Video Article

Using the *FishSim* Animation Toolchain to Investigate Fish Behavior: A Case Study on Mate-Choice Copying In Sailfin Mollies

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Abstract

Over the last decade, employing computer animations for animal behavior research has increased due to its ability to non-invasively manipulate the appearance and behavior of visual stimuli, compared to manipulating live animals. Here, we present the *FishSim* Animation Toolchain, a software framework developed to provide researchers with an easy-to-use method for implementing 3D computer animations in behavioral experiments with fish. The toolchain offers templates to create virtual 3D stimuli of five different fish species. Stimuli are customizable in both appearance and size, based on photographs taken of live fish. Multiple stimuli can be animated by recording swimming paths in a virtual environment using a video game controller. To increase standardization of the simulated behavior, the prerecorded swimming path may be replayed with different stimuli. Multiple animations can later be organized into playlists and presented on monitors during experiments with live fish.

In a case study with sailfin mollies (*Poecilia latipinna*), we provide a protocol on how to conduct a mate-choice copying experiment with *FishSim*. We utilized this method to create and animate virtual males and virtual model females, and then presented these to live focal females in a binary choice experiment. Our results demonstrate that computer animation may be used to simulate virtual fish in a mate-choice copying experiment to investigate the role of female gravid spots as an indication of quality for a model female in mate-choice copying.

Applying this method is not limited to mate-choice copying experiments but can be used in various experimental designs. Still, its usability depends on the visual capabilities of the study species and first needs validation. Overall, computer animations offer a high degree of control and standardization in experiments and bear the potential to 'reduce' and 'replace' live stimulus animals as well as to 'refine' experimental procedures.

Video Link

The video component of this article can be found at https://www.jove.com/video/58435/

Introduction

Recently, utilizing modern techniques for the creation of artificial stimuli, such as computer animations and virtual reality, has garnered popularity in research[1]. These methods provide several advantages compared to classic experimental approaches with live stimulus animals[1,2]. Computer animation enables non-invasive manipulation of the appearance (size, color) and behavior of virtual stimulus animals used in experiments. For example, the surgical removal of the sword in male green swordtails (*Xiphophorus helleri*) to test mate preferences in females[3] was rendered unnecessary by using computer animation in a later study on this species[4]. Furthermore, computer animations can create phenotypes that are only rarely encountered in nature[5]. Morphological features of virtual animals may even be altered beyond the natural range of that species[4]. Particularly, the possible systematic manipulation of behavior is one major advantage of computer animation, since it is almost impossible with live animals[6,7].

Various techniques exist to date for creating computer animations. Simple two-dimensional (2D) animations typically derive from a picture of a stimulus moving in only two dimensions and can be created with common software like MS PowerPoint[8] or Adobe After Effects[9]. Three-dimensional (3D) animations, which require more sophisticated 3D graphics modelling software, enable the stimulus to be moved in three-dimensions, increasing possibilities for realistic and complex physical movement[6,7,10,11,12]. Even virtual reality designs that simulate a 3D environment where live animals navigate have been used[13,14]. In a recent review Chouinard-Thuly *et al.* [2] discuss these techniques one by one and highlight advantages and disadvantages on their implementation in research, which notably depends on the scope of the study and the visual capacities of the test animal (see “Discussion”). Additionally, Powell and Rosenthal[15] give advice on appropriate experimental design and what questions may be addressed by employing artificial stimuli in animal behavior research.

