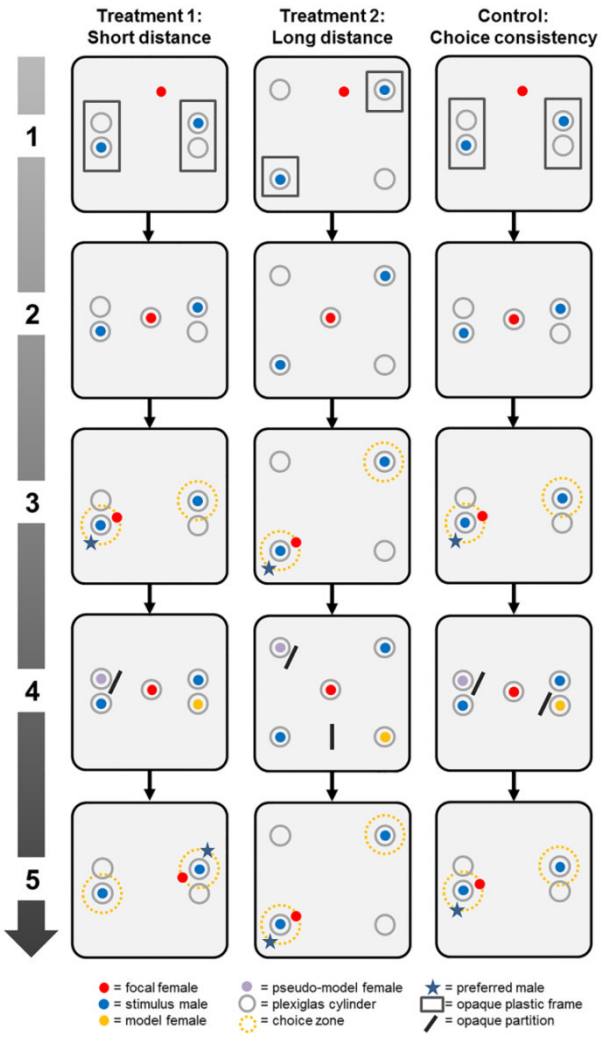

Figure 33. General overview of the experimental procedure for Treatment 1, Treatment 2 and the control. The figure describes the presence and position of fish in the experimental pool per treatment, depending on the experimental phase: (1) acclimatization period; (2) focal female watches stimulus males; (3) First mate-choice test (M1); (4) observation period; (5) Second mate-choice test (M2). The position of the preferred male is shown exemplarily for each treatment.

Observation and data recording were done by a single experimenter (MK) during all experiments therefore no blinded methods were used. The experimenter was sitting 100 cm away from the pool to reduce disturbance of the fish. Observation of obscured areas in the pool (e.g., by the pool wall) was provided by a mirror positioned in 80 cm height at the upper rear of the pool (see supplemental material, Fig. S5 in Appendix 7). Per stimulus male, we measured the absolute association time (in seconds) a focal female spent within a choice zone with a stopwatch. Association time is a well-established measure to determine mate choice in sailfin mollies when no direct contact is provided (Witte and Noltemeier 2002; Witte and Klink 2005; Nöbel and Witte 2013; Gierszewski et al. 2017). Association time is an indirect measure of female mate preference and several studies showed that the time females spent with a male was positively correlated with the probability of copulation with that same male in different species of fish (Bischoff et al. 1985; Forsgren 1992; Berglund 1993; Kodric-Brown 1993). After Pruett-Jones (1992), focal females were considered to copy the choice of the model female if they showed a significant increase in time spent with a prior non-preferred male after the observation

period, since an increase in association time directly results in a higher probability of mating with that same male.

If a focal female spent more than 90 % of the total time in only one choice zone, even if males were switched, her choice was judged to be side biased and the experiment was terminated. All females with a side bias were retested once after two days with different stimulus males and further excluded from the analysis if their side bias persisted. Focal females that spent less than 10 % of the total time of a mate-choice trial (20 min total) in both choice zones combined were excluded due to lack of interest in the stimulus males. Focal females that were too stressed during mate-choice trials and only stayed in one corner of the pool were excluded from analysis due to stress. To evaluate the effect of distance between the model female and the non-preferred stimulus male on MCC, we performed two different treatments and a control as described below.

Treatment 1: Short distance - model female next to non-preferred male

During the observation period in Treatment 1, cylinders containing the prior non-preferred male and the model female were set at a distance of 1 cm (short distance), measured between the outer rims of the cylinders (see Fig. 36 and supplementary Fig. S6 in Appendix 7). Same was true for the distance between the prior preferred male and the pseudo-model female. This distance is common in most MCC experiments and also describes a typical distance between male and female during courtship with direct contact (Parzefall 1969; Baird 1974; Gierszewski, personal observation). The cylinder of the focal female was 38 cm apart from stimulus males and model females. We tested whether females copy the mate choice of other females under these experimental conditions. We assumed that focal females would copy the mate choice of the model female, as shown in previous studies.

Treatment 2: Long distance - model female apart from non-preferred male

During the observation period in Treatment 2, cylinders containing the prior non-preferred male and the model female were set at a distance of 40 cm (long distance), measured between the outer rims of the cylinders (see Fig. 36 and supplementary Fig. S7 in Appendix 7). The same was true for the prior preferred male and the pseudo-model. The focal female was 43 cm apart from stimulus males and model females. The pseudo-model female was covered with a white opaque barrier and hence not visible to the focal female inside her clear cylinder. We made sure that, although the pseudo-model female was 40 cm apart from the previously preferred male and covered to the focal female, the male could see the pseudo-model female (see Fig. 36). Additionally, we added a second barrier to block the line of sight between the prior preferred male and the model female on the opposite side of the pool (Fig. 36).

So far, this distance was never tested in copying experiments in the lab before, however, this distance between conspecifics can be commonly observed in natural sailfin molly shoals (Witte, personal observation). Here, we tested whether females copy the mate choice of other females although model females were 40 cm apart from stimulus males. We assumed that focal females would not copy the mate choice of the model female due to the lack of close contact with the stimulus male.

Control for choice consistency

We performed a control for choice consistency in which the general procedure of the copying experiment was identical to that of Treatment 1 with the exception that both the model female and the pseudo-model female were not visible to the focal female during the observation period and, hence, no public information was provided for MCC (see Fig. 36 and supplementary Fig. S8 in Appendix 7). This procedure is a common control to test mate choice consistency in studies on MCC (Witte and Ryan, 1998; Witte and Noltemeier 2002; Witte and Massmann, 2003; Witte and Ueding, 2003; Heubel et al. 2008) and it is important to show that a change in preference can be linked to the absence/presence of a model female and, hence, the absence/presence of public information provided during observation. Here, we assumed that focal females would choose consistently and not alter their preference for the prior preferred male in M2.

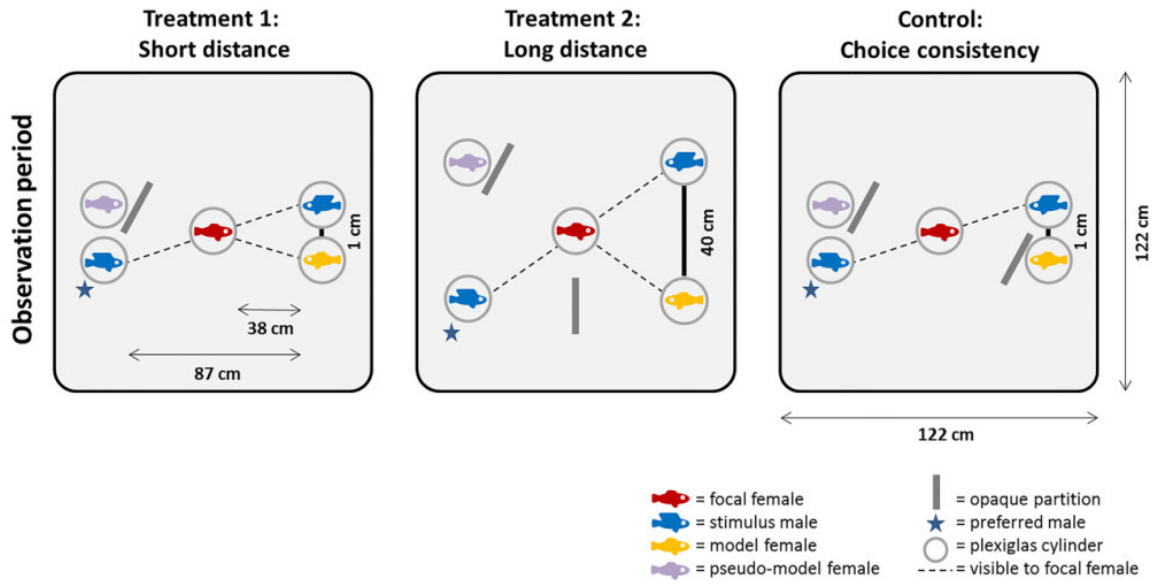

Figure 34. Position of fish during the observation period in Treatment 1, Treatment 2 and the control. The figure describes the position of each fish in the experimental pool during the observation period for each treatment and the control.

In all experiments, we used females (focal, model and pseudo-model females) that were around the same size (see supplementary Tables S10-S13 in Appendix 7). Stimulus males used in the same trial were of similar size as well (see supplementary Tables S10-S12 in Appendix 7). Stimulus males were unknown to the respective focal females and chosen to be similar in fin sizes and color. Stimulus males were always presented simultaneously in a binary choice situation and the position of the Plexiglas cylinders containing the stimulus males was alternated between trials. Due to the limited amount of fish available for experiments, we re-used stimulus males but always together with a different male a second time. In each experiment and trial, females were used first and only once as the focal female but were later re-used as model or pseudo-model female. Standard length of each fish participating in a trial was measured after testing. All fish were later returned to their home tanks.

9.3.3 Statistical analysis

R 3.2.2 (R Development Core Team 2015) and SPSS v. 24 (IBM Statistics) were used for data analyses. Descriptive statistics for sizes and association time are given as mean ± SD or median with first and third quartile. Per experiment, we compared sizes of focal females, models and pseudo-models using a Kruskal-Wallis rank sum test and sizes of the two stimulus males using a Wilcoxon rank sum test for unpaired samples.

For each treatment and the control, we analyzed whether the overall choosing motivation (total time spent in both mate-choice zones within a mate-choice test) differed between mate-choice tests using a paired Wilcoxon signed-rank test. When choosing motivation differed in any experiment, all further analysis was done using relative values (preference scores) rather than absolute association times to ensure comparability within and between treatments. Association time, i.e., time spent within mate-choice zones, was used as a measure of mate choice for a given stimulus male. For each mate-choice test (M1 and M2), a preference score for the preferred and non-preferred male was calculated as the absolute time spent with a male divided by the total time spent with both males. To analyze whether mate choice for either stimulus male differed from chance, we tested the preference score for either stimulus male against a 50 % expectation using a one-sample t test. Preference scores for the prior non-preferred stimulus male were further used to test whether these scores changed between the first and second mate-choice test when public information was provided, compared to the control treatment in which public information was absent. Since it was not possible to gain normally distributed data by using common transformation methods, we used paired Wilcoxon signed-rank tests. A Holm-Bonferroni correction was applied when multiple testing occurred and adjusted p-values were calculated using the *p.adjust* function in R.

To compare both distance treatments, we analyzed whether choosing motivation in M2 (after observation) was different across treatments, as a results of the varying distances in which the model female was presented using a Mann-Whitney U test. Furthermore, we tested whether a change of preference for the prior non-preferred stimulus male (copying score = score for prior non-preferred male in M2 – score for non-preferred male in M1) was different between treatments using a Mann-Whitney U test. Since we were also interested in whether a change of preference (copying score) for the prior non-preferred male was correlated with focal female's standard length (SL) we performed a Spearman rank correlation.

Additionally, Fisher's exact test was calculated to test whether the number of focal females that changed their mate preference in M2, and hence, copied the choice of a model female differed between the control and both distance treatments. P-values were considered significant if $p \leq 0.05$. All p-values are two-tailed. Data are available in the supplementary material (Tables S10-S12 in Appendix 7).

9.4 Results

Detailed information on standard length of all fish used, absolute time spent of focal females with stimulus males and relative time spent (preference scores) for all experiments can be found in the supplemental material (Tables S10-S13 in Appendix 7). Although we only used preference scores for analyzing a potential change of focal female's preference for the prior non-preferred male, a graphical overview of the

absolute time spent with each stimulus male for all treatments and the control can be found in supplementary Fig. S9 in Appendix 7.

Treatment 1: Short distance - model female next to non-preferred male

In Treatment 1, we tested 16 females. Three females showed a side bias and were all successfully re-tested. One female had to be excluded due to stress. We could analyze data from 15 focal females. Focal females (n = 15) spent 840 ± 293 s in both choice zones in M1 and 851 ± 306 s in M2. Overall choosing motivation did not differ between M1 and M2 (Wilcoxon signed-rank test: V = 51, p = 0.639).

During M1, focal females (n = 15) spent on average 644 ± 316 s with the preferred male and 203 ± 176 s with the non-preferred male, resulting in a preference score of 0.68 (0.55, 0.98) and 0.32 (0.02, 0.45) respectively. Focal females showed a significant preference for one stimulus male (one-sample t test: T = 4.937, df = 14, p < 0.001). In M2, focal females spent on average 510 ± 303 s with the prior preferred male and 343 ± 213 s with the prior non-preferred male [scores: 0.49 (0.41, 0.68) and 0.51 (0.32, 0.59)]. Preference scores for the prior non-preferred male significantly increased from M1 to M2 (Wilcoxon signed-rank test: V = 0, p = 0.003; Fig. 37). Preference for the prior preferred male did not differ from chance in M2 (one-sample t test: T = 1.122, df = 14, p = 0.281). We found no correlation between the copying scores for the prior non-preferred male and focal female's SL (Spearman rank correlation: n = 15, r_S = -0.095, p = 0.736). Nine out of 15 focal females (60 %) reversed their initial mate choice for a male and copied the choice of the model female in the short distance treatment.

Treatment 2: Long distance - model female apart from non-preferred male

In Treatment 2, we tested 20 females. Four females showed a side bias, three females were successfully re-tested and one had to be excluded from analysis. Four females were excluded due to lack of interest. Overall, focal females (n = 15) spent 722 ± 293 s in both choice zones in M1 and 736 ± 302 s in M2. Choosing motivation did not differ between preference tests (Wilcoxon signed-rank test: V = 52, p = 0.67).

In M1, focal females (n = 15) spent on average 527 ± 308 s with the preferred male and 193 ± 131 s with the non-preferred male [scores: 0.6 (0.57, 0.83) and 0.4 (0.16, 0.43)]. Focal females showed a significant preference for one stimulus male in the first mate-choice test (one-sample t test: T = 4.197, df = 14, p = 0.001). In M2, focal females spent on average 390 ± 302 s with the prior preferred male and 346 ± 200 s with the prior non-preferred male [scores: 0.47 (0.34, 0.64) and 0.53 (0.36, 0.66)]. Preference scores of time spent with the initially non-preferred male significantly increased from M1 to M2 (Wilcoxon signed-rank test: V = 16, p = 0.025; Fig. 37). The initially preferred male of the first mate-choice test was not preferred anymore in the second test (one-sample t test: T = -0.042, df = 14, p = 0.967). Copying scores for the prior non-preferred male were not correlated with focal female's SL (Spearman rank correlation: n = 15, r_S = 0.054, p = 0.847). Nine out of 15 focal females (60 %) reversed their initial mate choice for a male and, hence, copied the choice of the model female in the long distance treatment.

Control for choice consistency

In the control, we tested 17 females. Two females were excluded from analysis, one due to lack of interest and one due to stress. Two females that showed a side bias were later successfully re-tested. Focal females (n = 15) spent 987 ± 167 s in both choice zones in M1 and 890 ± 241 s in M2. Choosing motivation significantly differed between mate-choice tests (Wilcoxon signed-rank test: V = 102, p = 0.015), with females spending less time in both choice zones in M2.

During M1 of the control, focal females (n = 15) spent on average 694 ± 240 s with the preferred male and 294 ± 151 s with the non-preferred male [scores: 0.68 (0.56, 0.76) and 0.32 (0.24, 0.44)]. Focal females showed a significant preference for one stimulus male in M1 (one-sample t test: T = 4.197, df = 14, p = 0.001). In M2, females spent on average 571 ± 232 s with the prior preferred male and 319 ± 165 s with the prior non-preferred male [scores: 0.62 (0.53, 0.71) and 0.38 (0.29, 0.47)]. There was no change in preference scores of time spent with the prior non-preferred male in M2 (Wilcoxon signed-rank test: V = 40, p = 0.554; Fig. 37). Preference for the initially preferred male in M1 was still significant in M2 (one-sample t test: T = 3.661, df = 14, p = 0.003). One out of 15 focal females (6.6 %) reversed her initial mate choice for a male in M2. Overall, focal females were consistent in their mate choice for a male when no opportunity for copying and, hence, no public information was provided during the observation period.

Comparison of distance treatments and control

There was no difference in the change of preference (copying score) for the prior non-preferred male from M1 to M2 across distance treatments (Mann-Whitney U test: Z = -0.104, p = 0.917). Further, choosing motivation in M2 (after observation of a model female) did not differ across treatments (Mann-Whitney U test: Z = 0.995, p = 0.317). The number of focal females that copied the choice of a model female and also reversed their initial preference in favor for the prior non-preferred male in M2 was significantly higher in both treatments than compared to the control for choice consistency (short distance vs. control: Fisher's exact test: p = 0.005; long distance vs. control: Fisher's exact test: p = 0.005; Fig. 38).