Since creating computer animation may be difficult and time consuming, the need for software to facilitate and standardize the process of animation design arose. In this study, we introduce the free and open-source *FishSim* Animation Toolchain[16] (short: *FishSim;* https://bitbucket.org/EZLS/fish_animation_toolchain/), a multidisciplinary approach combining biology and computer science to address these needs. Similar to the earlier published tool *anyFish*[17,18], the development of the toolchain followed the goal to provide researchers with an easy-to-use method for implementing animated 3D stimuli in experiments with fish. Our software consists of a set of tools that can be used to: (1) create 3D virtual fish (*FishCreator*), (2) animate the swimming paths of the virtual fish with a video game controller (*FishSteering*), and (3) organize and present prerecorded animations on monitors to live focal fish (*FishPlayer*). Our toolchain provides various features that are especially useful for testing in a binary choice situation but also applicable to other experimental designs. Moreover, the possible animation of two or more virtual fish enables the simulation of shoaling or courtship. Animations are not bound to a specific stimulus but may be replayed with other stimuli making it possible to change the appearance of a stimulus but keep its behavior constant. The open-source nature of the toolchain, as well as the fact that it is based on the robot operation system ROS (www.ros.org), provide high modularity of the system and offer nearly endless possibilities to include external feedback devices (as the controller or a tracking system) and to adapt the toolchain to one's own needs in research. In addition to the sailfin molly, four other species are currently usable: the Atlantic molly *Poecilia mexicana*, the guppy *Poecilia reticulata*, the three-spined stickleback *Gasterosteus aculeatus* and a cichlid *Haplochromis* spp. New species can be created in a 3D graphics modelling tool (*e.g.,* Blender, www.blender.org). To exemplify the workflow with *FishSim* and to provide a protocol on how to conduct a mate-choice copying experiment with computer animation, we performed a case study with sailfin mollies.

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. They may rely on personal information when evaluating potential mating partners independently, possibly according to predetermined genetic preferences for a certain phenotypic trait[19, 20]. However, they may also observe the mate choice of conspecifics and thereby utilize public information[21]. If the observer then decides to choose the same mate (or the same phenotype) as the observed conspecific — the "model" — chosen previously, this is termed mate-choice copying (hereafter abbreviated as MCC)[22,23]. Mate-choice copying is a form of social learning and, hence, a non-independent mate-choice strategy[24], which has been observed in both vertebrates[25,26,27,28,29] and invertebrates[30,31,32]. So far, MCC was predominantly studied in fish and is found both under laboratory conditions[33,34,35,36,37,38] and in the wild[39,40,41,42]. 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[43]. The quality of a model female herself can affect whether focal females copy her choice or not[44,45,46,47]. 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[44,45,46], or by her being a conspecific or a heterospecific[47]. In sailfin mollies that copy the mate choice of conspecifics[39,48,49,50,51], it was found that focal females even copy the rejection of a male[52]. Since MCC is considered to play an important role in the evolution of phenotypic traits as well as speciation and hybridization[21,23,53,54], the consequences of copying a "false" choice may be tremendous in reducing the fitness of the copier[55]. 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'). This darkly pigmented area in their anal region derives from melanization of the tissue lining the ovarian sac[56]. The size and presence of the gravid spot are variable across conspecific females and may further individually change during the progression of ovarian cycles[56,57]. Gravid spots may serve to attract males and facilitate gonopodial orientation for internal insemination[58] or as a means of advertising fertility[59,60]. 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[59], 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.*[60], 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. So far, the role of the gravid spot for MCC in sailfin molly females has never been tested, nor experimentally manipulated.

We used *FishSim* to perform an MCC experiment by presenting virtual stimulus males and virtual model females on computer monitors instead of using live stimulus and model fish as used in the classic experimental procedure[49,50,51,61]. The general usability of our software has previously been validated for testing hypotheses about mate choice in sailfin mollies[12]. Here, we tested whether the absence or presence of a gravid spot in virtual model females affects the mate choice of observing live focal females. We first let focal females acclimate to the test tank (**Figure 1.1**) and let them choose between two different virtual stimulus males in a first mate-choice test (**Figure 1.2**). Afterwards, during the observation period, the prior non-preferred virtual male was presented together with a virtual model female (**Figure 1.3**). In a subsequent second mate-choice test, focal females chose again between the same males (**Figure 1.4**). We analyzed whether focal females had copied the mate choice of the observed model female by comparing her mate-choice decision in the first and second mate-choice test. 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 (1) together with a virtual model female with a gravid spot ("spot" treatment); or (2) together with a virtual model female without a gravid spot ("no spot" treatment). Additionally, in a control without any model female, we tested whether focal females chose consistently when no public information was provided.