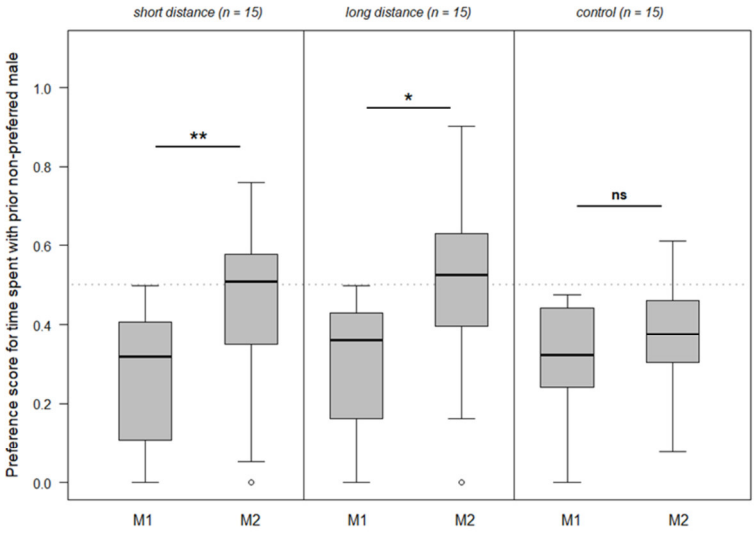

Figure 35. Preference scores for Treatment 1, Treatment 2 and the control. Boxplots of median, quartiles and whiskers (1.5 x interquartile range) are shown for preference scores for the time spent with the prior non-preferred stimulus male in mate-choice test 1 (M1) and mate-choice test 2 (M2). The grey dotted line represents a preference score of 0.5. Circles indicate outliers. N = 15; * $p \leq 0.05$, ** $p \leq 0.01$, ns = not significant

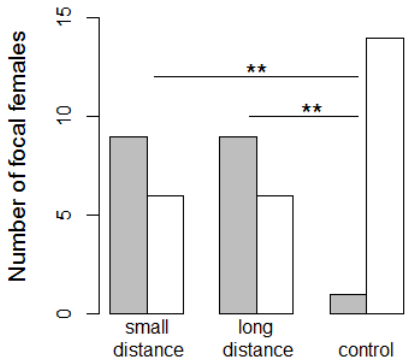

Figure 36. Number of focal females that reversed their initial mate choice after observation. Bar plots show the absolute number of females (total of n = 15 for each treatment and the control) that either did copy and reversed their mate choice (grey bars) or did not copy (white bars) the choice of a model female in both distance treatments and the control. ** = $p < 0.01$

9.5 Discussion

In the present study, we investigated whether the distance between a model female and a male would affect the use of public information and alter the mate choice of an observing female in favor of that male. As predicted, focal females significantly increased time spent with the prior non-preferred male after the observation period and copied the mate choice of the model female when the model female was in close distance (1 cm) to the stimulus male. Regarding the distance between the model female and the stimulus male, the short distance treatment resembled the classic procedure in experiments on MCC and illustrates a natural distance between male and female during courtship (Parzefall 1969; Baird 1974). Nevertheless, our experimental design differed from the classical MCC experiment in a way that focal females had much more space to swim around during mate-choice tests, and hence was more similar to the situation in the field

in this aspect. Yet, focal females copied the mate choice of the model. This result supports the previous finding of MCC in sailfin molly females. Focal females originally came from a population from Mustang Island near Corpus Christi. This is the third population of sailfin mollies from Texas exhibiting MCC (Comal River: Witte and Ryan 2002; Witte and Noltemeier 2002; Witte and Massmann 2003; Witte and Ueding 2003; San Marcos River: Schlupp and Ryan 1997; Witte and Ryan 1998).

In contrast to previous studies on MCC in fish, where model female and stimulus male could either directly interact (Bierbach, Kronmarck, et al. 2011; observations from the wild: Witte and Ryan 2002; Goulet and Goulet 2006; Alonzo 2008, Godin and Hair 2009) or at least had close range contact through a clear wall or partition (Schlupp et al. 1994; Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Massmann 2003; Widemo 2006; Frommen et al. 2009; Moran et al. 2013; Auld and Godin 2015), we here increased the distance between the model female and the prior non-preferred stimulus male during observation, and, as a result, prevented close range sexual interactions. Surprisingly and contrary to our prediction, focal females significantly increased time spent with the prior non-preferred male when the model female was positioned 40 cm away from the stimulus male (long distance treatment) as well. Despite prevention of close-range contact between the model female and the stimulus male during the observation period, public information was still available to affect the mate choice of observing focal females who copied the choice of the model.

Since focal females were consistent in their mate choice when no model female was visible and, hence, no public information was provided during the observation period (control for choice consistency), we conclude that the change in preference in favor of the prior non-preferred male in both distance treatments was due to MCC. Additionally, in all previous standard experiments testing MCC in the sailfin molly we never found any effect of local enhancement or shoaling that might have explained a change in preference in focal females (Witte and Ryan 1998; Witte and Noltemeier 2002; Witte and Ryan 2002; Witte and Massmann 2003; see also Schlupp et al. 1994; Heubel et al. 2008).

The presence of a model female simulating a choice for the prior non-preferred male in both distance treatments altered the mate choice of focal females irrespective of the distance provided. Experiments on MCC already showed that it is not necessary that an actual copulation is observed to elicit copying behavior in the observer (classic experimental procedure without direct contact), but our results further show that even interactions in distance provide enough information to affect the mate choice of an observer. Sailfin molly females were shown to perceive and respond to dummy males in a distance up to 68 cm (MacLaren 2006), therefore, it can be assumed that the focal female, the model female as well as the non-preferred male were able to see each other and respond to each other as potential mates. For this reason, it was important to block the view of the prior preferred male to the model female on the other side of the pool. In zebra finches, Kniel, Schmitz, et al. (2015) found that females copy the choice for a male phenotype when the model female and the stimulus male could acoustically and visually interact through a transparent barrier. Zebra finch females, however, did not copy the mate choice of a model female when an opaque barrier was inserted between the model female and the stimulus male, preventing visual interactions. This shows that visual cues or signals between the model female and stimulus male provides important public information used in mate choice by the observing female.

Although behavior of stimulus and model fish was not quantified in this study, all fish were observed to be visually interacting during all experiments (Keil, personal observation). The fact that focal females showed copying behavior indicates that distant visual interactions between stimulus male and model female were still perceived as sexually motivated. We assume that sexual interest between the stimulus male and the model female was expressed by an increase in swimming activity. During experiments, fish were observed actively swimming up and down the walls of the clear Plexiglas cylinders (Keil, personal observation). It was also observed that stimulus males and model females were facing each other while visually communicating (Keil, personal observation) which suggests that focal females might have assessed to whom the sexual interest was directed. A list of possible behavioral patterns of interacting sailfin mollies, which might have been visible and, therefore, influencing to an observing female, can be found in the supplementary material (see “Behavioral patterns” in Table S14 in Appendix 7).

In both treatments, the focal female was farther away from the stimulus male and the model female than they were to each other. In Treatment 1, the cylinder of the focal female was 38 cm away from stimulus males and model females whereas model female and stimulus male were only 1 cm apart. In Treatment 2, the cylinder of the focal female was 43 cm away from the stimulus males and model females, whereas model female and stimulus male were 40 cm away. If focal females were able to assess these distances, then the model female was always perceived as being nearer and, hence, more closely interacting with the stimulus male than the focal female herself. However, it is, so far, not known whether the behavior of the stimulus male or that of the model female is more important for an observing sailfin molly female’s decision to copy the choice of the model or not.

We did not find a difference in strength of preference (copying scores) between the two distance treatments, meaning that an increase in distance from 1 cm to 40 cm between the prior non-preferred male and the model female did not weaken the strength of copying behavior in our study. As our analysis shows, focal female’s motivation to choose between stimulus males was also not affected. A weakening effect of distance between sender and receiver on information transfer could, for instance, be found in a foraging context in starlings, *Sturnus vulgaris* (Fernández-Juricic and Kacelnik 2004). Fernández-Juricic and Kacelnik (2004) used two distance treatments from 0 m to 3 m within the natural range of starling flocks and showed that individual foraging and scanning behavior was less affected when birds were farther away. To our knowledge, comparable results for public information use in mate choice over distance are not apparent.

In our experimental setup, visual conditions were very good and might have facilitated public information use. The experimental pool was well illuminated and the shallow water was clear, providing excellent visibility that was also stable over time. Long and Rosenqvist (1998) showed that guppy males vary their courting distance depending on the light environment with a two- to three-times larger distance (6-9 cm) at higher light levels. Therefore, courting distance may vary without losing information for the receiver and, presumably, for an observer in the case of MCC. It is, however, likely that under natural conditions, the perception of distant interactions and, hence, public information use over distance, is limited by individual visual capacities and environmental factors like water turbidity, which has e.g., shown to affect mate choice in sailfin mollies (Heubel and Schlupp 2006) and in three-spined sticklebacks (Engström-Öst and Candolin 2007). Therefore, it can be assumed that, depending on the respective habitat and particularly

depending on differences in visual conditions, it is likely that different populations of sailfin mollies differ in public information use. There may exist a high variability of public information use within populations of sailfin mollies, due to e.g., seasonal and geographical differences in turbidity and, hence, visibility as well (see Heubel 2004). Turbidity can lead to different mate choice decisions in different populations of the same species. Basolo (2002) found a preference for males with an artificial sword in sailfin molly females of a population in Louisiana living in murky water. Witte and Klink (2005) tested a latent preference for males with an artificial sword in sailfin mollies of a population from the Comal River in New Braunfels, Texas, living in clear water. In contrast to Basolo (2002), Witte and Klink (2005) found no latent preference for artificially sworded males in sailfin molly females. This difference in female mate choice between these two populations may be due to adaptations to the environment with bad or good visual conditions.

9.5.1 Conclusion

In our study, we could show that close range contact between a model female and a male is no prerequisite for MCC in sailfin mollies. Public information gained from interactions between two heterosexual conspecifics at distance still provides public information to affect the mate choice of an observing female. Our results indicate a much wider transfer and use of public information in sailfin molly groups than previously thought as 40 cm exceed the, for fish typically described, social interaction distance for group members of four body lengths (Croft et al. 2008). Further, our results raise the question of what specific behavioral features communicate sexual interest in sailfin mollies at distance, apart from commonly described courtship displays in this species. Future studies should evaluate this aspect in more detail. Since MCC is regarded as an important evolutionary driver for both the transmission of phenotypic traits as well as the preference for those (Danchin et al. 2004), our findings underline even more far-reaching consequences of MCC for the evolution of sexually selected traits in sailfin mollies. This is especially important when regarding the presumably high variability of public information use due to varying environmental constraints. The use of public information from distance does not lower the risk of the detection of an observer by the interacting individuals, which can lead to the audience effect. Thus, apart from MCC, a wider information transfer might also lead to implications for other behavioral phenomena like e.g., audience effects (reviewed in Plath and Bierbach 2011) and should be evaluated in more detail in the future.

9.6 Acknowledgements

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Chapter 10
Synopsis

In my thesis, I investigated the use of public information for MCC in female sailfin mollies with focus on differences in public information content, to increase common knowledge of the underlying mechanism of MCC. Here, I will evaluate the use of computer animation as an alternative experimental approach for studying MCC and I will summarize and discuss my thesis' results in light of the relevance of information content for MCC in sailfin mollies. Further, I will suggest prospects for future research, which derived from my own research, and I will give possible new lines and advances for future application of computer animation (e.g., *FishSim*) in behavioral experiments.

10.1 Evaluating the use of *FishSim* for behavioral studies on MCC

The heart of my thesis described a series of experiments, in which I studied how differences in visual information content affect the use of public information by female sailfin mollies in the context of MCC (**Chapters 6, 7, 8** and **9**). My thesis was thereby intended to increase the body of knowledge on MCC to get a better understanding of its underlying mechanism. I used a newly developed research tool for design, animation and presentation of virtual fish stimuli during experiments, which was far beyond the state-of-the art regarding previous experimental studies on MCC.

As described in detail in **Chapter 4**, my PhD project included the development and implementation of a novel and innovative new approach for testing questions related to MCC in sailfin mollies, using computer-animated fish stimuli. *FishSim* is the result of a successful interdisciplinary collaboration between computer-scientists and biologists and its use in research allowed me to follow new approaches in the study of MCC compared to classic experimental designs. With the help of *FishSim*, I was able to perform automated MCC experiments using virtual fish stimuli instead of live ones. By this, I was able to manipulate the visual information provided during the observation period of an MCC experiment in a non-invasive and highly standardized way.

Following the initial software development, but preceding all further testing (with regard to MCC), I needed to confirm the usability of our newly developed research tool in a series of validation experiments that I designed with respect to previous studies using computer animation (e.g., Qin et al. 2014; see Chapters 3.5). In **Chapter 5**, I describe the positive validation of *FishSim* for binary mate-choice experiments with sailfin mollies. In this study, I showed that there was no difference in attractiveness of a virtual male stimulus (created with *FishSim*), presented to live focal females, compared to the presentation of live or video recorded male stimuli. Additionally, focal females were attracted to moving stimuli compared to stationary ones and, overall, preferred to associate with a fish-shaped stimulus compared to a geometrical box. Live focal males were further observed to perform courtship behavior (lateral displays and gonopodial thrusting) towards virtual female conspecifics which demonstrates the high degree of realism the animation must have had for them (Gierszewski et al. 2017). The results of my validation indicate that live fish readily identified virtual fish as conspecifics as well as mating partners, which justified the use of *FishSim* to test questions related to mate choice in general.

After confirming the general acceptance and attraction towards virtual fish stimuli by live fish (i.e., association behavior), as well as their ability to provoke sexual interest in observing live fish, I needed to test the usability of our toolchain for the procedure of MCC experiments. In a comparative study, presented in **Chapter 6**, I performed the identical MCC experiment by either (I) exclusively using live stimulus fish, or (II) computer animated stimuli created with *FishSim.* Here, I demonstrated that *FishSim* can be used to adopt the experimental procedure of a classic MCC experiment to conduct virtual MCC experiments in an easy-to-use and highly controlled way. Using *FishSim*, I was able to control for a specific body size in virtual fish, which can be quite difficult when relying on one pool of live fish in which natural size ranges can be quite variable. For example, live fish used in Experiment 1 (**Chapter 6**) needed to fulfill specific criteria for body size. For this, all fish were measured prior to experiments, to ensure that live stimulus males were of similar body size (size difference < 5mm), or that a live model female was larger or smaller than the focal female. Live fish were, therefore, exposed to handling-stress already prior to experiments. Moreover, I was dependent on the distribution of body sizes present in the lab population at the time the experiment took place. In contrast, by using *FishSim* (Experiment 2, **Chapter 6**), I was able to create virtual fish stimuli of specific body sizes that were identical throughout, without measuring and inflicting stress to fish prior to experiments. Virtual fish showing a specific behavioral pattern (i.e., an animated swimming path) further prevented natural variation in behavior, as e.g., expressed by individual live stimulus fish, which overall increased the reliability of the results gained from experiments.

Notably, I was able to show that live focal females copied the choice of a live model female for a prior non-preferred male and, likewise, that of a virtual model female as well. Therefore confirming the possibility of providing public information via computer-animated scenes (created with *FishSim*), that can be assessed by live focal females. Overall, there was no difference in focal female's copying behavior across presentation types (live or virtual). Over the course of the experiment, focal female's relative choosing motivation was not affected by whether live or virtual fish stimuli were presented during the different stages of the MCC experiment. Therefore, the results of this study justified the use of *FishSim* to test questions related to MCC, which was continued in the following **Chapters 7 and 8**.

In Chapter 7, I provide a classic example for the possibility to manipulate the appearance of a virtual stimulus to investigate the role of certain visual traits. Here, I artificially manipulated the absence or presence of the gravid spot in virtual model females. In contrast to a rather invasive procedure performed by Benson (2007), who injected tattoo ink into the abdominal region of live female green swordtails to generate a gravid spot, my study was completely non-invasive. By using *FishSim*, I was not only able to present a gravid spot of specific size, but I was also able to keep its size and position identical across all experimental trials. By this, the trait under testing remained constant but otherwise natural variation in virtual model female phenotypes was assured.

Clearly, the possibility to manipulate behavior is another key advantage of using artificial stimuli such as computer-animated animals. In **Chapter 8**, I profited from the functions of *FishSim* for independent manipulation of courtship behavior performed by the virtual model female and the virtual male. During the observation period, I varied the extent and direction of courtship behavior by presenting either (I) mutual driven, (II) female driven or (III) male driven courtship sequences. Using *FishSim* I was able to control for individual behavioral differences by keeping the behavior of virtual fish constant per treatment, across each trial. In this study, I could show that focal females were able to assess

differences in behavior created with *FishSim* (i.e., copulation events, presence or absence of male and female sexual behavior) and altered their mate-choice decisions accordingly. These results demonstrate the high value of *FishSim* for behavioral studies with sailfin mollies, and presumably other fish species as well. Especially, since behavioral manipulations are almost impossible to achieve in live animals.

10.2 New insight into MCC in sailfin mollies

Following previous research that postulated an important role of the model female for MCC to occur (e.g., Dugatkin and Godin 1993; Amlacher and Dugatkin 2005; Godin et al. 2005; Hill and Ryan 2006; Kniel et al. 2017), I manipulated visual information content regarding features that possibly characterize the quality of the model female (**Chapters 6 and 7**). Additionally, I aimed to manipulate the sexual interaction of the model female and the prior non-preferred male, that is courtship behavior, to investigate how this might affect public information use for MCC in observing focal females (**Chapters 8 and 9**). Manipulation of the behavioral interaction between model female and male posed a novel angle on MCC research, which has so far not been followed-up by scientists in great detail (but see Witte and Ueding 2003).

For my study, I tested descendants of wild sailfin mollies who can be considered the ideal study organism to investigate questions on MCC. As a group-living fish, sailfin mollies have ample opportunity to observe others and their mate choice and both male and female alike deploy MCC as an alternative mate-choice strategy, in both the laboratory and in the wild (Witte and Ryan 1998; Witte and Ryan 2002; see Chapter 2). In addition to previous studies using inland populations of sailfin mollies (Comal River: Witte and Ryan 2002; Witte and Noltemeier 2002; Witte and Massmann 2003; Witte and Ueding 2003; San Marcos River: Schlupp and Ryan 1997; Witte and Ryan 1998), I was able to confirm the existence of MCC in a third population of sailfin mollies from Texas (USA), the coastal Mustang Islands; a population which has not been used in experiments on MCC before.

In **Chapter 6**, I first tested whether the body size of the model female affects MCC in observing focal female sailfin mollies. In guppies, model female size was suggested to serve as a means of quality by providing information on their experience in mate choice. With larger and consequently older females being more experienced in mate choice, hence, their choice being more valuable to observing conspecific females (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005). Female size was shown to be correlated to fecundity in poeciliid fishes, with males preferring to associate with larger females (e.g., Ptacek and Travis 1997; Gabor 1999). Therefore, I hypothesized that, model female body size also poses an important sign for quality to observing female sailfin mollies, when it comes to copying the choice of said model female. For this, I used a comparative approach performing the same MCC experiment deploying either (I) a classic experimental setup exclusively using live fish, or (II) a novel approach using virtual fish stimuli created with *FishSim*. Both experimental approaches (only live fish vs. live focal fish with virtual stimulus fish) revealed that live focal females copied the choice of the model female (live or virtual) regardless of whether she was larger or smaller than the focal female herself. This result is in contrast to previous research in guppies (Dugatkin and Godin 1993; Amlacher and Dugatkin 2005), suggesting that underlying rules or mechanisms describing MCC may not necessarily be the same across species. Moreover, it can be assumed that species-specific life-history traits and/or different

socio-ecological factors (Chouinard-Thuly and Reader 2019; Smolla et al. 2019; Webster et al. 2019) may define social information use in general which, further, needs to be flexible across different contexts. Therefore, underlying rules for the use of MCC as an alternative mate-choice strategy may as well differ between and within species (Heubel et al. 2008) or even between the sexes (Smolla et al. 2019). Here, future research is necessary to identify the nature of these defining factors (see also Chapter 10.3 below).