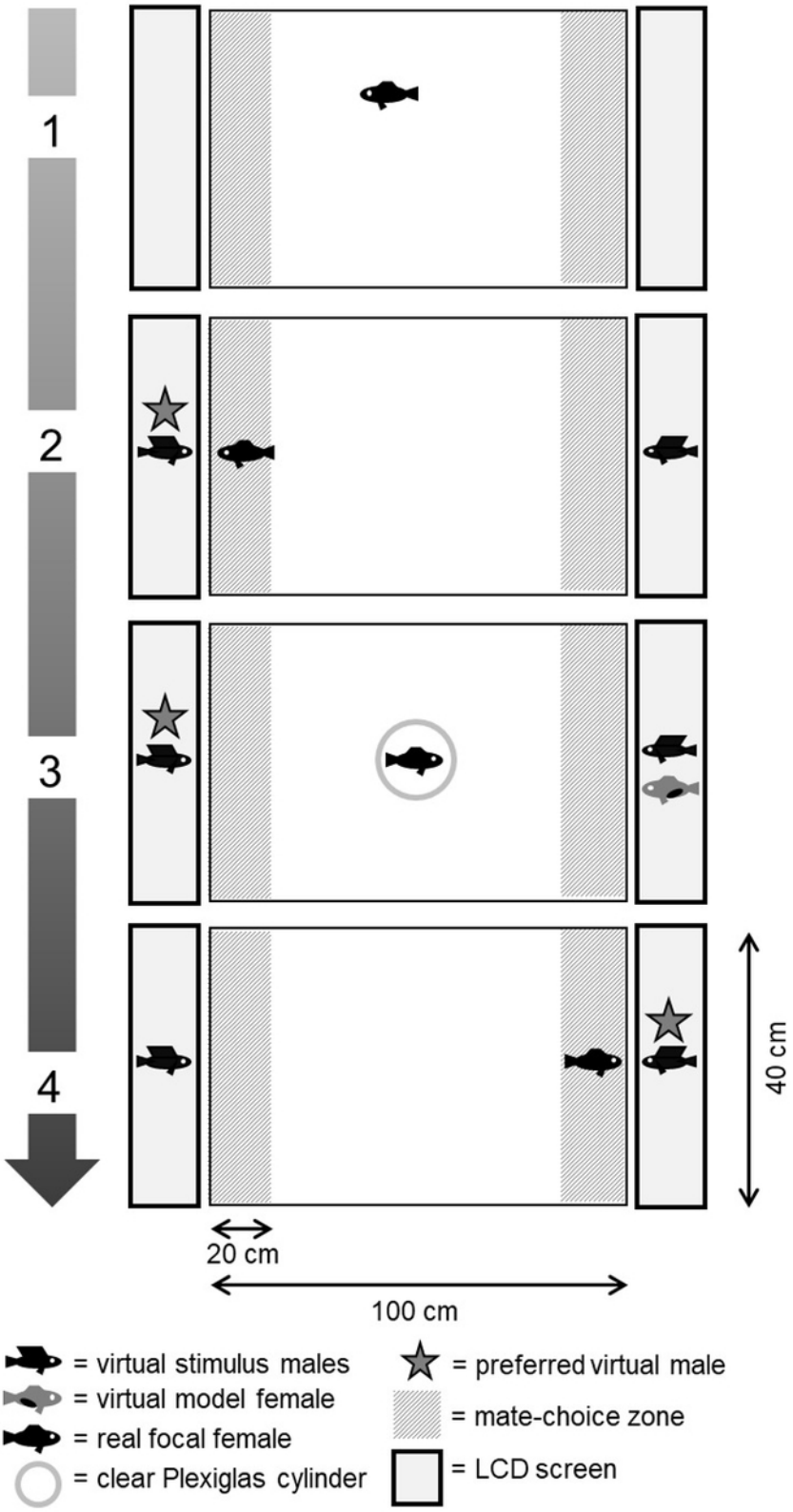

Figure 1. General overview of the most important experimental steps for a MCC experiment using virtual fish stimuli. (1) Acclimatization period. **(2)** First mate-choice test: live focal female chooses between virtual stimulus males. **(3)** Observation period: focal female watches the prior non-preferred 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 model. Please click here to view a larger version of this figure.

Protocol

The performed experiments and handling of the fish were in line with the German Animal Welfare legislation (Deutsches Tierschutzgesetz), and approved by the internal animal welfare officer Dr. Urs Gießelmann, University of Siegen, and the regional authorities (Kreisveterinäramt Siegen-Wittgenstein; Permit number: 53.6 55-05).

1. Virtual Fish Design

Note: Find a list of the required hardware and software in the supplementary materials list. 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/).

1. **Preparation of female body textures with and without gravid spot**
    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.
    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 2**)!
    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.
    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.
    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 2**).
    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.

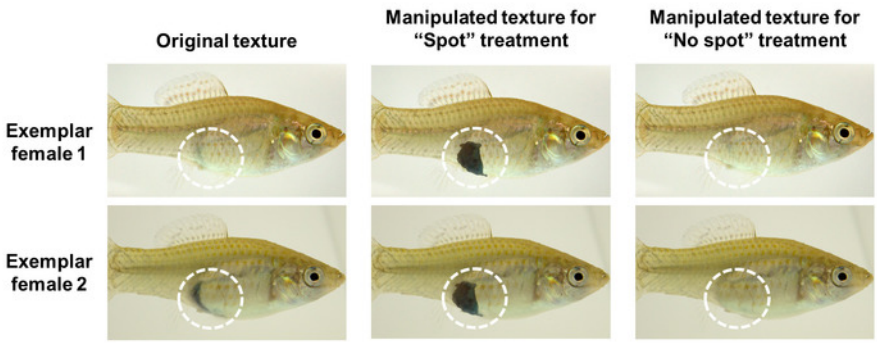

Figure 2: Exemplar pictures of female body textures prior to (original) and after manipulation for the "spot" and "no spot" treatment using the picture editing tool GIMP. The dotted circle marks the area that was manipulated. Please click here to view a larger version of this figure.

2. **Adjusting the viewpoint and setting the "scene" for animation**
    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).
    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.
    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.
    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 3A**). Adjusting the scene has to be done only once.