Another feature that possibly contributes as an indicator for model female quality is one providing information on her reproductive state and, consequently, her motivation to mate. Similar to other poeciliids, female sailfin mollies show a distinctive dark spot in their urogenital region, the "gravid spot", which is discussed to serve as a fertility advertisement towards males (e.g., Sumner et al. 1994). To investigate whether the gravid spot also provides information on model female quality to observing focal females, I manipulated its visual presence or absence in virtual model females using *FishSim* (**Chapter 7**). However, the results of this study do not indicate that the gravid spot of a virtual model female has any relevance for model female quality in the context of MCC. Observing live focal females copied the choice of a virtual model whether she had a gravid spot or not. As postulated by Farr and Travis (1986), the gravid spot indicates sexual maturity in general, which consequently includes females that just reached maturity and are not experienced in mate choice yet. Overall, the expression of a gravid spot can be very different between individual females, at least in sailfin mollies, and might, therefore, not be considered a reliable source of information in observing females. Further, additional olfactory cues might indicate a female's reproductive state towards males (Sumner et al. 1994). The very same olfactory cues might hence also provide information to observing females. However, such cues were missing in the present study and I can only speculate about their importance for MCC. I conclude that, at least by visual presence alone, the gravid spot does not contribute as a source of information for model female quality in sailfin mollies in the context of MCC. Nevertheless, it might be interesting to test whether this conclusion is also true for female guppies or other poeciliids who express gravid spots, accompanying their reproductive cycle (see also Chapter 10.3 below for future research ideas).

Not only visual features, resulting from specifics in morphology or appearance, may contribute as a source of information for MCC, but also the behavior of each protagonist involved. Here, in particular, differences in courtship behavior of the interacting pair of model female and stimulus male is a most interesting aspect which has, so far, not received a lot of interest in research. This might be due to the fact that it is impossible to manipulate animal behavior in a non-invasive way, especially, if you want to create or promote one specific behavioral pattern. Not to mention a behavior which is normally not natural to the species or sex under study (e.g., reversed roles during courtship). Witte and Ueding (2003) had previously shown that live focal females spent less time with a prior-preferred video-recorded male after they had observed the video of that same male together with a model female who was fleeing from him. Focal females were obviously paying attention to the fleeing behavior of the model female and used this information in their own mate choice, by increased avoidance of that male. Here, the question arises, whether focal females generally tend to give more credit to the behavior of the model female compared to that of the male.

In **Chapter 8**, I tried to decipher whose behavior of the interacting pair provides most relevant information for copying to observing focal females. With the help of *FishSim*, I varied the extent and direction of courtship behavior by presenting either (I) mutual, (II) female driven or (III) male driven courtship sequences during the observation period.

Further, I tested whether (IV) presence or (V) absence of male courtship affected observing females' mate choice without public information (no model female visible). In all three treatments (mutual, female driven and male driven courtship), live focal females copied the choice of the virtual model female for a prior non-preferred virtual male. However, I found that focal female copying behavior was the weakest after having observed the 'mutual courtship' scenario, in which both model female and male showed high sexual activity and simulated copulations. In contrast, copying behavior was strongest in the 'male driven' scenario, when only the male was sexually active and no copulations occurred. When focal females were not able to rely on the choice of a model, lack of male courtship behavior during the observation period resulted in focal females spending less time with a prior preferred male, compared to when that male was actively courting. These results indicate for the first time, that besides providing general information for male assessment, a model female may actually also be perceived as a competitor to observing females. I could show that focal females were able to assess differences in behavior created with *FishSim* (i.e., copulation events, presence or absence of male and female sexual behavior) and altered their mate-choice decisions accordingly. To my knowledge, this study is the first of its kind and it highlights the importance of including questions related to behavioral aspects in future research on MCC (see also Chapter 10.4.1 below).

Aside from the behavior itself, an important factor influencing the sexual interaction between a male and a female is the distance at which the pair is communicating to each other. So far, it has never been investigated whether variation in distance of an interacting pair (towards each other) alters the information another individual might gather from observing this pair. In **Chapter 9,** I studied the effect of distance of the interacting pair on focal female MCC, using live sailfin mollies only. Natural sailfin molly courtship is described to act at very close distance between a male and a female (only few centimeters apart; e.g., Parzefall 1969; Baird 1974). I assumed that increasing the distance between the pair would impede courtship and an observing female would hence not be able to gather information on the sexual interaction of the pair. Not to mention, using that information for MCC. However, the results of my study clearly show, that focal females copied the choice of a model female at close proximity to the male (1 cm) as well as when she was at distance (40 cm apart) from that male. Observation shows that the majority of behavioral patterns involved in sailfin molly courtship may also be performed at distance (see "Behavioral patterns" in Table S14, Appendix 7). Additionally, intentional gaze and attentive behavior in direction of the opposing partner presumably favor the impression of sexual interest in an observing female even over distance. My results suggest that as long as a positive sexual intention of the model female is visible to observing focal females, her choice is copied. Obviously, close proximity of an interacting pair poses no prerequisite for MCC but also sexual interactions at long distance provide valuable public information. This new finding implies a much wider range of public information use than previously thought. This is especially important when considering variation in public information use due to varying environmental conditions possibly affecting behavior (Long and Rosenqvist 1998; Heubel and Schlupp 2006; Engström-Öst and Candolin 2007; Candolin and Wong 2019; Mobley et al. 2019).

To conclude the outcome of my thesis, I was able to (I) emphasize the importance of the model female for observing conspecific females, and to (II) draw attention to the importance of the behavioral interaction that is to be observed. Investigating potential effects of behavioral differences on MCC in sailfin mollies poses a new line which should clearly be focused on in future research (see Chapter 10.4.1 below). Further, it became clear that sexual interaction at distance indicates a much wider transfer and use of public

information in sailfin molly groups than previously thought. MCC remains a complex and fascinating mate-choice strategy that, if utilized, depends on the specific interplay between quality and quantity of public information. Overall, my thesis' research succeeded in adding valuable new information to the growing body of knowledge on the underlying mechanism of MCC, not least due to the use of an innovative and novel research tool, that is, *FishSim* Animation Toolchain.

10.3 Limitations and strengths of *FishSim* for behavioral studies on MCC

There are certain pitfalls and limitations that need to be kept in mind while using *FishSim*, and computer animation in general, which we discussed in detail in **Chapter 3**. Further, we address several critical steps that need specific attention to ensure the correct handling of our toolchain and the success of the experiment in Gierszewski, Baker, et al. (2018). It is important to note that we wanted to provide a fast and easy-to-create animation process by using a video-game controller. Here, the general swimming behavior of virtual fish is automatically generated, based on videos of swimming live sailfin mollies (Smielik et al. 2015) and therefore represents real life data of behaving sailfin mollies. However, the visual expression of individual behavioral patterns in an animated scene (e.g., courtship) is subject to the interpretation of the experimenter who creates an animated scene and to his or her practice in using the controller.

Notably, my thesis demonstrates a highly standardized procedure for visual manipulation of public information for MCC experiments with fish. A prominent advantage of using computer animation is the easy implementation of the 3R's principle – *reduce, replace, refine* – in research (Russell and Burch 1959; ASAB 2018). Using *FishSim*, I was able to *reduce* the use of live fish during MCC experiments by 80% (one live fish instead of five), compared to the classic experimental design. I was able to *replace* all live stimulus fish used in experiments presented in **Chapters 6** (Experiment 2), **7 and 8** by virtual fish (virtual stimulus males and virtual model females) and, since I was able to control the specific behavior of virtual stimulus males, there was no need for an additional pseudo-model female [e.g., compare **Chapter 6** (Experiment 1) and **Chapter 9**]. Further, I was able to *refine* the experimental procedure by reducing handling-stress inflicted to live fish and by increasing standardization. For example, there was no handling-stress inflicted towards stimulus males by switching their position during mate-choice tests [side bias control for focal females; compare **Chapter 6** (Experiment 1) and **Chapter 9**]. In addition, the whole experimental procedure was automated and could easily be controlled from the operating computer, to keep general handling and disturbance of the experimental setup to a minimum. Whereas the view of live stimulus fish needed to be obscured by inserting opaque screens at multiple times during the experiment (e.g., Experiment 1 in Chapter 6), this procedure was not needed when using *FishSim*. Instead, the sequence of animated scenes was carefully planned prior to experiments, including "pause" screens that did not show any stimulus during handling times. By this, overall disturbance of focal fish during experiments was decreased. Additionally, the overall value of the data gained by using *FishSim* is increased due to highly standardized and controlled experimental procedures.

Arguably, *FishSim* even enables a 4th R regarding the *reproducibility* of studies due to its high degree of standardization and the possibility to share virtual fish models and created animations (i.e., swimming paths) between research groups. Since we ensured *FishSim* to be open-source and freely available for everyone, we further support the 5th R

responsibility as suggested by members of the Max Planck Society[17]. By providing access to our research tool to the scientific community, we actively promote and facilitate increasing the degree of animal welfare standards for behavioural experiments.

10.4 Outlook

10.4.1 Discovering new leads for research on MCC

To date, the fascination for studying social learning in animals, with regard to MCC, has still not faded. New findings related to MCC across the whole animal kingdom give reason to assume that MCC is more of a rule than the exception. From mammals over birds and fish to even invertebrates, MCC seems to be prevalent in nature (as reviewed by Witte et al. 2015 and Jones and DuVal 2019). Who would have thought that even the tiniest fruit fly may rely on social information to facilitate mate choice (Danchin et al. 2018; Nöbel, Allain, et al. 2018)? New evidence is further adding up to suggest social learning taking part during mate choice in spiders (Fowler-Finn et al. 2015; Dion et al. 2019; Scott et al. 2019; Gilman et al. 2019).

So far, the majority of studies focused on examining one single source type of social information, that is, mostly visual cues. Only few studies tried to follow a multimodal approach regarding MCC (visual and/or auditory cues: Kniel, Schmitz, et al. 2015). Surely this may be due to a decrease in experimental control with increasing number of cues taken into account. However, multimodal stimuli represent the natural setting an animal has to deal with when facing mate-choice decisions (e.g., Reding and Cummings 2017) and a possible trade-off between the validity of different sources depending on the respective social environment may be assumed. Chemical cues have so far been neglected in this context as well (but see Kavaliers et al. 2017; Scott et al. 2019). It would be interesting to test if and how an animal's emotional state (e.g., stressed vs. relaxed) or its reproductive state (e.g., receptive vs. non-receptive), represented by differences in hormonal levels, might alter the use or strength of MCC (see Gonçalves and Oliveira 2010 for a review on hormones and sexual behaviour in teleost fishes). This might be equally interesting to test from the observer's perspective (both sexes) as well as from the perspective of the individual that is being observed, i.e., the model. Gabor and Contreras (2012) were able to extract and measure water-borne hormones of male and female sailfin mollies. Evidence suggests that, at least concerning social competition, hormones seem to play a role during mate choice in this species (Kim et al. 2019).

In line with the increasing amount of research on the existence of repeatable individual differences in behavior (i.e., personality types) and their postulated significance regarding sexual selection (Schuett et al. 2010) and evolution in general (Wolf and Weissing 2012), it would be worthwhile to study possible effects regarding the expression of MCC. It can be assumed that varying degrees in social learning in general and MCC in particular, may also be subject to differences in personality. In the context of mate choice, it was indeed found that male sailfin molly courtship rate was positively correlated with boldness (Seda et al. 2012). Further, individual differences in anxiety of sailfin mollies seem to be related to differences in learning performance and cognitive style (Gibelli et al. 2019) and might, hence, also affect MCC. Boldness and sociability were demonstrated to affect decisions on using social over private information in wild

[17] See statement of the Max Planck Society here: https://www.mpg.de/10973438/4rs (last accessed February 12th, 2020)

female guppies (Trompf and Brown 2014; see also Smit and van Oers 2019) while sociability was later linked to predict MCC intensity in this species by White, Watts et al. (2017).

Regarding future research in sailfin mollies, it is of particular interest to investigate whether differences in personality traits might also contribute to the expression of MCC, for example if differences in personality of focal females affect their tendency to copy the choice of a model or regarding a possible link between differences in a female's personality and her quality as a model female for observing conspecifics. Moreover, the interaction of the model female and the male might be affected by this as well and I cannot emphasize enough the importance of studying the possible effects of behavioural differences on MCC. With regard to our findings of potential long-distance effects of public information use in sailfin molly mate choice (Gierszewski, Keil et al. 2018; **Chapter 9** in my thesis), future research might benefit from using experimental settings that grant more space when using live fish. Near-natural group settings allowing for detailed network analysis, with regard to sociality and its implications for public information use in sailfin mollies, could even be possible (Krause et al. 2007; Wey et al. 2008; Makagon et al. 2012). As a group-living fish, it can be assumed that group composition per se, as well as an individual's relationships within a social network (e.g., dominant-subordinate relationships) could possibly affect MCC in sailfin mollies. Socio-ecological constraints, such as the simulated presence or absence of predators and/or competitors (e.g., the Amazon molly) could potentially affect the strength of copying in sailfin mollies. For this, technical advances for behavioural tracking of individual animals and the implementation of interactive systems, regarding closed-loop computer animation (e.g., virtual reality), will be particularly valuable in the future (see Chapter 10.4.2 below).

Still, a lot of questions on the nature of MCC and its underlying mechanism remain unanswered. Especially regarding its implications for speciation in a world that is subject to fast (anthropogenic) change, that surely also affects visual communication and hence mate choice as well (Candolin and Wong 2019). We are not even close to understanding the full spectrum of this fascinating strategy but, hopefully, future studies will continuously widen our view on the evolution of sexual traits to cultural transmission of traditions in animal groups based on social learning such as MCC.

10.4.2 What does the future hold for computer animation in research?

The use of *FishSim,* as described in my thesis, is not limited to my study design on MCC in sailfin mollies, but can generally be applied to other study systems as well. We provide an evaluation for a more universal application of *FishSim* for behavioral testing of other fish species in Gierszewski and Baker et al. (2018). In general, computer animation tools offer a wide variety of solutions for studying fish behaviour, such as mate choice, shoaling decisions, predator-prey detection or general learning abilities.

Even though modern techniques, such as computer animation and virtual reality, are not anymore completely new, more advanced methods are still on the rise and are continuously refining. The need for such techniques has, to date, not receded and research is longing for controlled and standardized methods which can be used to artificially alter an animal's sensory environment. As clearly visible from the field of robotics, the trend goes towards studying social interactions and communication in animal groups (e.g., collective movement) using systems that are responsive or

interactive towards the behavior of the focal animal (as reviewed by Romano et al. 2019; see also Landgraf et al. 2008; Landgraf et al. 2011; Landgraf et al. 2014; Landgraf et al. 2016; Bierbach, Landgraf, et al. 2018; Bierbach, Lukas, et al. 2018; Spinello et al. 2019). As with robotics, also computer animation frameworks can be capable of simulating interactive stimuli and even environments (e.g., Bian et al. 2018), as can be seen by the implementation of sophisticated virtual reality solutions (e.g., Naik et al. 2019; Stowers et al. 2017). As already mentioned in **Chapter 4.7**, we had this trend in mind while developing *FishSim* and already set foot for the future integration of interactive stimuli. Thanks to the modularity of the underlying ROS, external devices, such as high-resolution cameras, can be integrated into the toolchain. A first successful attempt showed that *FishSim* can be used to simulate interactive virtual fish stimuli by extension of a 3D real-time tracking system (Müller et al. 2014; Müller, Gierszewski, et al. 2016; Müller et al. 2018). During the science communication event "*Molly knows best*", we were able to demonstrate that virtual fish can be programmed to follow live focal fish on screen and perform courtship behavior according to predefined algorithms. Interactivity increases a more natural appearance of virtual stimuli for observing live fish and paves the way for the study of social communication, in which a virtual animal may respond directly to the behavior expressed by a live animal.

To achieve this, future development should clearly emphasize on implementing and refining algorithms for automated detection and interpretation of additional features of live animals apart from general movement in space, such as body posture and specific behavioral patterns (e.g., Swierczek et al. 2011). This can be achieved with the help of tracking systems, which are currently also under continuous development with several solutions already available and specifically tailored for the use in behavioral experiments (Spink et al. 2001; Crall et al. 2015; Müller, Gierszewski, et al. 2016; Bierbach, Lukas, et al. 2018). Here, the integration of deep learning procedures, which now gain more and more popularity in ecology (Christin et al. 2019) could lead to interesting new lines of research.

In conclusion, the use of computer animation in animal behavior research is a promising approach when conventional methods would require invasive treatment of live animals to manipulate the expression of a visual trait or behavioral pattern. Manipulating computer animations allows for a high degree of control and standardization compared to using live test fish, especially, since it also offers solutions to manipulate behavior which is very limited or even impossible in induce in live fish and other animals. In times when the successful integration of the 3Rs-principle (see Chapter 10.3), and similar guidelines for the use of animals in research and teaching, is required more than ever. This technique not only bears the potential to increase animal welfare but it may simultaneously increase a study's overall validity and public value. Obviously, we have not yet reached the peak of technical innovation and, surely, the future has a lot on hold for the study of animal behavior.

Summary

The strategy of mate-choice copying has fascinated scientist for decades but still, little is known about its underlying mechanism. Investigating what aspects of public information are relevant for triggering copying behavior and how these might be intertwined is, therefore, a central objective in current research. During my PhD project, I investigated the use of public information for mate-choice copying in the livebearing sailfin molly *Poecilia latipinna*. Both males and females were shown to copy the mate choice of conspecifics which makes the sailfin molly the ideal model species. Aside from using a classic experimental approach with live fish stimuli to study copying, I also used a novel methodological approach, that is, computer animation. For this, my PhD project also involved the development of "*FishSim* Animation Toolchain" within the interdisciplinary "Virtual Fish Project" at University of Siegen. *FishSim* is a software framework for the creation, animation and presentation of virtual sailfin mollies during experiments. A positive validation of the new method showed that focal females were equally attracted to virtual males as to live males or those presented as videoplayback. In a series of copying experiments with live and virtual stimulus fish, I manipulated visual public information content provided by either a model female and/or her sexual interaction with a male during the observation period.

A model female's body size is assumed to indicate her quality as a model with respect to her age and, consequently, her experience in mate choice. With larger models being older and, hence, more experienced in mate choice. In a comparative study using live and virtual fish stimuli, I found that the size of a model female did not affect copying behavior in observing female sailfin mollies. They copied the choice of a model female (live and virtual) for a prior non-preferred male irrespective of whether the model female was larger or smaller than the focal female. These results were in contrast to previous studies that demonstrated size-dependent copying behavior in the closely related guppy. I assume that possible rules underlying mate-choice copying may be species-specific and vary between different socio-environmental conditions.

In another study, I assumed that the gravid spot, which is a common feature of female poeciliids, might provide information about a model female's quality with respect to her reproductive status. However, I could show that neither the presence nor absence of a visible gravid spot in virtual model females affected copying behavior in focal females. They copied the choice of a virtual model female in both conditions, indicating that the gravid spot does not provide a reliable source of information for observing females. At least not in sailfin mollies.

To investigate whether the behavioral interaction of the model female and the male affects copying behavior in observing focal females, I manipulated courtship behavior of the interacting pair. I used *FishSim* to either present courtship behavior that was mutual, only female driven or only male driven. I could show that focal females copied the choice of a virtual model female in all three conditions. The strength of copying, however, was affected by differences in courtship behavior. Copying behavior was strongest when only the male was actively engaged in courtship and weakest when both male and female were actively engaged and also copulated. When no model female was present, a lack of male courtship behavior resulted in focal females losing interest in a prior preferred male. My results indicate that, albeit providing valuable information for the assessment of a male, a sexually active model female may nevertheless be perceived as a competitor which overall weakens copying behavior. This study, demonstrates a first attempt for a

systematic manipulation of behavior to investigate its possible effect on mate-choice copying, which has not been done before in such a controlled way.

In a final experiment using live fish only, I manipulated the behavioral interaction of the model female and male by increasing the distance between them. I could show that focal females showed copying behavior when model and male were at close proximity (1 cm) to each other but also when they were at apart from each other (40 cm) and only able to communicate over distance. These results were in contrast to my prediction that increasing the distance between the pair would impede courtship or any sexual interaction, which is described to naturally occur at close distance. This study implies an even wider transfer of public information for mate choice than previously thought.

In summary, my findings support previous knowledge on the importance of the model female and further highlight the importance of including aspects about the behavioral interaction of model female and male in future research on mate-choice copying. Further, I was able to demonstrate a highly controlled and standardized approach for future studies on mate-choice copying using *FishSim*, which also has the ability to increase animal welfare in research.

Zusammenfassung

Die Strategie des Kopierens der Partnerwahl fasziniert Forscher seit Jahrzehnten, aber dennoch ist nur wenig über seinen grundlegenden Mechanismus bekannt. Die Erforschung davon, welche Aspekte der sozialen Information relevant sind, um Kopierverhalten auszulösen und wie diese miteinander verknüpft sein könnten, ist ein zentrales Ziel derzeitiger Forschung. In meiner Doktorarbeit untersuchte ich den Nutzen sozialer Information für das Kopieren der Partnerwahl bei dem lebendgebärenden Breitflossenkärpfling *Poecilia latipinna.* Es wurde gezeigt, dass sowohl die Männchen, wie auch die Weibchen, die Partnerwahl von Artgenossen kopieren, was den Breitflossenkärpfling zu einer idealen Modellart macht. Neben der klassischen, experimentellen Vorgehensweise mit echten Stimulusfischen um das Kopieren zu untersuchen, verwendete ich auch einen methodisch neuen Ansatz, nämlich den mittels Computeranimation. Dafür beinhaltete meine Doktorarbeit auch die Entwicklung von „*FishSim* Animation Toolchain“, innerhalb des interdisziplinären Projekts „Virtueller Fisch“ an der Universität Siegen. *FishSim* ist eine Softwareoberfläche für die Erstellung, Animation und Präsentation von virtuellen Breitflossenkärpflingen für Experimente. Eine positive Validierung der neuen Methode konnte zeigen, dass Testweibchen an virtuellen Männchen gleichermaßen interessiert waren, wie an echten Männchen oder solchen die als Videoaufnahmen gezeigt wurden. In einer Reihe von Kopierexperimenten mit echten und virtuellen Stimulusfischen, manipulierte ich innerhalb der Beobachtungsphase den Inhalt sichtbarer sozialer Information, der entweder durch ein Modellweibchen und/oder ihre sexuelle Interaktion mit einem Männchen zur Verfügung stand.

Es wird vermutet, dass die Körpergröße eines Modellweibchens auf ihre Qualität als Modell hinweist, im Hinblick auf ihr Alter und, in der Konsequenz, auf ihre Erfahrung bei der Partnerwahl. Größere Modelle seien älter und daher erfahrener in der Partnerwahl. In einer Vergleichsstudie mit echten und virtuellen Stimulusfischen fand ich heraus, dass die Körpergröße eines Modellweibchens das Kopierverhalten von beobachtenden Testweibchen nicht beeinflusste. Sie kopierten die Wahl des Modellweibchens (echt oder virtuell) für ein vormals nicht präferiertes Männchen unabhängig davon, ob das Modellweibchen größer oder kleiner als das Testweibchen war. Diese Ergebnisse stehen im Kontrast zu früheren Studien, die größenabhängiges Kopierverhalten bei dem nahverwandten Guppy demonstrierten. Ich vermute, dass grundlegende Regeln für das Kopieren der Partnerwahl artabhängig sind und zwischen verschiedenen sozialen und ökonomischen Begebenheiten variieren können.

In einer anderen Studie vermutete ich, dass der Trächtigkeitsfleck, welcher ein typisches Merkmal von weiblichen, lebendgebärenden Zahnkarpfen ist, möglicherweise Informationen über die Qualität eines Modellweibchens im Hinblick auf ihren Fortpflanzungsstatus verrät. Ich konnte allerdings zeigen, dass weder das Vorhandensein, noch die Abwesenheit des Trächtigkeitsflecks bei einem virtuellen Modellweibchens das Kopierverhalten der Testweibchen beeinflusste. Sie kopierten die Wahl eines virtuellen Modellweibchens in beiden Fällen, was andeutet, dass der Trächtigkeitsfleck keine Quelle zuverlässiger Information für beobachtende Weibchen darstellt. Zumindest nicht für Breitflossenkärpflinge.

Um zu untersuchen, ob die Verhaltensinteraktion zwischen dem Modellweibchen und dem Männchen das Kopierverhalten von beobachtenden Testweibchen beeinflusst, manipulierte ich das Balzverhalten des interagierenden Paares. Ich verwendete *FishSim*, um Balzverhalten zu präsentieren, das entweder einvernehmlich war oder jeweils nur

von dem Männchen oder dem Modellweibchen ausging. Ich konnte zeigen, dass die Testweibchen die Wahl eines virtuellen Modellweibchens in jeder der drei Situationen kopierten. Die Stärke des Kopierens wurde jedoch durch Unterschiede im Balzverhalten beeinflusst. Das Kopierverhalten war am stärksten, wenn nur das Männchen aktiv balzte und am schwächsten, wenn Männchen und Weibchen beide aktiv balzten und sogar sichtbar kopulierten. Wenn kein Modellweibchen anwesend war, sorgte ein Fehlen des männlichen Balzverhaltens dafür, dass die Testweibchen das Interesse an einem vormals präferierten Männchens verloren. Meine Ergebnisse zeigen, dass obwohl ein Modellweibchen wertvolle Information für die Beurteilung eines Männchens liefern kann, ein sexuell aktives Modellweibchen nichts desto trotz auch als Konkurrentin wahrgenommen werden könnte, was das Kopierverhalten insgesamt vermindert. Diese Studie beschreibt einen ersten Versuch für eine systematische Manipulation von Verhalten, um dessen mögliche Effekte auf das Kopieren der Partnerwahl zu untersuchen, was bisher noch nicht unter solch kontrollierten Bedingungen versucht wurde.

In einem letzten Experiment mit realen Fischen manipulierte ich die Verhaltensinteraktion des Modellweibchens und dem Männchen, indem ich die Distanz zwischen ihnen vergrößerte. Ich konnte zeigen, dass Testweibchen Kopierverhalten zeigten wenn sich Modellweibchen und Männchen in unmittelbarer Nähe zueinander befanden (1 cm) aber auch, wenn sie voneinander entfernt waren (40 cm) und nur über Distanz kommunizieren konnten. Diese Ergebnisse stehen im Kontrast zu meiner Vermutung, dass eine Vergrößerung der Distanz zwischen dem Paar das Balzverhalten und jegliche sexuelle Interaktion verhindern würde, welche normalerweise als in kurzer Distanz stattfindend beschrieben wird.

Zusammengefasst unterstützen meine Ergebnisse das bisherige Wissen über die Wichtigkeit des Modellweibchens und heben zudem die Wichtigkeit hervor, Aspekte zur Verhaltensinteraktion des Modellweibchens und des Männchens in zukünftige Forschung zum Kopieren der Partnerwahl zu integrieren. Zusätzlich konnte ich eine hoch kontrollierte und standardisierte Vorgehensweise für zukünftige Forschung zum Kopieren der Partnerwahl mit *FishSim* vorstellen, die außerdem die Möglichkeit bereithält das Tierwohl in der Forschung zu verbessern.

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Appendices

Appendix 1

Table S1. Measurements of *Poecilia latipinna* males in the lab. Body measurements according to the measuring scheme in Figure 9 (Chapter 4.2) are shown for different male individuals (M; n = 22). Values were calculated with Image J (except for MP1).

M	Measuring point (MP)																	
	1 (mm)	2 (mm)	3 (mm)	4 (mm)	5 (mm)	6 (mm)	7 (mm)	8 (mm)	9 (mm)	10 (mm)	11 (mm)	12 (mm^2)	13 (mm)	14 (mm)	15 (mm^2)	16 (mm)	17L	17R
1	43	55.4	3.9	11.6	3.4	17.8	14.0	6.0	22.1	7.4	14.3	140.4	9.1	12.3	193.7	13.0	3	4
2	33	46.0	3.2	10.2	3.0	15.3	11.0	4.5	18.8	7.0	12.0	99.7	7.5	10.1	126.2	8.2	4	3
3	46	60.1	4.4	13.9	3.7	17.7	13.1	9.9	27.2	13.3	14.7	282.4	9.4	13.8	240.2	11.6	3	4
4	40	50.9	2.9	10.2	3.1	16.1	12.1	4.9	20.1	7.6	12.1	116.4	8.7	11.3	163.1	8.9	4	4
5	31	40.0	2.5	8.6	2.8	12.6	9.8	6.3	14.1	4.7	8.8	49.9	5.9	8.3	86.5	6.8	3	3
7	37	46.8	2.8	10.1	3.1	16.2	11.4	7.2	17.3	5.9	11.1	77.0	7.7	9.9	107.6	8.4	3	3
9	40	50.8	3.6	11.4	3.7	15.7	12.2	8.1	20.2	7.5	13.3	125.7	8.0	10.4	135.5	8.4	4	4
10	25	31.7	2.4	7.6	2.3	10.1	8.4	4.6	11.4	3.3	7.9	28.5	4.9	6.9	54.4	4.9	0	0
12	51	66.2	4.3	13.5	4.0	18.3	16.1	9.5	33.1	12.1	18.4	290.2	11.9	15.8	266.9	9.0	4	4
13	36	45.8	3.2	9.6	2.9	15.0	12.0	6.1	17.8	5.7	10.9	86.8	7.7	9.9	129.0	7.6	3	3
14	51	65.8	4.7	14.3	4.2	20.2	16.2	9.3	27.7	9.9	17.0	216.0	10.6	14.8	277.2	12.3	2	3
15	39	50.1	3.4	11.2	3.6	17.5	12.5	7.6	17.9	6.2	11.0	88.5	8.6	11.1	161.6	8.8	3	3
16	34	42.6	2.7	9.1	2.8	14.1	11.0	6.9	17.0	6.6	10.5	102.1	7.5	8.4	121.6	7.9	4	4
18	39	48.8	3.5	10.7	3.3	15.5	11.5	8.6	18.9	5.8	12.1	79.5	8.3	9.4	127.8	7.0	4	4
19	31	39.3	2.6	9.1	2.9	14.1	9.6	6.0	12.8	4.5	8.4	43.0	6.3	8.2	84.0	6.7	4	4
20	35	44.4	2.6	9.2	3.1	14.8	10.3	6.1	15.5	6.3	9.7	80.8	7.4	9.4	107.3	7.7	4	5
21	33	43.3	2.5	9	3	15.5	11	5.9	13.2	5.7	8.8	62.5	6.8	10.3	117.2	7.8	4	3
22	40	51.8	3.3	10.6	3.1	17.8	13.2	7.8	18.5	6.9	11.1	104	8.3	11.3	152.3	8	2	3
23	47	59.0	3.4	11.8	3.5	18.9	15.2	9.9	23.1	10.3	15.3	182.9	9.8	12.1	170.4	9.6	0	0
24	37	47.2	2.5	9.6	3.1	17.9	13.1	5.4	14.1	4.7	10.1	54.1	7.1	10.2	118.9	6.8	3	4
25	37	46.8	2.6	9.7	3.1	17.9	13.1	5.1	12.9	5.4	9.8	61.7	7.4	10	102.7	6.9	4	4
26	40	50.8	2.9	10	3.1	18.4	13.7	5.8	15.8	5.1	10.9	67.7	8.1	10.7	140.2	7.6	4	3
Mean	38.4	49.2	3.2	10.5	3.2	16.2	12.3	6.9	18.6	6.9	11.7	110.9	8.0	10.7	144.7	8.4	3.1	3.3
SD	6.4	8.3	0.7	1.7	0.4	2.3	2.0	1.7	5.4	2.5	2.8	71.8	1.5	2.1	56.9	1.9	1.2	1.2
%CV	16.8	16.9	21.1	16.4	13.1	14.2	16.4	24.9	29.0	36.0	23.6	64.7	19.3	20.0	39.3	22.7	38.5	36.7

Table S2. Measurements of *Poecilia latipinna* females in the lab. Body measurements according to the measuring scheme in Figure 11 (Chapter 4.2) are shown for different female individuals (F; n = 23). Values were calculated with Image J (except for MP1).

F	Measuring point (MP)																			
	1 (mm)	2 (mm)	3 (mm)	4 (mm)	5 (mm)	6 (mm)	7 (mm)	8 (mm)	9 (mm)	10 (mm)	11 (mm)	12 (mm^2)	13 (mm)	14 (mm)	15 (mm^2)	16 (mm)	17 (mm)	18 (mm)	19 (mm)	20 (mm^2)
1	38	47.7	2.9	10.8	3.2	19.2	13.3	7.3	12.9	4.1	9.5	47.7	7.1	9.7	122.8	2.7	5.0	2.5	4.3	14.9
2	53	67.2	4.5	16.0	4.0	25.5	17.2	10.5	18.2	6.6	13.3	108.7	10.3	14.2	233.2	4.2	7.1	1.6	3.8	0.0
3	33	41.3	3.3	10.3	3.0	17.2	11.8	6.3	10.5	4.0	8.4	35.0	6.7	8.4	101.0	2.2	4.3	1.4	3.3	0.0
4	45	56.7	3.0	12.6	3.6	20.8	12.6	9.3	17.4	5.8	13.5	83.2	8.9	11.4	184.2	2.5	5.7	2.9	4.5	0.0
5	36	45.8	3.1	9.7	3.1	17.6	11.9	6.8	11.8	4.7	8.6	49.6	6.9	9.8	116.3	2.7	5.8	1.5	4.5	3.0
6	41	52.0	4.0	11.6	3.4	20.3	13.1	7.2	14.2	4.8	10.9	57.3	7.3	10.9	117.2	2.7	4.6	1.6	2.8	0.0
7	39	48.8	3.2	11.4	3.3	20.1	13.2	8.9	12.8	4.4	9.6	44.8	7.3	10.1	119.3	2.4	4.6	2.0	4.6	0.0
8	41	50.3	3.2	11.4	3.4	21.2	14.6	8.1	13.2	4.9	9.9	54.6	7.9	9.3	104.3	2.3	4.8	2.2	3.5	11.5
9	36	45.5	3.0	10.2	3.3	18.6	12.4	6.8	11.7	4.6	8.6	49.2	6.3	9.5	138.5	2.1	4.5	1.7	2.2	4.4
10	33	41.7	2.8	9.2	3.0	17.1	11.2	7.1	10.5	4.3	7.4	39.1	5.9	9.0	106.5	2.0	3.9	1.1	2.5	5.2
11	36	45.3	2.9	9.7	3.1	19.0	11.9	6.8	10.8	4.7	8.0	42.9	6.4	9.1	119.5	1.9	4.5	1.7	4.0	6.2
12	42	49.8	3.4	12.1	3.6	21.7	13.0	9.3	13.4	2.1	4.1	51.1	7.3	7.6	86.6	2.7	5.8	5.0	9.7	0.0
13	34	43.1	3.0	9.5	3.1	18.3	10.9	7.2	11.1	3.9	8.1	33.9	6.2	9.0	93.6	2.0	4.5	1.7	3.7	0.0
14	43	53.7	3.9	12.2	3.5	21.1	13.7	8.7	14.4	4.7	11.4	55.3	8.3	10.6	171.0	2.4	6.1	2.1	3.2	0.0
15	38	47.6	3.3	10.4	3.3	19.5	12.3	8.2	13.0	5.9	9.3	53.2	6.6	9.4	128.0	2.8	5.0	1.9	2.2	4.3
19	41	51.5	3.1	10.9	3.6	21.2	14.8	8.1	13.3	5.1	10.7	55.8	8.1	10.9	130.4	2.6	5.1	1.9	1.9	21.3
20	34	43.4	3.2	10.0	3.0	18.5	12.6	5.5	10.8	4.4	8.1	41.2	6.6	9.3	117.0	2.4	3.9	1.0	3.1	4.6
22	35	44.4	3.1	10.1	3.3	19.1	12.3	7.9	10.3	4.0	8.5	35.2	6.4	9.4	92.0	2.1	4.8	1.3	2.9	6.3
23	30	37.1	2.7	9.1	2.7	16.5	10.5	5.9	9.1	3.7	7.1	23.4	5.2	7.5	62.7	1.9	3.7	1.3	3.0	5.4
24	37	45.8	3.6	11.2	3.5	19.5	14.1	8.1	12.1	4.4	9.8	45.5	7.1	9.1	122.4	1.9	4.2	1.5	3.1	2.7
25	40	50.3	3.5	11.6	3.6	20.6	13.2	8.7	12.8	5.2	9.7	55.7	7.3	10.7	118.0	2.5	5.1	2.0	3.8	13.0
26	35	44.8	3.0	10.2	3.3	19.3	12.4	4.8	10.3	4.0	8.1	33.7	6.4	9.6	100.8	2.1	4.4	1.7	3.3	4.6
27	33	42.3	3.1	9.8	3.3	17.9	10.9	6.3	9.9	3.9	7.6	31.9	5.9	9.3	88.9	2.2	4.6	1.2	2.4	5.3
Mean	38.0	47.7	3.2	10.9	3.3	19.6	12.8	7.6	12.4	4.5	9.1	49.0	7.1	9.7	120.6	2.4	4.9	1.9	3.6	4.9
SD	5.0	6.2	0.4	1.5	0.3	1.9	1.5	1.4	2.2	0.9	2.0	17.9	1.1	1.4	35.7	0.5	0.8	0.8	1.5	5.6
%CV	13.2	13.0	12.9	13.7	8.5	10.0	11.6	17.9	18.1	19.8	22.0	36.4	15.6	14.1	29.6	20.1	16.3	43.6	43.0	113.8

Table S3. Options for animating the virtual fish models and the respective buttons/joysticks of the Playstation 3 controller. Swimming and turning speed was defined based on video analysis and personal behavioral observations, and specified for the use of sailfin mollies as models.