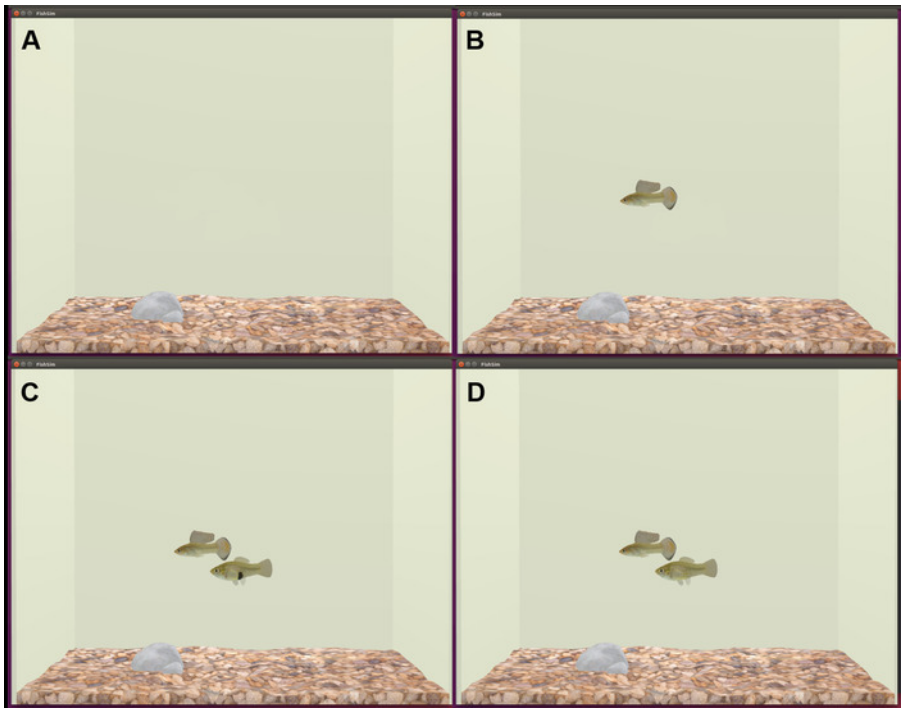

Figure 3: Screenshots of a scene in *FishSim*. (A) The empty default scene without a fish, **(B)** a scene showing a male alone, **(C)** a scene showing that same male together with a model female with a spot, and **(D)** a scene showing the identical male and the identical model female without a spot. Please click here to view a larger version of this figure.

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. If not already open, start *FishSim*. Press **F1** to enter the editing mode.
   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.**
   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.
   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.
   5. Alter the **Scale** (dimensions) of the male by changing the values for x, y, and z, if needed and click **Apply**.
   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.
   7. Click on a texture displayed in the list (right), and it will directly appear and replace the prior texture on the fish.
   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.fish" in the **models** folder.
   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 3B**).
   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.

4. **Design of virtual model female fish for presentation during the observation period**
   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.
   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.
   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.
   4. Click **Apply** under **Config** in the **Edit Toolbox**, then save fish to disk by clicking on **Save** and create a file "Female_1S.fish" (S = spot).
   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.fish" (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.

6. Click **File >> Load scene** to load the empty default scene. Click **File >> Load fish model** to insert a virtual male "Male_A.fish" from the **models** folder. Click **File >> Load fish model** again to add the virtual model female "Female_1S.fish" 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.
7. **Save** the scene including male and model female by clicking **File >> Save Scene** as "Male_A_with_Female_1S.scene" (**Figure 3C**).
8. Repeat steps 1.4.6 to 1.4.7 to create three additional scenes for: (1) Male_A with Female_1NS (see **Figure 3D**), (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).

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.
   1. Plug in the gaming controller (*e.g.,* Sony Play Station 3) into the USB port of the operating computer.
   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.
   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**.
   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).
   5. Click **Start placing** and use the controller to place the fish at any starting position in the virtual tank. Click **Stop placing**.
   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.
   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.
   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).
   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.
   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. **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.
   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. Select male and female alternately to place them (by clicking **Start/Stop placing**) in the virtual tank.
   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.
   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 1**). The tool consists of a main window showing the record playlist for each monitor (**Figure 4**) and a separate animation window for each presentation monitor.

1. **General functionality and arrangement of scenes and records**
   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.

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. 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.**
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.

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.
   1. When running the experiment, first, prepare a playlist for the first mate-choice test.
   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. **Specific playlist arrangement for the "spot" treatment**
   1. For the first mate-choice test in Treatment 1, order the playlist exactly as depicted in **Figure**
   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. Arrange the playlist for observation and the following second mate-choice test according to **Figure 5**.
   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.
   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.