Animation	Controller button / joystick	Possible range of animation	Speed
Swim forward/backward	Push left joystick up / down	indefinitely	Swimming speed from 0 to 40 cm/s
Turn up/down	Push right joystick down / up	360°	Turning speed for up and down swimming from 0°/s to 30°/s.
Turn left/right	Push right joystick left / right	360°	Turning speed for curved swimming and on-spot turning from 0°/s to 200°/s
Raise dorsal fin (default down)	Press L1 (infinitely variable)	0 ... 90° up	/
Gonopodial thrusting	Press R1 to hinge down	approx. 45°down	/
	Press R2 to swing forward	170° to side	/

Appendix 2

Table S4. List of studies cited in the main text, as well as additional selected exemplar studies (to complement the use of study species) representing the use of computer animations and virtual reality since the early 1990s. For each study, information on the used method, the taxonomic study group, and the research topic are highlighted. For brevity, only the first author is given in the first column. 2D = two-dimensional computer animation, 3D = three-dimensional computer animation, VR = virtual reality, M = mammals, B = birds, R = reptiles, A = amphibians, F = fish, I = insects, S = spiders. Different shades of grey are used to visually distinguish between 'method', 'taxonomic study group' and 'research topic'.

Reference	Method			Taxonomic study group							Research topic			
				Vertebrates					Invertebrates					
	2D	3D	VR	M	B	R	A	F	I	S	Sexual signaling	Group forming	Navigation	Perception/ recognition
Abaid, 2012														
Amcoff, 2013														
Baldauf, 2009, 2010, 2011														
Butkowski, 2011														
Campbell, 2009														
Clark, 1999														
Culumber, 2013														
Dolins, 2014														
Egger, 2011														
Fischer, 2014														
Fry, 2008														
Gerlai, 2009														
Gray, 2002														
Harland, 2002														
Hess, 2016														
Hiermes, 2016														
Hölscher, 2005														
Ioannou, 2012														
Künzler, 1998														
Levy, 2014														
Makowicz, 2010b														
Mazzi, 2003														
McKinnon, 1996														
Mehlis, 2008														
Moffat, 1998														

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Reference	Method			Taxonomic study group							Research topic			
				Vertebrates					Invertebrates					
	2D	3D	VR	M	B	R	A	F	I	S	Sexual signaling	Group forming	Navigation	Perception/ recognition
Morris, 2003														
Nakayasu, 2014														
Neave, 2011														
Nelson, 2006														
Nelson, 2010														
Ord, 2002														
Parr, 2008														
Pather, 2009														
Peckmezian, 2015														
Peters, 2007														
Qin, 2014														
Reichert, 2014														
Robinson, 2010														
Rosenthal, 1998														
Rosenthal, 2004														
Rosenthal, 2005														
Roster, 1995														
Tedore, 2013														
Tedore, 2015														
Thurley, 2014														
Thünken, 2014														
Van Dyk, 2008														
Watanabe, 2006														
Wong, 2006														
Woo, 2012, 2015														
Zbinden, 2004														
Total	**21**	**24**	7	7	1	5	3	**28**	2	5	**22**	6	4	**23**

Table S5. **List of selected programs (current as of 16 March 2016) for creating and presenting 2D and 3D stimuli as well as VR.** Freely available software is printed in italics.

Software	Website	Short information
Design and animation of 2D stimuli		
Adobe Photoshop[1,2]	http://www.adobe.com/	State-of-the-art raster graphics editing program for print and web; elaborate layer processing; support for RAW files; *30-day free trial version*
Adobe After Effects[1] **or Adobe Animate (formerly Adobe Flash)**	http://www.adobe.com/	Animation program for 2D .psd image files prior modified with Adobe Photoshop; video editing in After Effects; *30-day free trial version*
GIMP* with *GIMP Animation Package[2]	http://www.gimp.org/	2D raster and vector graphics editing program; *GIMP animation package* is a plugin that enables animation of images as a sequence of single frames; video editing (frame by frame); offers many of the same features as Adobe Photoshop at no costs using much less memory capacities; working with different layers possible; supports many file formats including RAW; cross-platform
MS PowerPoint[3]	https://www.microsoft.com/	Presentation program with limited 2D raster and vector graphics editing and animation capabilities
Pencil2D[4]	http://www.pencil2d.org	Realistic sketching program suited also for 2D animation; allows both creation and animation of raster and vector graphics based on keyframing timeline; free alternative to Adobe Flash
Design and animation of 3D stimuli (generally also applicable for 2D animation)		
3D Studio Max	http://www.autodesk.com/	Most common used professional 3D modelling tool to design, visualize, and animate 3D objects and environments; animation by keyframing; more user-friendly interface than Maya; available for Windows only; *free 30-day trial version*
anyFish 2.0[5]	http://swordtail.tamu.edu/anyfish/	Generate, animate (keyframing), and share 3D fish models, for fish biologists; currently supports models for sticklebacks and poeciliid fishes
Blender[6,7]	https://www.blender.org/	Design of 3D objects and environments; free alternative to Maya; offers most tools that available elsewhere; keyframing animation; supports Python scripts; game engine included (see below); cross-platform; open-source
LightWave 3D[8]	https://www.lightwave3d.com/	Professional modelling and animation tool; supports Python scripts; LightWave's flocking system for simulating coordinated animal motion; needs less resources and memory compared to 3D Studio Max; *free 30-day trial version* and reduced educational version for students and faculty staff
Maya	http://www.autodesk.com/	Professional software for 3D modelling, rendering and animation of objects and environments; animation by keyframing; supports Python scripts; *free 30-day trial version; 3 years free for students*
Unity 3D Personal Edition[9]	http://unity3d.com/	Design of 3D objects and environments; game engine included (see below)

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Game engine		
Blender game engine[6]	https://www.blender.org/	2D/3D game engine written in the programming languages C, C++ and Python; included in *Blender* main software (see above); supports real-time rendering and external input devices
Irrlicht[6]	http://irrlicht.sourceforge.net/	2D/3D game engine developed by a small and independent developer team; written in C++; cross-platform using D3D, OpenGL; enables real-time rendering and external input devices
Unity 3D Engine[10]	http://unity3d.com/	Advanced 2D/3D and VR development platform written in C#, JavaScript or Boo; included in *Unity 3D Personal Edition*; supports real-time rendering and external input devices; game development for mobile devices; cross-platform
Design and animation of VR		
Unity 3D Engine	http://unity3d.com/	Advanced 2D/3D and development platform for VR (head mounting devices) included in *Unity 3D Personal Edition* (see above).
WorldViz[7]	http://www.worldviz.com/	Professional distributor for VR software and entire VR setup solutions
Tracking software		
BIOBSERVE[11]	http://www.biobserve.com/	Company that develops software and hardware for behavioral experiments including tracking, recording and analyzing of behavior and path tracking in 2D and 3D
EthoVision XT (Noldus)[12]	http://www.noldus.com/	Software for automated video tracking (2D) and behavior analysis software specifically designed for animal behavior research; Track3D add-on available for 3D tracking; *free 30-day trial version*

[1] Tedore and Johnsen (2015); [2] Baldauf et al., (2011); [3] Fischer et al., (2014); [4] Makowicz et al., (2016); [5] Culumber and Rosenthal (2013); [6] Müller et al., (2017); [7] Thurley et al., (2014); [8] Woo (2007); [9] Ingley et al., (2015); [10] Peckmezian and Taylor (2015); [11] Butkowski et al., (2011); [12] Fry et al., (2008).

Table S6. List of useful online resources concerning the issue of latency with VR (written by experts).

The John Carmack Blog (well-known American game developer)
• http://oculusrift-blog.com/john-carmacks-message-of-latency/682/ • https://www.twentymilliseconds.com/post/latency-mitigation-strategies/
Occulus and Valve Blogs (prominent VR and game development studios)
• http://blogs.valvesoftware.com/abrash/latency-the-sine-qua-non-of-ar-and-vr/ • https://developer.oculus.com/blog/the-latent-power-of-prediction/
Gizmondo
• http://gizmodo.com/the-neuroscience-of-why-vr-still-sucks-1691909123

Box S1. Software example for design and animation of 3D fish stimuli for biologists.

***anyFish* 2.0**
One obstacle to using animations in behavioral research is the difficulty and financial cost often associated with using advanced animation software programs (e.g., Maya). Recently, efforts have been made to create a free, open-source, user-friendly software platform for generating animations of fish for behavioral research (Veen et al., 2013; Ingley et al., 2015). '*anyFish*' is the result of this effort, and provides a model for transparency, repeatability, and collaboration in the field of animal behavior. *anyFish* provides an excellent means to create high-quality fish stimuli for behavioral research, requires only basic computational equipment to rapidly and repeatedly create animations, and is completely free and open-source, facilitating user guided changes to improve or customize the program according to their needs. To create an animation, *anyFish* uses lateral images of fish as an input, and incorporates modern **geometric morphometric** methods to accurately model fish shape. Images are used to quantify body shape and provide a texture for the final 3D model. *anyFish* allows users to map fin and body textures independently, providing added flexibility in experimental design, e.g., the appearance of the fins can be manipulated independently of the appearance of the body (Culumber and Rosenthal, 2013). The shape of the model can be manipulated easily by changing the position of digitized landmarks that are applied to lateral images of the fish. By doing so, the user can manipulate body and fin shape even beyond morphological variation found in nature. The shape of the model can also be determined by generating an average body shape of a subsample of fish, and different sets of fish could be used to create multiple stimuli and avoid **pseudoreplication**. The *anyFish* platform allows users to create animations of behavioral sequences using three different approaches. First, users can create an animation de novo by **keyframing** the animated rig in the X, Y, and Z-axes. Second, users can use a **rotoscoping** technique, wherein the model is matched to a video of a behavioral sequence frame by frame. Finally, motion capture data from third-party tracking systems can be used to determine the animated fish's swimming path. A major benefit of the *anyFish* workflow is that project folders can easily be shared amongst collaborators or via online data repositories (e.g., Dryad). This increases the ease of collaboration and provides added transparency and repeatability. In summary, *anyFish* provides a unique, albeit not always user-friendly, approach to creating animations for behavioral studies for a specific taxonomic group, and serves as an example of repeatability, transparency, and collaboration.

Appendix 3

Table S7. Association times measured for each experiment (E), total number of test fish and those showing side biases as well as all size measurements of test fish and used stimuli.

E	Number of tested fish	Number of fish with side bias	Number of analyzed fish	Sex	SL [cm], mean ± *SD*	Stimulus		Stimulus size [cm], SL (TL)		Absolute association time per stimulus [s], median (*1. quartile, 3. quartile*)	
						A	B	A	B	A	B
1	18	2	16	f	4.2 ± *0.6*	Video male CRT	Video male LCD	4.8 (6.3)	4.8 (6.3)	131.5 (*99.5, 208.3*)	241 (*188.8, 284.5*)
2.1	18	3	15	f	3.9 ± *0.8*	3D-male	Empty 3D-tank	5.3 (6.9)	/	350 (*277.5, 430*)	106 (*43, 136.5*)
2.2	18	2	16	f	4.2 ± *0.5*	Video male	Video empty tank	5.3 (6.9)	/	385.5 (*320.3, 432.3*)	82 (*51.8, 119.8*)
2.3	25	8	17	f	3.9 ± *0.6*	Live male	Real empty tank	4.9 (6.3)	/	420 (*211, 507*)	93 (*39, 182*)
3.1	23	8	15	f	4 ± *1.2*	Moving 3D-male	Static 3D-box	4.5 (5.5)	/ (5.5)	310 (*205.5, 368*)	90 (*58.5, 151.5*)
3.2	22	5	17	f	3.9 ± *0.5*	Static 3D-male	Moving 3D-box	4.5 (5.5)	/ (5.5)	224 (*153, 277*)	187 (*119, 265*)
4	24	9	7	m_{pale}	3.5 ± *0.6*	3D-male	3D-female	4.2 (5.2)	4.2 (5.2)	133 (*94, 223*)	235 (*159.5, 244.5*)
			8	$m_{colored}$	4.1 ± *0.7*	3D-male	3D-female	4.2 (5.2)	4.2 (5.2)	368.5 (*252.8, 476.5*)	137 (*26.3, 154.8*)

Appendix 4

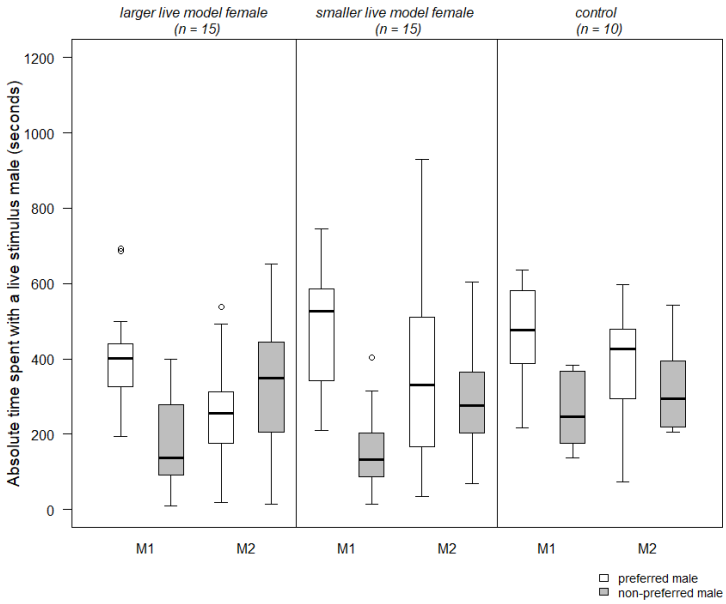

Figure S1. Absolute time spent of focal females with a live stimulus male for Treatments 1.1, 1.2 and C1 of Experiment 1. Boxplots of median, quartiles and whiskers (1.5 x interquartile range) are shown for absolute time spent (seconds) with a live stimulus male. White boxes represent absolute time of focal females spent in front of the preferred male in M1 and the prior preferred male in M2, whereas grey boxes always describe absolute times for the non-preferred male in M1 and the prior non-preferred male in M2. Circles indicate outliers. N = 15 in each treatment and n = 10 in the control; M1 = 1st mate-choice test; M2 = 2nd mate-choice test.

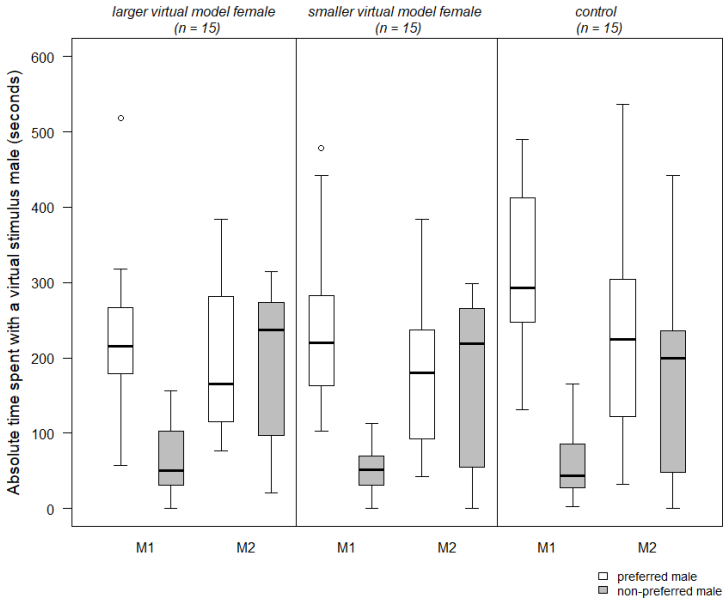

Figure S2. Absolute time spent of focal females with a virtual stimulus male for Treatments 2.1, 2.2 and C2 of Experiment 2. Boxplots of median, quartiles and whiskers (1.5 x interquartile range) are shown for absolute time spent (seconds) with a virtual stimulus male. White boxes represent absolute time of focal females spent in front of the preferred male in M1 and the prior preferred male in M2, whereas grey boxes always describe absolute times for the non-preferred male in M1 and the prior non-preferred male in M2. Circles indicate outliers. N = 15 in each treatment and the control; M1 = 1st mate-choice test; M2 = 2nd mate-choice test.

Appendix 5

***FishSim* PROTOCOL (as published in Gierszewski, Baker, et al. 2018):**

1. **Virtual Fish Design**
Note: Find a list of the required hardware and software in the supplementary materials list (Table S8). A detailed description of the general functionality of *FishSim* and additional tips and tricks can be found in the User Manual (https://bitbucket.org/EZLS/fish_animation_toolchain/).

Table S8. Materials list of required hardware and software.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Hardware			
2x 19" Belinea LCD displays	Belinea GmbH, Germany	Model 1970 S1-P	1280 x 1024 pixels resolution
1x 24" Fujitsu LCD display	Fujitsu Technology Solutions GmbH, Germany	Model B24-8 TS Pro	1920 x 1080 pixels resolution
Computer	/	/	Intel Core 2 Quad CPU Q9400 @ 2.66GHz x 4, GeForce GTX 750 Ti/PCIe/SSE2, 7.8 GiB memory, 64-bit, 1TB; keyboard and mouse
SONY Playstation 3 Wireless Controller	Sony Computer Entertainment Inc., Japan	Model No. CECHZC2E	USB-cable for connection to computer
Glass aquarium	/	/	100 cm x 40 cm x 40 cm (L x H x W)
Plexiglass cylinder	custom-made	/	49.5 cm height, 0.5 cm thickness, 12 cm diameter; eight small holes (approx. 5 mm diameter) drillt close to the end of the cylinder lower the amount of water disturbance while releasing the fish
Gravel	/	/	/
2x OSRAM L58W/965	OSRAM GmbH, Germany		Illumination of the experimental setup
2x Stopwatches	/	/	/
Software			
ubuntu 16.04 LTS	/	/	Computer operating system; Download from: https://www.ubuntu.com/
FishSim Animation Toolchain v.0.9	/	/	Software download and user manual from: https://bitbucket.org/EZLS/fish_animation_toolchain
GIMP Gnu Image Manipulation Program (version 2.8.22)	/	/	Download from: https://www.gimp.org/

1.1. **Preparation of female body textures with and without gravid spot**
1.1.1. Start **GIMP** and **click File→Open** to open the female body texture image "PLF_body_6.png" from the folder **models** in the directory "**fishsim_animation_toolchain**". Use this picture as a reference for all new created female body textures with gravid spot. Select the dark gravid spot area of the reference picture with the **free select tool** and cut it (click **Edit→Cut**).

Note: GIMP (available at www.gimp.org) is a free picture editing tool, similar to Adobe Photoshop, which can be used to manipulate digital pictures and graphics.

1.1.2. Open a second female body texture file in GIMP (*e.g.,* "PLF_body_7.png") and transfer the spot area onto the second body texture by inserting (**Edit→Paste Into**) the prior cut spot area as a new floating layer. Adjust the position of the gravid spot in the second picture and merge layers by clicking **Layer→Anchor Layer**.

Note: Ensure that the area of the gravid spot has the same size and identical position on each virtual model female (Figure 28)!

1.1.3. Export (**Edit→Export As**) the new "spot" texture under a new name (*e.g.,* PLF_body_7_S. png) in the **models** folder. Close all open picture windows in GIMP.

Note: Do not make any other changes (*e.g.,* scaling) to the texture files since they are specifically edited to be later mapped onto the 3D fish.

1.1.4. Create a second body texture without a gravid spot, using the same original female body texture file a second time (*e.g.,* "PLF_body_7.png"). Now, cover already existing gravid spots in the original file with the help of GIMP.

1.1.5. Open the female body texture in GIMP and select the **clone tool**. Select the pattern of the surrounding abdominal area (without dark pigmentation) by pressing **Ctrl + left-click** and use this selection to cover existing dark pigmentation by painting over it with the clone tool (Figure 28).

1.1.6. Export the newly created "no spot" texture under a new name (*e.g.,* PLF_body_7_NS.png) in the **models** folder. Close GIMP.

1.2. **Adjusting the viewpoint and setting the "scene" for animation**
1.2.1. Start *FishSim* by selecting the ***FishSim*** icon in the launcher on the left side of the desktop. Configure the resolution for the presentation monitors and click **Launch**.

Note: It is recommended to make the following adjustments (steps 1.2.2–1.2.4) on screen of one of the presentation monitors (if monitor dimensions and resolutions differ).

1.2.2. Press **F1** on the keyboard to change from viewing mode to editing mode (toggle between viewing and editing mode by repeatedly pressing **F1**).

Note: Switching to editing mode enables the editing toolbar at the top of the window. The scene as seen in the viewing mode depicts what will be presented on screen during the experiments.

1.2.3. Adjust the viewpoint to match the dimensions of the presentation monitors by adjusting the camera angle. Rotate the camera by holding the left mouse button and move the cursor. Pan the camera by holding the right mouse button and moving the cursor. Zoom in and out by holding the middle mouse (or both mouse buttons) and moving the cursor.

1.2.4. Click **Camera settings** in the editing toolbar (camera icon) and click **Copy to static cam** to set the viewpoint. Click **File→Save scene** to save the adjusted scene as the new default scene. For this, **override** the file "default_scene.scene" in the **scenes** folder of the *FishSim* directory.

Note: The default scene will appear at each start of *FishSim* and as the starting scene in *FishPlayer*. In *FishPlayer* the default scene also serves as a pause during experiments (Figure 29A). Adjusting the scene has to be done only once.

1.3. **Design of a virtual male stimuli for presentation during mate-choice tests**
Note: Prepare virtual male stimuli which will later be animated and presented to live focal females during mate-choice tests.

1.3.1. If not already open, start *FishSim*. Press **F1** to enter the editing mode.

1.3.2. Click **File→Load fish model** from the drop-down menu and load the default male sailfin molly template "**default_PLM.x**" by selecting it from the folder **models.**

1.3.3. Left double-click on the loaded fish to select it. It will be highlighted in a mesh. Click the gear icon in the toolbar to open the fish toolset. A box will pop up with the editing options used to customize the virtual male. Untick **Show mesh** for a better view of the fish.

1.3.4. Change the **Name** to **male**.

Note: The **Name** of the male is important and represents the "role" it will later play during the animation. This **Name** must be identical for every newly-created virtual male that will be used later during the experiments.

1.3.5. Alter the **Scale** (dimensions) of the male by changing the values for x, y, and z, if needed and click **Apply**.

1.3.6. Edit the male's texture by clicking **Textures** in the **Edit Toolbox**. Click on a feature of the fish (body, dorsal, caudal) to change it.

Note: The **Choose a texture for** box will pop up with all .png-files that may be used as textures. Textures will appear with names as given in the **models** folder.

1.3.7. Click on a texture displayed in the list (right), and it will directly appear and replace the prior texture on the fish.

1.3.8. When the desired male is created, click **Apply** under **Config** in the **Edit Toolbox**, and click **Save fish to disk**. Save the new male as "Male_A.x" in the **models** folder.

1.3.9. Additionally, save the whole scene (**File→Save scene**) including that one male in the **scenes** folder. Here, it is recommended to use the name "Male_A_alone.scene" (Figure 29B).

1.3.10. Click **File→Load scene** to load the empty default scene and repeat steps 1.3.2 to 1.3.9 to create as many different virtual males as needed and save each newly created male under a unique name in the **models** folder and as a new .scene-file in the **scene** folder.

1.4. **Design of virtual model female fish for presentation during the observation period**
1.4.1. Click **File→Load scene** to load the default scene. Follow step 1.3.1 and click **File→Load fish model** to load the default female template "default_PLF.x" from the **models** folder.

1.4.2. Left double-click on the loaded female to select it and open the fish toolset. Change the name to "female". Scale the female if needed as described in step 1.3.5.

Note: Name and scaling should be identical for all females for the purposes of this experiment.

1.4.3. Replace the default female body texture with the previously created "spot"-body texture (listed in the box on the right) as described in steps 1.3.6 to 1.3.7.

1.4.4. Click **Apply** under **Config** in the **Edit Toolbox**, then save fish to disk by clicking on **Save** and create a file "Female_1S.x" (S = spot).

1.4.5. Click **File→Load scene** to load the default scene. Repeat steps 1.4.1 to 1.4.4 to create at least one (or as many as needed) identical model female but without the gravid spot and name it "Female_1NS.x" (NS = no spot). Save each fish in the **models** folder**.**

Note: For the observation period of the MCC experiment, scenes including one male and one female have to be created and saved.

1.4.6. Click **File→Load scene** to load the empty default scene. Click **File→Load fish model** to insert a virtual male "Male_A.x" from the **models** folder. Click **File→Load fish model** again to add the virtual model female "Female_1S.x" to the scene. Change the position of each fish by altering their x-, y-, and z-coordinates so that their bodies do not overlap.

Note: Delete a fish from the scene by double-clicking on it and pressing **Delete** on the keyboard.

1.4.7. **Save** the scene including male and model female by clicking **File→Save Scene** as "Male_A_with_Female_1S.scene" (Figure 29C).

1.4.8. Repeat steps 1.4.6 to 1.4.7 to create three additional scenes for: (1) Male_A with Female_1NS (see Figure 29D), (2) Male_B with Female_1S, and (3) Male_B with Female_1NS.

2. **Animation of Virtual Fish Stimuli**
Note: Each type of animation needed for the experiment needs to be prepared only once using one exemplary male scene and one exemplary observation scene (male and female animated together). During the animating process, a swimming path for each fish is created which can later be replayed by any fish, as long as the name is identical (see step 1.3.4).

2.1. **Virtual male animations for presentation during mate-choice tests**
Note: Prepare two animations of a virtual male: (1) a swimming path with a duration of 7.5 min, and (2) a swimming path with a duration of 5 min.

2.1.1. Plug in the gaming controller (*e.g.,* Sony Play Station 3) into the USB port of the operating computer.

2.1.2. Open *FishSim* and click **File→Load scene** to load the scene of one male from the folder **scenes**, *e.g.,* "Male_A_alone.scene". Start *FishSteering* by clicking on the ***FishSteering*** icon.

2.1.3. Configure the controller settings in a separate window.

Note: *FishSim* and *FishSteering* run simultaneously and fish can either be steered in viewing mode, as shown during experiments, or in editing mode by pressing **F1**.

2.1.4. To animate the (male) fish, select it from the drop-down menu of the **steering** panel. Model names here correspond to the name given in the **Edit Toolbox** (see step 1.3.4).

2.1.5. Click **Start placing** and use the controller to place the fish at any starting position in the virtual tank. Click **Stop placing**.

2.1.6. Start recording the fish's swimming path by clicking **Start recording**. Use the controller to move the fish around the scene.

Note: The duration of the recording is given in the lower right corner of the steering panel.

2.1.7. Click **Stop recording**. Click **Save** to save the swimming path as a **.bag-file** (a "record") on the drive (*e.g.,* on the desktop). Choose the name of the file to represent the duration of the record, *e.g.,* "7_30_min_male_alone.bag".

Note: Once the recording is stopped, it is not possible to edit the total duration again.

2.1.8. Edit the recording to add movement of the male's dorsal fin to mimic male courtship behavior during mate-choice tests. Select the dorsal fin from the drop-down menu in the **Edit** feature (only one feature can be edited at a time).

2.1.9. Select **Start editing** and the complete swimming path will be replayed. Press the L1 button on the controller to raise the dorsal fin at specific points in time. Click **Save** to save the edited version of the swimming path as a new **.**bag-file.

2.1.10. Repeat steps 2.1.8 and 2.1.9 but select the gonopodium to add its movement. Save the final version for later use in *FishPlayer*. Close *FishSteering.*

Note: It is recommended to save bag-files for each editing step under a unique name. By this, it is always possible to come back to an earlier version of the animation if something in the editing process goes wrong.

2.2. **Virtual male and model female animation for presentation during observation period**

Note: Prepare one animation with a virtual male and the virtual model female in courtship, thus sexually interacting with each other, with a total duration of 10 min.

2.2.1. Open *FishSim.* Press **F1** to enter the editing mode and click **File→Load scene** to load a scene with male and female, *e.g.,* "Male_A_with_Female_1S.scene". Start *FishSteering.*

2.2.2. Select male and female alternately to place them (by clicking **Start/Stop placing**) in the virtual tank.

2.2.3. For the recording, select the female fish first from the drop-down menu of the **steering** panel and create a swimming path with duration of 10 min following steps 2.1.5 to 2.1.6.

Note: The swimming path of only one fish at a time can be recorded. After animating the first fish, the swimming path of the second fish can be included using the **Edit** function while the previously steered fish will be replayed alongside for the whole duration of the animation.

2.2.4. Click **Stop recording** and **Save** the swimming path on the drive, *e.g.,* as "10_00_min_male_with_female.bag". Then successively edit the male's swimming path, dorsal fin movement and gonopodium movement as described in steps 2.1.8 to 2.1.10. **Save** the final version for later use in *FishPlayer*.

3. **Preparing Animation Playlists for the MCC Experiment**
Note: Use *FishPlayer* to present animations on two monitors to live focal females. Arrange the playlist for each monitor separately to simulate the procedure of the MCC experiment (Figure 26). The tool consists of a main window showing the record playlist for each monitor (Figure S3) and a separate animation window for each presentation monitor.

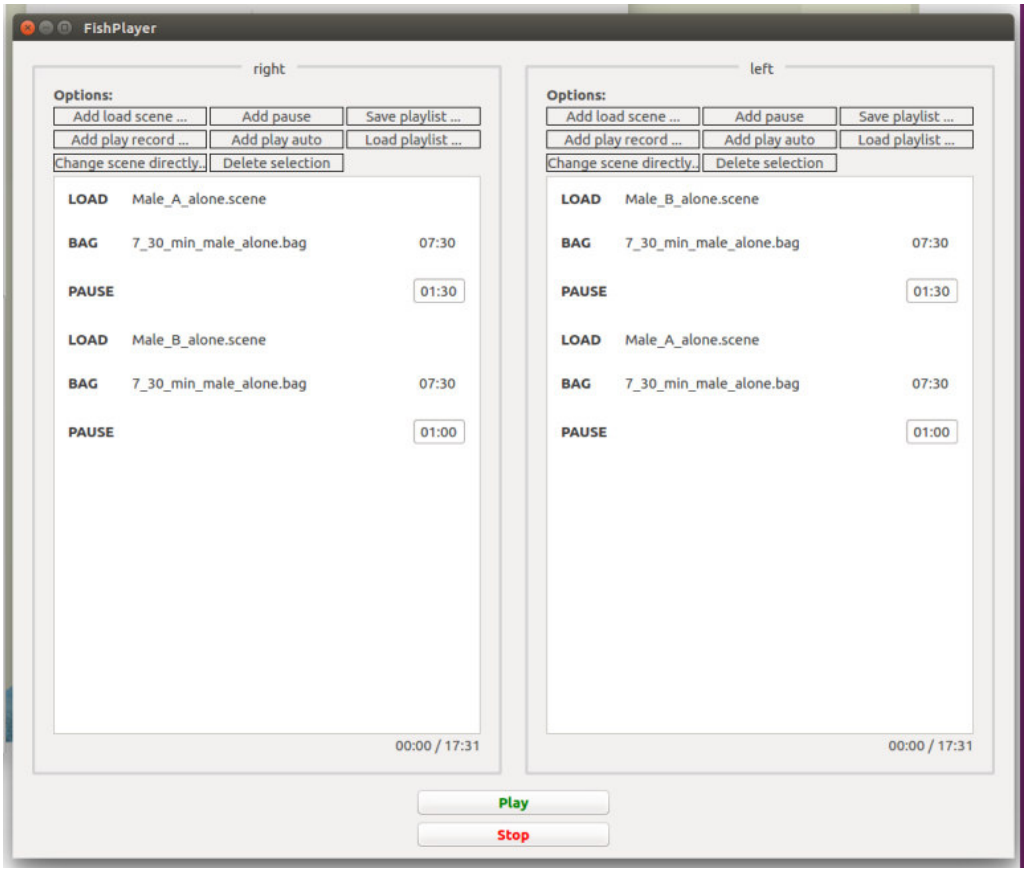

Figure S3. Screenshot showing the *FishPlayer* playlists for the left and right monitors in the first part (i.e., the first mate-choice test) of the MCC experiment. Playlist entries are ordered as needed for the first mate-choice test in Treatment 1.

3.1. **General functionality and arrangement of scenes and records**
3.1.1. Close all windows and open *FishPlayer* by clicking the corresponding icon. Configure the setup for the use with two monitors for presentation (left and right) and click **Launch**.

Note: The default scene created in step 1.2.4 (saved in *scenes/default_scene.scene*) will always be loaded and displayed on both monitors as the starting scene and during a pause command.

3.1.2. Add entries to the playlist for each monitor separately. Click **Add load scene** to add the scene of *e.g.,* Male A, from the *scenes* folder in the *FishSim* directory. Click **Add play record** to add a record from the drive, *e.g.,* the 7.5 min record for a male alone.

Note: The scene and the following record will then be linked by the software and the virtual male depicted in the scene will be animated as defined in the corresponding record.

3.1.3. Click **Add pause** to add a pause command of a specific duration (minutes/seconds) showing the default scene without fish as a break for fish handling between records.

Note: Pause duration should generally depend on the time needed for fish handling. Click an entry and drag to change its order in the list. Selected entries are marked in red. Delete an entry from the playlist by selecting the entry and clicking **Delete selection.**

3.1.4. Click **Play/Stop** to start and stop a presentation. **Stop** will always finish the complete playlist, *e.g.,* there is no way to pause at the middle of a playlist once running.

Note: Playlists will always start from the first entry and run from top to bottom. Therefore, the correct order of all entries has to be set prior to the experiment and cannot be changed afterwards

without stopping the presentation. A timer at the bottom of the window shows the duration and actual time of the current playlist.

3.2. **Playlist arrangement for the two treatments and the control of the MCC experiment**
Note: In terms of the entry arrangement, the MCC experiment is split into two parts: (1) the first mate-choice test, and (2) the observation period followed by the second mate-choice test. Therefore, for each treatment and the control, playlists have to be arranged in two different orders.

3.2.1. When running the experiment, first, prepare a playlist for the first mate-choice test.

3.2.2. Second, in the process of the running experiment, change the arrangement of the playlist for the subsequent observation period and the second mate-choice test according to which virtual male was preferred by the focal female in the first mate-choice test.

3.3. **Specific playlist arrangement for the "spot" treatment**
3.3.1. For the first mate-choice test in Treatment 1, order the playlist exactly as depicted in **Figure**

3.3.2. After the first mate-choice test, take break for calculating which virtual male was preferred (see step 5.9 below). Then rearrange the playlists for the observation period, in which public information is provided to the focal female by showing the prior non-preferred male together with the model female.