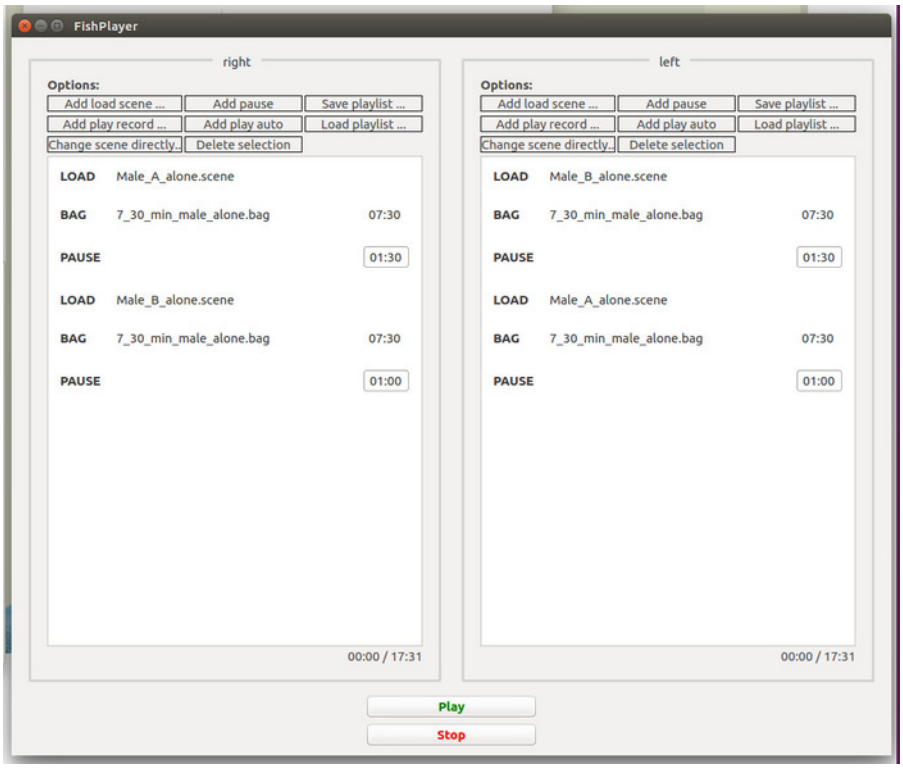

Figure 4: Screenshot showing the *FishPlayer* playlists for the left and right monitors in the first part (*i. e.,* the first mate-choice test) of the MCC experiment. Playlist entries are ordered as needed for the first mate-choice test in Treatment 1. Please click here to view a larger version of this figure.

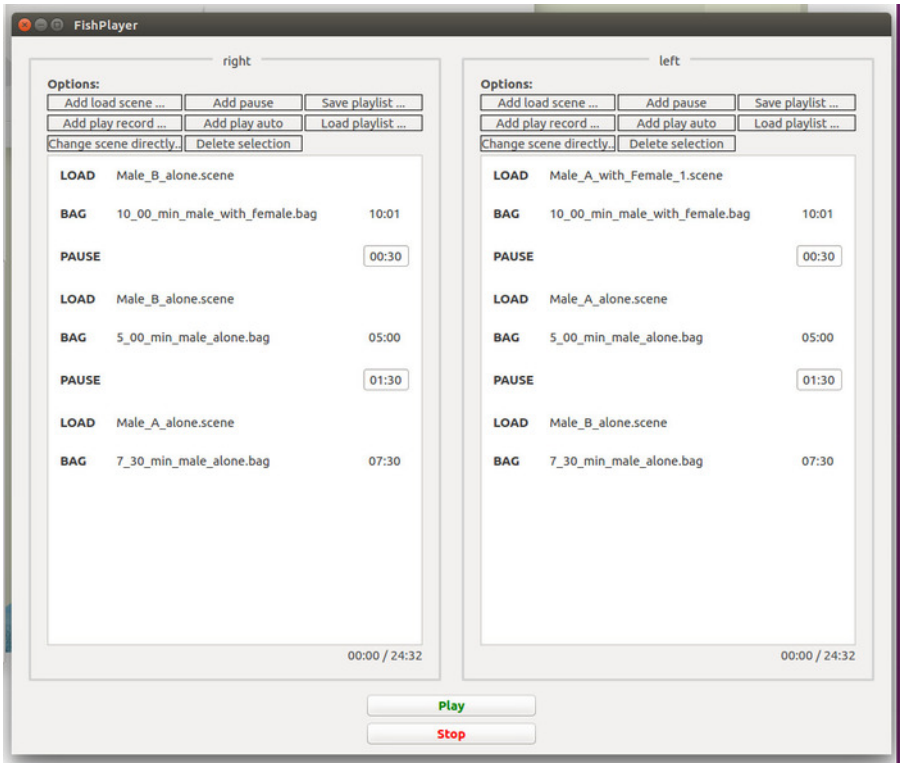

Figure 5: Screenshot showing the *FishPlayer* playlists for the left and right monitors in the second part (observation period and second mate-choice test) of the MCC experiment. Playlist entries are ordered as needed for the observation period and the second mate-choice test in Treatment 1. Please click here to view a larger version of this figure.