3.3.3. Arrange the playlist for observation and the following second mate-choice test according to Figure S4.

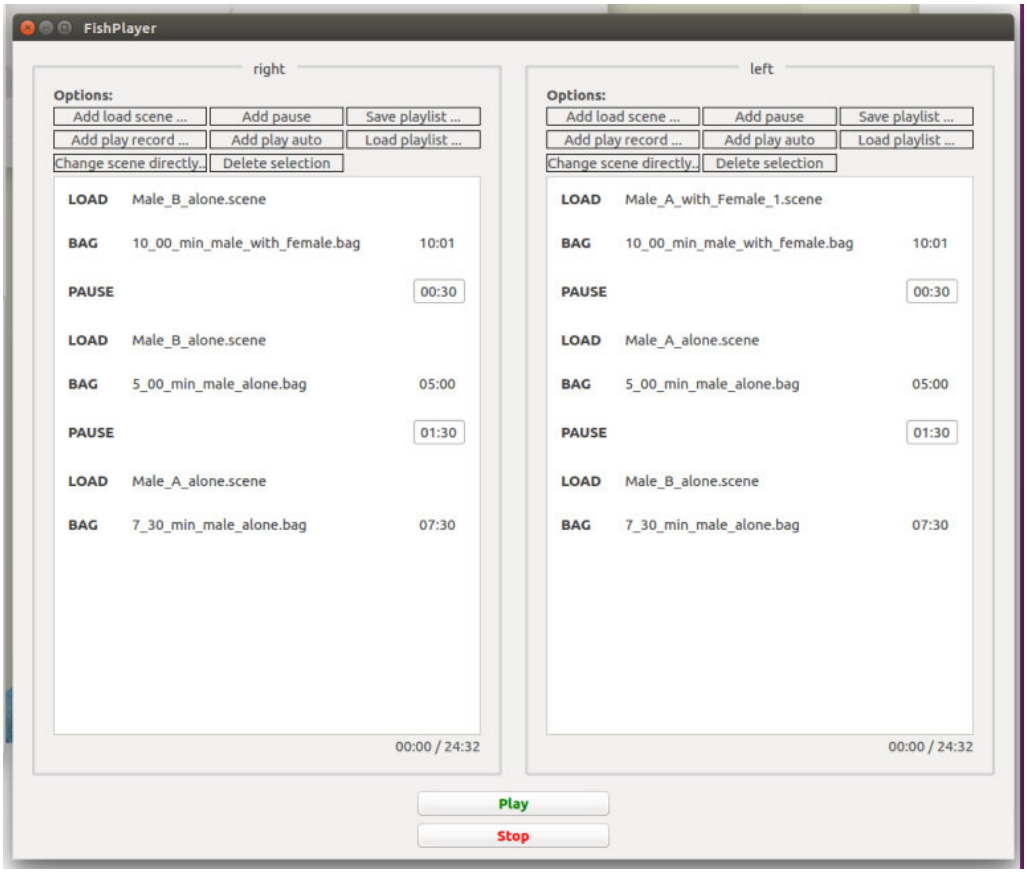

Figure S4. Screenshot showing the *FishPlayer* playlists for the left and right monitors in the second part (observation period and second mate-choice test) of the MCC experiment. Playlist entries are ordered as needed for the observation period and the second mate-choice test in Treatment 1.

3.3.4. For the observation period, link the 10-min record (male and model female together) with a scene showing the prior preferred male alone.

Note: In this case, only the swimming path of the male will be displayed and, because it is missing in the scene, the virtual model female will be absent.

3.3.5. For the playlist featuring the non-preferred male, link the 10-min record to the scene including the prior non-preferred male together with the model female. Choose the scenes including a model female with a gravid spot (S) for this treatment.

Note: In contrast to 3.3.2, here, the identical record will be replayed but now the model female is visible.

3.4. **Specific playlist arrangement for the "no spot" treatment**
3.4.1. For Treatment 2, order the playlists as described for Treatment 1 (Figures S3 and S4), but instead use the scene including the virtual model female without a gravid spot (NS) during observation.

3.5. **Specific playlist arrangement for the control for choice consistency**
Note: In the control for choice consistency, playlist entries for the mate-choice test are identical to Treatments 1 and 2 (Figure S3). During the observation period, however, no public information is provided to focal females and, hence, no model female is visible.

3.5.1. Order the playlists as shown in Figure S4 but combine the scenes for each male alone together with the 10-min record.

Note: In this case, only the swimming path of the male fish will be displayed and, because it is missing in the scene, the model female will be absent on both sides.

4. **Experimental Setup**
4.1. Place two computer screens each at opposite ends of a test tank. Adjust the screens to cover most of the glass walls of the test tank and to have 1.5 cm of space between the screens and the tank walls. Provide illumination to the tank from above.

4.2. Cover the tank bottom with a thin layer of gravel and fill it with water appropriate for live fish to the height of the screens. Mark a choice zone with a vertical line at 20 cm depth from each end on the outside of the tank. Have an acrylic glass cylinder and two stopwatches at hand.

4.3. Connect the monitors to the power supply and to the operating computer, placed at least 1 m away from the test tank, *e.g.,* on a small table (Figure 25).

5. **Running the MCC Experiment**
Note: Follow the experimental procedure below to perform one trial of Treatment 1, Treatment 2 or the control MCC experiment using one live focal female (see Figure 26).

5.1. Open *FishPlayer* on the operating computer and arrange the playlists for the **first mate-choice test** as *e.g.,* described for Treatment 1 (Figure S3). Check that the monitors for presentation are running and that they are showing the empty default scene.

5.2. Place a live focal female inside the test tank. Let her swim freely and acclimate to the tank and the presentation of the empty tanks on both monitors for a period of 20 min.

5.3. After acclimatization, place the focal female in a clear acrylic cylinder in the middle of the test tank to ensure an equal distance to both monitors and run the playlists on both monitors simultaneously by clicking **Play**, starting with the first mate-choice test.

Note: The focal female is allowed to watch both virtual stimulus males from inside the cylinder for around 2.5 min.

5.4. Before the timer reaches 02:30 min, go slowly to the experimental tank and release the focal female from the cylinder by gently lifting it up straight out of the water, *e.g.,* at 02:15 min.

Note: Here, the exact timing depends on the distance from the operating computer to the test tank and should be determined during prior test runs. It is critical to act slowly and gently to avoid stressing the fish. Since fish may act very fast, it is recommended to already have one stopwatch at hand while releasing the female to directly start measuring association time (see step 6.1).

5.5. Return to the operating computer. Observe the focal female and have two stopwatches at hand to **measure association time** with each virtual stimulus male (see step 6.1).

Note: The focal female is allowed to swim freely and choose between both males for 5 min.

5.6. Stop measuring association time when the timer reaches 07:30 min. The pause entry will then run for 1.5 min. Use the pause as handling time to, again, place the focal female inside the cylinder and write down the time for each virtual male on a data sheet.

Note: After the pause, the second 07:30 minutes entry will start and the focal female can watch both males for 02:30 minutes. Male position is now switched between left and right to control for a possible side bias in focal females (see step 6.3).

5.7. Before the timer reaches 11:30 min, release the focal female from the cylinder. Measure association time for the next 5 min.

5.8. Stop measuring association time when the timer reaches 16:30 min. The pause entry will run for 1 min. Use this handling time to place the focal female inside the cylinder.

5.9. Write down association times for the second measurement. For each male, sum up association times of both halves of the first mate-choice test (before and after males were switched). Calculate whether the focal female had a side bias and which male was preferred by the focal female (see steps 6.1 to 6.3).

Note: It is no problem if the pause is finished before the calculation is done, since proceeding to the next step needs the playlist to reach its end and stop.

5.10. Rearrange the playlists (do **not** close *FishPlayer*!) as shown in Figure S4 (depending on the current treatment) so that the prior preferred male will be animated alone during the observation period and the prior non-preferred male will be animated together with the virtual model female.

Note: Changes made to the playlists are not visible to the focal female.

5.11. Click **Play** to continue the second part of the experiment and the entries will be replayed from top to bottom starting with the **10-min observation period**.

Note: During the observation period, the focal female remains inside the cylinder but is able to watch both presentations.

5.12. After the observation period, a pause of 0.5 min starts. Before the timer reaches 10:30 min, release the focal female from the cylinder and start the **second mate-choice test** with the 5-min record for each male. Measure association times for the next 5 min.

5.13. Stop measuring association time when the timer reaches 15:30 min. The pause entry will then run for 1.5 min. Place the focal female inside the cylinder and write down the measured time for each virtual male.

Note: After the pause, the next 7:30 min entry will start and the focal female can watch both males (whose position has again switched between left and right) for 2.5 min.

5.14. Before the timer reaches 19:30 min, release the focal female from the cylinder and measure association time for the last 5 min.

5.15. Stop measuring association time when the timer reaches 24:30 min and **terminate the experiment**. Write down association times for both virtual males and proceed with analysis.

6. **Data measurement**
6.1. Measure association time during the first and the second part (prior to and after stimuli are switched) of each mate-choice test, when the focal female is allowed to choose between the two males.

Note: Start measuring when the female crosses the line confining the choice zone with her head and operculum. Stop measuring when her head and operculum are outside the choice zone.

6.2. Sum up association time measured for each male in the first and second part of a mate-choice test and determine which male was preferred.

Note: The preferred male is determined as the one the focal female spent more than 50% of the total time she spent in both choice zones within a mate-choice test. For analysis, association time is often translated into preference scores (relative mate-choice value), which is defined as the time a focal female spent with a male divided by the time she spent with both males in the mate-choice zones.

6.3. Calculate whether focal females show a side bias during the first mate-choice test and exclude biased females from the final analysis.

Note: Focal females are considered to be side-biased if they spent more than 90% of the total time (both halves of the first mate-choice test) in the same choice zone, even after the male position was switched. Her choice for a male is then considered as side biased and the trial is terminated.

6.4. Measure each focal female's standard length (SL).

Note: To prevent fish from being stressed during experiments, measurements are always taken after the termination of an experimental trial.

Appendix 6

Table S9. Descriptive statistics for M1 and M2 of all treatments. Absolute times (seconds) are given as mean ± SD; preference scores and change of preference (copying score) are given as median (1st, 3rd quartile). M1 = 1st mate-choice test; M2 = 2nd mate-choice test; P = preferred virtual male; NP = non-preferred virtual male; PP = prior preferred virtual male; PNP = prior non-preferred virtual male; MR = mate-choice reversals; T = treatment. N = 15 for all treatments.

T	M1 (2 x 5 minutes)					M2 (2 x 5 minutes)					Change of preference PNP	MR
	Absolute time both males	Absolute time P	Absolute time NP	Score P	Score NP	Absolute time both males	Absolute time PP	Absolute time PNP	Score PP	Score PNP		
Public information												
T1[1]	447 ± 124	354 ± 162	93 ± 64	0.74 (0.59, 0.98)	0.26 (0.02, 0.41)	455 ± 126	267 ± 106	188 ± 87	0.52 (0.49, 0.69)	0.48 (0.31, 0.51)	0.16 (0.04, 0.31)	4
T2[2]	430 ± 93	337 ± 116	94 ± 50	0.73 (0.67, 0.92)	0.27 (0.08, 0.34)	470 ± 81	256 ± 109	214 ± 116	0.52 (0.50, 0.65)	0.48 (0.36, 0.51)	0.22 (0.12, 0.36)	5
T3[3]	405 ± 113	335 ± 122	70 ± 57	0.87 (0.69, 0.96)	0.13 (0.05, 0.31)	489 ± 78	227 ± 117	262 ± 99	0.48 (0.32, 0.52)	0.52 (0.48, 0.68)	0.37 (0.14, 0.53)	8
No public information											**Change of preference PP**	
T4[4]	423 ± 109	309 ± 120	114 ± 60	0.65 (0.59, 0.86)	0.35 (0.14, 0.41)	446 ± 124	243 ± 115	203 ± 98	0.53 (0.48, 0.72)	0.48 (0.28, 0.52)	-0.14 (-0.37, 0.01)	6
T5[5]	444 ± 109	360 ± 144	83 ± 54	0.78 (0.61, 0.91)	0.22 (0.09, 0.39)	460 ± 96	251 ± 128	210 ± 115	0.51 (0.34, 0.75)	0.49 (0.25, 0.66)	-0.27 (-0.35, -0.14)	6

[1]Mutual courtship; [2]Female driven courtship; [3]Male driven courtship; [4]Male alone, courting; [5]Male alone, not courting

Appendix 7

Table S10. Detailed overview of the standard length of all fish used in Treatment 1, as well as absolute time and relative time (preference scores) spent of focal females with each stimulus male.

Treatment 1: short distance (1 cm)										
	Fish sizes					1st mate-choice test (M1)				
	Focal female	Model female	Pseudo-model female	Male 1	Male 2	Association time			Scores	
No.	SL (mm)	SL (mm)	SL (mm)	SL (mm)	SL (mm)	Absolute time (s) preferred male	Absolute time (s) non-preferred male	Total time (s) with both males	Score preferred male	Score non-preferred male
1	31	32	36	27	28	1126	0	1126	1.00	0
2	32	31	33	35	36	733	184	917	0.90	0.10
3	28	29	33	37	36	482	127	609	0.79	0.21
4	32	33	36	38	35	74	64	138	0.54	0.46
5	34	32	29	35	37	405	155	560	0.72	0.28
6	35	36	37	39	36	1050	0	1050	1.00	0
7	35	34	31	38	39	1175	0	1175	1.00	0
8	39	37	34	37	38	664	374	1038	0.64	0.36
9	37	39	34	36	37	716	413	1031	0.60	0.40
10	41	40	36	40	38	892	14	906	0.98	0.02
11	35	31	35	36	37	653	339	992	0.66	0.34
12	47	43	48	37	36	285	133	418	0.68	0.32
13	31	35	36	37	36	512	416	928	0.55	0.45
14	36	35	31	27	28	386	317	703	0.55	0.45
15	42	43	47	41	45	507	503	1010	0.50	0.50

Focal females (Kruskal-Wallis test: $\chi^2(2) = 0.021$; $p = 0.989$), as well as stimulus males (Wilcoxon rank sum test: $W = 112$, $p = 1$), did not differ in standard length.

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Treatment 1: short distance (1 cm) *(…continued from previous page.)*						
2nd mate-choice test (M2)						
Association time			**Scores**			
Absolute time (s) prior preferred male	**Absolute time (s) prior non-preferred male**	**Total time (s) with both males**	**Score prior preferred male**	**Score prior non-preferred male**	**Strength of preference prior non-preferred male**	**Male preference changed**
573	596	1169	0.49	0.51	0.51	yes
430	613	1043	0.41	0.59	0.49	yes
508	243	751	0.68	0.32	0.12	no
30	95	125	0.24	0.76	0.30	yes
406	242	648	0.63	0.37	0.10	no
1168	0	1148	1.00	0	0	no
1122	62	1184	0.95	0.05	0.05	no
426	450	876	0.49	0.51	0.15	yes
444	389	833	0.53	0.47	0.07	no
674	165	839	0.80	0.20	0.18	no
449	507	956	0.47	0.53	0.19	yes
128	185	313	0.41	0.59	0.27	yes
491	502	993	0.49	0.51	0.06	yes
314	466	780	0.40	0.60	0.15	yes
479	628	1107	0.43	0.57	0.07	yes

Table S11. Detailed overview of the standard length of all fish used in Treatment 2, as well as absolute time and relative time (preference scores) spent of focal females with each stimulus male.

Treatment 2: long distance (40 cm)										
	Fish sizes					1st mate-choice test (M1)				
	Focal female	Model female	Pseudo-model female	Male 1	Male 2	Association time		Scores		
No.	SL (mm)	SL (mm)	SL (mm)	SL (mm)	SL (mm)	Absolute time (s) preferred male	Absolute time (s) non-preferred male	Total time (s) with both males	Score preferred male	Score non-preferred male
1	38	40	35	36	37	253	193	446	0.57	0.43
2	35	33	34	28	27	417	326	743	0.56	0.44
3	38	39	34	39	37	814	155	969	0.59	0.41
4	41	39	36	36	37	390	265	655	0.60	0.40
5	35	35	37	36	37	123	24	147	0.84	0.16
6	35	35	38	28	27	286	107	393	0.73	0.27
7	38	34	36	36	37	1048	0	1048	1.00	0
8	36	35	38	46	45	494	365	859	0.58	0.42
9	36	38	41	36	37	1124	0	1124	1.00	0
10	37	36	38	39	36	752	294	1046	0.72	0.28
11	34	35	31	35	34	750	64	842	0.92	0.08
12	41	38	37	39	37	191	186	377	0.51	0.49
13	37	37	40	37	36	526	394	920	0.57	0.43
14	36	39	41	41	42	494	278	772	0.64	0.36
15	38	41	39	37	38	246	245	491	0.50	0.50

... continued overleaf ...

Focal females (Kruskal-Wallis test: $\chi^2(2) = 0.043$, $p = 0.979$), as well as stimulus males (Wilcoxon rank sum test: $W = 111$, $p = 0.966$), did not differ in standard length.

Treatment 2: long distance (40 cm) *…continued from previous page.*						
2nd mate-choice test (M2)						
Association time			**Scores**			
Absolute time (s) prior preferred male	**Absolute time (s) prior non-preferred male**	**Total time (s) with both males**	**Score prior preferred male**	**Score prior non-preferred male**	**Strength of preference prior non-preferred male**	**Male preference changed**
187	275	462	0.40	0.60	0.16	yes
291	569	860	0.34	0.66	0.22	yes
499	387	886	0.56	0.44	0.03	no
42	281	323	0.13	0.87	0.47	yes
158	48	206	0.77	0.23	0.07	no
785	152	937	0.84	0.16	-0.11	no
496	584	1080	0.46	0.54	0.54	yes
236	637	873	0.27	0.73	0.30	yes
515	572	1087	0.47	0.53	0.53	yes
1180	0	1180	1.00	0	-0.28	no
352	322	674	0.52	0.48	0.40	no
183	208	391	0.47	0.53	0.04	yes
58	534	592	0.10	0.90	0.47	yes
597	332	929	0.64	0.36	0	no
271	288	559	0.48	0.52	0.02	yes

Table S12. Detailed overview of the standard length of all fish used in the control, as well as absolute time and relative time (preference scores) spent of focal females with each stimulus male.

Control: choice consistency										
	Fish sizes					1st mate-choice test (M1)				
	Focal female	Model female	Pseudo-model female	Male 1	Male 2	Association time			Scores	
No.	SL (mm)	SL (mm)	SL (mm)	SL (mm)	SL (mm)	Absolute time (s) preferred male	Absolute time (s) non-preferred male	Total time (s) with both males	Score preferred male	Score non-preferred male
1	31	34	35	36	37	1181	0	1181	1.00	0
2	34	33	36	37	36	747	323	1070	0.70	0.30
3	37	36	38	35	37	321	289	610	0.53	0.47
4	31	33	34	28	27	1182	0	1182	1.00	0
5	37	38	33	35	33	696	332	1028	0.68	0.32
6	39	35	38	46	45	514	443	957	0.54	0.46
7	33	35	31	36	38	630	505	1135	0.56	0.44
8	38	35	37	34	35	674	164	838	0.80	0.20
9	31	31	32	35	36	868	269	1137	0.76	0.24
10	31	28	31	28	27	729	239	968	0.75	0.25
11	31	27	32	37	36	659	449	1108	0.59	0.41
12	35	31	32	38	36	581	459	1040	0.56	0.44
13	32	33	35	34	35	385	348	733	0.53	0.47
14	33	32	34	38	36	624	266	890	0.70	0.30
15	29	32	29	33	34	613	317	930	0.66	0.34

... continued overleaf ...

Female fish (Kruskal-Wallis-test: $\chi^2(2) = 0.515$, $p = 0.773$), as well as stimulus males (Wilcoxon rank sum test: $W = 113$, $p = 0.983$), did not differ in standard length.

Control: choice consistency *…continued from previous page.*					
2nd mate-choice test (M2)					
Association time			**Scores**		
Absolute time (s) prior preferred male	**Absolute time (s) prior non-preferred male**	**Total time (s) with both males**	**Score prior preferred male**	**Score prior non-preferred male**	**Male preference changed**
645	471	1116	0.58	0.42	no
530	521	1051	0.50	0.50	no
311	146	457	0.68	0.32	no
1084	91	1175	0.92	0.08	no
522	432	954	0.55	0.45	no
400	242	642	0.62	0.38	no
389	611	1000	0.39	0.61	yes
555	334	889	0.62	0.38	no
911	89	1000	0.91	0.09	no
586	314	900	0.65	0.35	no
534	473	1007	0.53	0.47	no
888	245	1133	0.78	0.22	no
376	343	719	0.52	0.48	no
564	369	931	0.60	0.40	no
263	106	369	0.71	0.29	no

Table S13. Detailed overview of the number of focal females used in Treatments 1-2 and the control for choice consistency.

	Focal females							
	Total number tested	**Number excluded due to lack of interest**	**Number excluded due to stress**	**Number with side bias**	**Number successfully retested**	**Total number excluded**	**Total number analyzed**	**SL (mm) analyzed, mean ± SD**
Treatment 1: short distance (1 cm)	16	0	1	3	3	1	15	35.7 ± 5
Treatment 2: long distance (40 cm)	20	4	0	4	3	5	15	37 ± 2.1
Control: choice consistency	17	1	1	2	2	2	15	33.5 ± 3.1

Table S14. Behavioral patterns of interacting sailfin mollies. Behavioral patterns of an interacting sailfin molly pair which might potentially be visible to an observer (depending on visibility and the distance of the encounter). Restricted means that fish are inside a clear Plexiglas cylinder:

	Physical contact	Restricted contact at close range (1 cm treatment)	Restricted contact at distant range (40 cm treatment)
Swimming activity	+	+	+
Swimming up and down the cylinder	-	+	+
Directional behaviour (facing the opposite fish)	+	+	+
Approaching	+	+ (limited to cylinder)	-
Following	+	+ (limited to cylinder)	-
Raised dorsal fin	+	+	+
Lateral display and raised dorsal fin	+	+	+
Sigmoid display and raised dorsal fin	+	+ (generally possible but not in front of female as usual)	+ (generally possible but not in front of female as usual)
Gonopore nipping	+	-	-
Gonopodial thrusting	+	+	+
Copulation	+	-	-

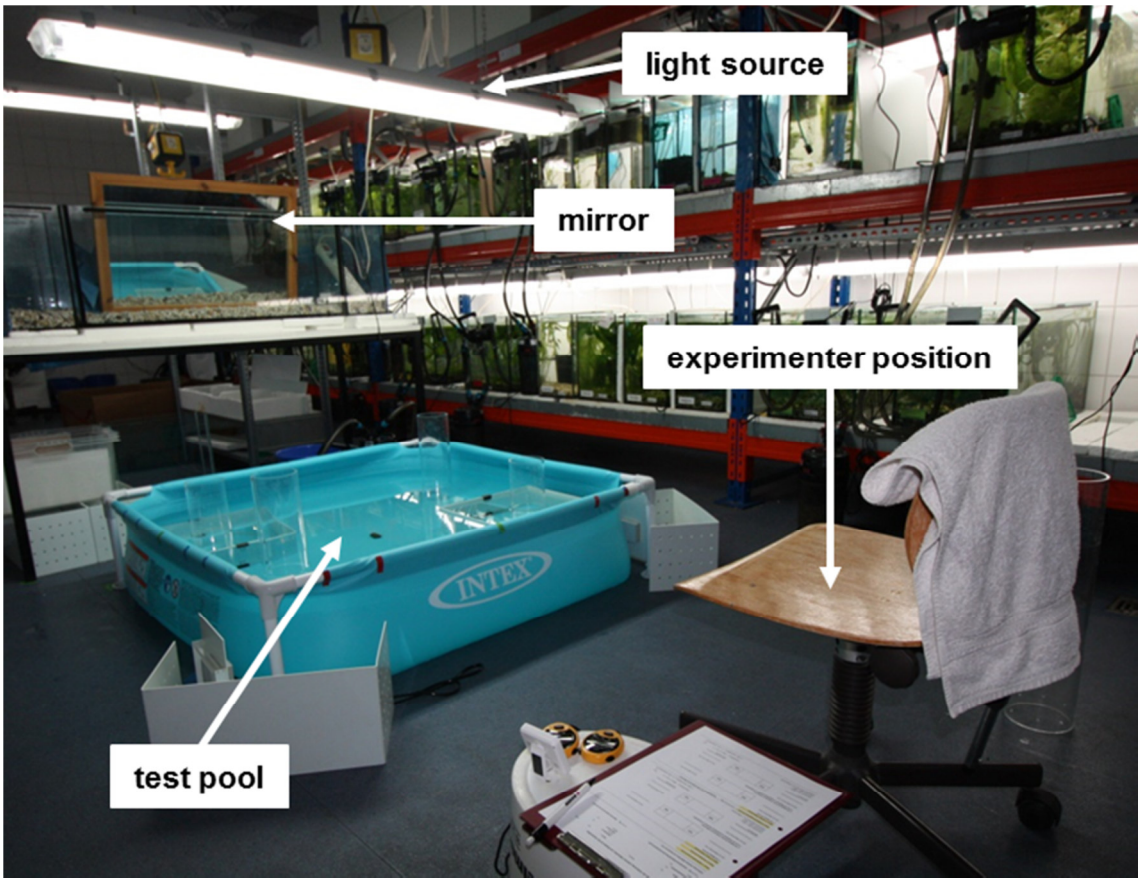

Figure S5. Test pool in the experimental room. The picture shows the experimental setup including the position of the light source above the pool, the position of the experimenter during the trials as well as the position of the mirror, which was used to observe the focal female when swimming in areas hidden by the pool walls. Picture by Melissa Keil.

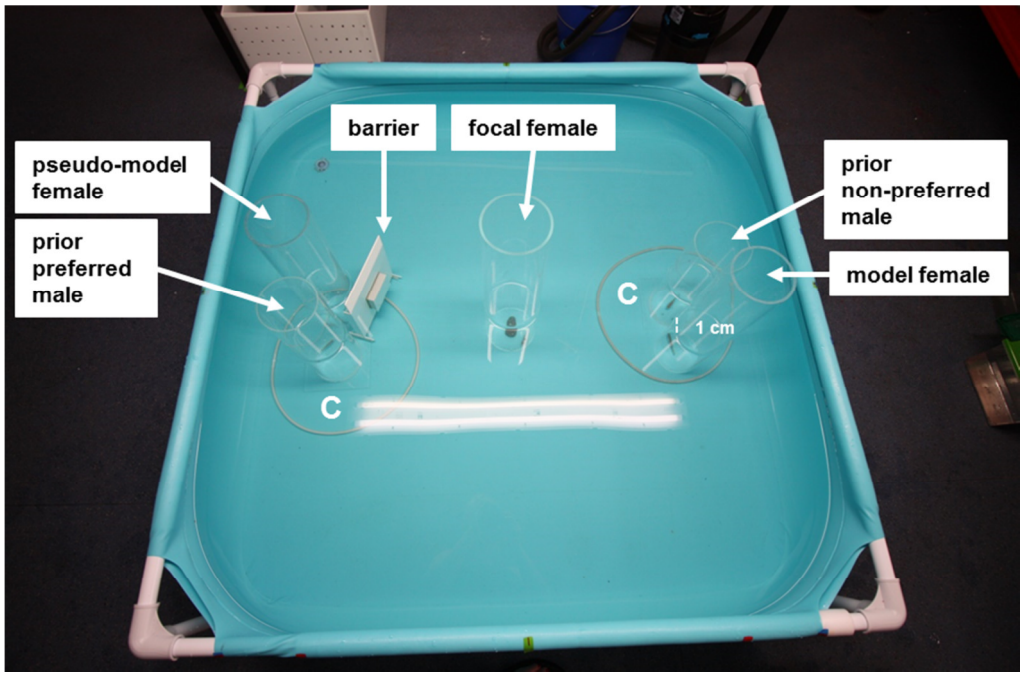

Figure S6. Top view of the experimental setup during the observation phase in Treatment 1. The picture shows the position of each cylinder containing a fish during the observation phase in the short distance treatment (1 cm). The barrier is used to hide the pseudo-model female next to the prior preferred male from the view of the focal female in the middle of the pool. The middle of the pool was marked with a small stone. Circular tubes filled with sand (C) mark the choice zones during preference tests. Picture by Melissa Keil.

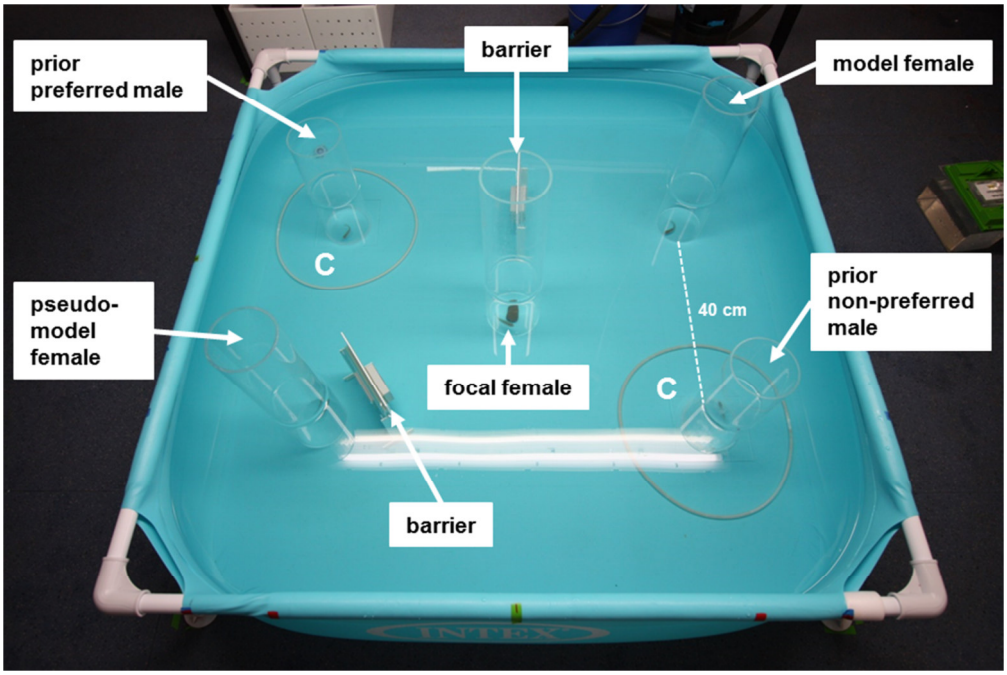

Figure S7. Top view of the experimental setup during the observation phase in Treatment 2. The picture shows the position of each cylinder containing a fish during the observation phase in the long distance treatment (40 cm). A stone marked the position of the cylinder for the focal female. One barrier is used to hide the pseudo-model female next to the prior preferred male from view of the focal female in the middle of the pool. A second barrier is used to block the view between the prior preferred male and the model female on the opposite side of the tank. Circular tubes filled with sand (C) mark the choice zones during preference tests. Picture by Melissa Keil.

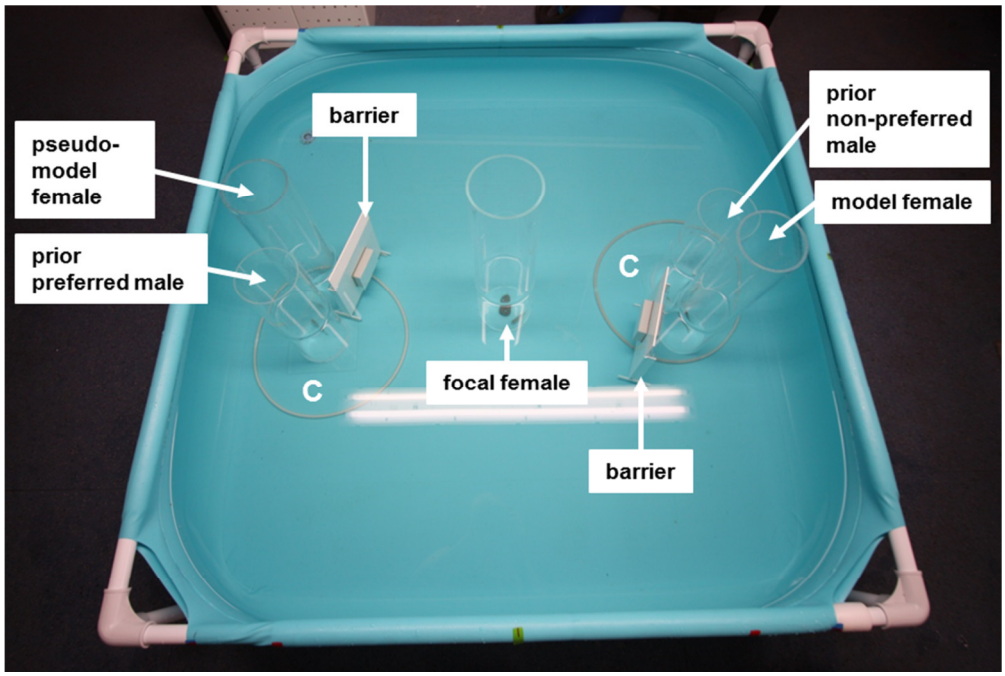

Figure S8. Top view of the experimental setup during the observation phase in the control. The picture shows the position of each cylinder containing a fish during the observation phase in the choice consistency control. A stone marked the position of the cylinder for the focal female. Two barriers are used to hide both the pseudo-model female as well as the model female from the view of the focal female in the middle of the pool. Circular tubes filled with sand (C) mark the choice zones during preference tests. Picture by Melissa Keil.

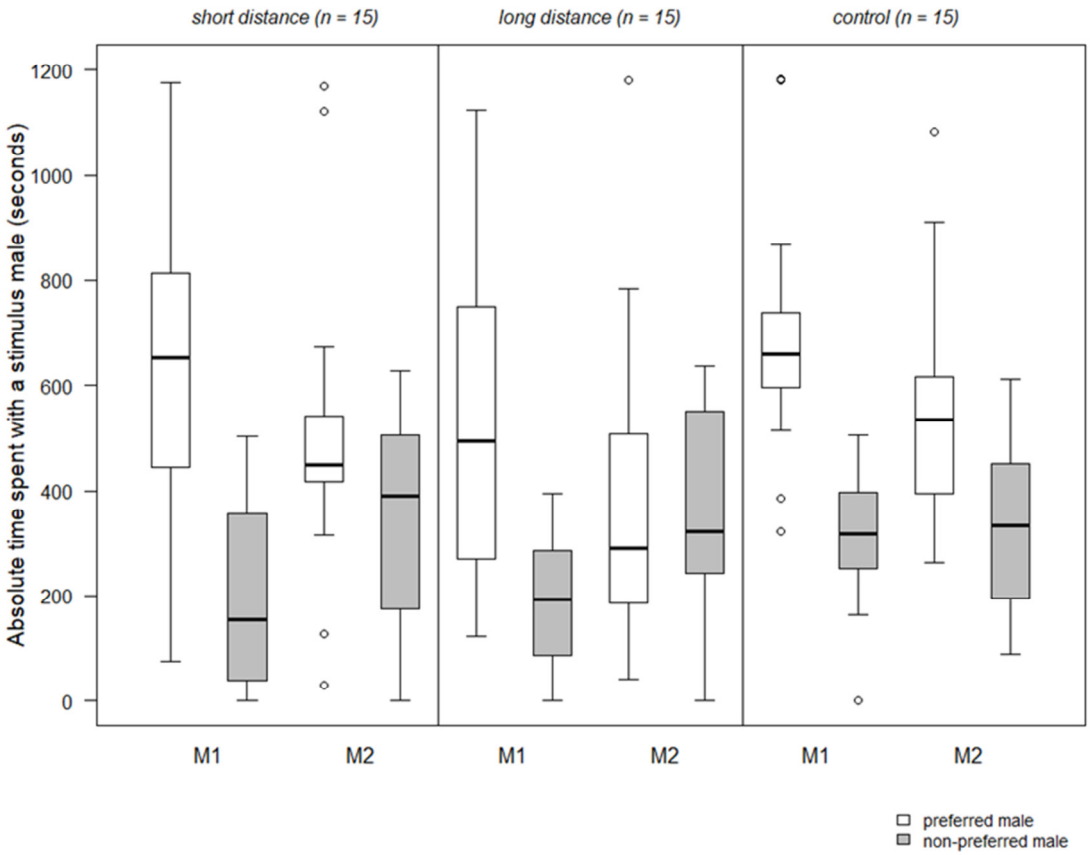

Figure S9. Absolute time spent of focal females with a stimulus male for Treatment 1, Treatment 2 and the control. Boxplots of median, quartiles and whiskers (1.5 x interquartile range) are shown for absolute time spent (seconds) with a stimulus male. White boxes represent absolute time of focal females spent in front of the preferred male in M1 and the prior preferred male in M2, whereas grey boxes always describe absolute times for the non-preferred male in M1 and the prior non-preferred male in M2. Circles indicate outliers. M1 = 1st mate-choice test; M2 = 2nd mate-choice test. *N* = 15 in each treatment and in the control.

Acknowledgements

EVERY INDIVIDUAL MATTERS. EVERY INDIVIDUAL HAS A ROLE TO PLAY.
EVERY INDIVIDUAL MAKES A DIFFERENCE.

Dr. Jane Goodall, DBE

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