4. **Specific playlist arrangement for the "no spot" treatment**
    1. For Treatment 2, order the playlists as described for Treatment 1 (**Figures 4** and **5**), but instead use the scene including the virtual model female without a gravid spot (NS) during observation.

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 4**). During the observation period, however, no public information is provided to focal females and, hence, no model female is visible.
    1. Order the playlists as shown in **Figure 5** 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

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.
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.
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 6**).

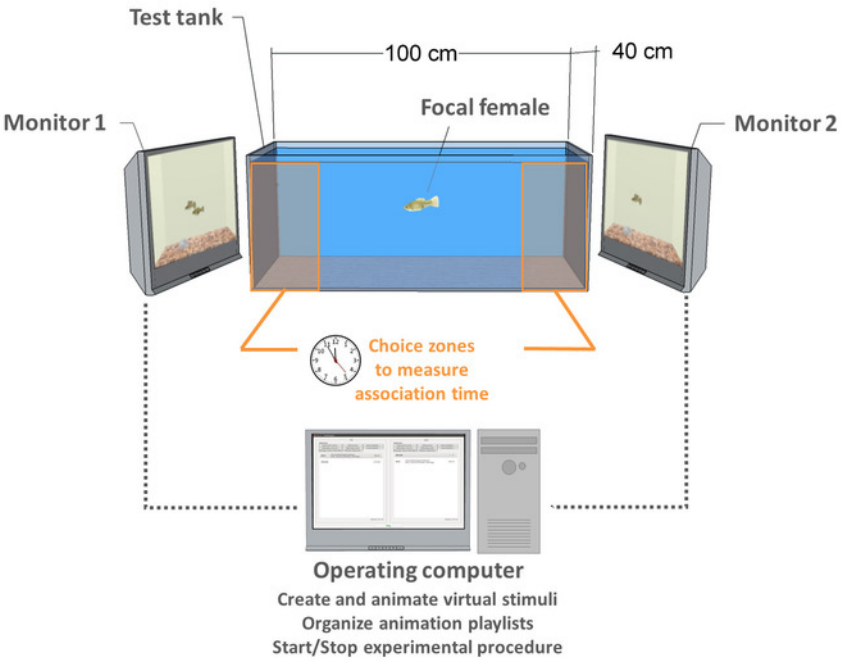

Figure 6: Experimental setup for the MCC experiment with computer animation. The operating computer connects to two presentation monitors (Monitor 1 and 2) which replay animations to live focal females inside the test tank. For illustration, both LCD monitors are angled to show an animated scene. Please click here to view a larger version of this figure.

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 1**).

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 4**). Check that the monitors for presentation are running and that they are showing the empty default scene.
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.
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.
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. 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.
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).
7. Before the timer reaches 11:30 min, release the focal female from the cylinder. Measure association time for the next 5 min.
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.
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.
10. Rearrange the playlists (do **not** close *FishPlayer*!) as shown in **Figure 5** (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.
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.

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

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.
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.
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.
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.

Representative Results

Following the protocol, we used *FishSim* to create computer animations of virtual sailfin molly males and females. We further used the toolchain to present animations to live focal females in a binary choice situation to perform an MCC experiment according to the experimental procedure described in **Figure 1** and step 5 of the protocol.

In order to determine whether focal females copied the choice of the virtual model female, we measured a focal female's association time for each male within the first and second mate-choice test during the experiments. Several parameters are typically analyzed using association time obtained in the first and the second mate-choice test for each treatment and the control for choice consistency. How the data are being analyzed is not bound to a specific statistical test but can be done in various ways (*e.g.,* parametric/nonparametric tests, repeated measures ANOVA, statistical models) and may depend on the final data structure. For our data analysis, we used R 3.2.2[62]. We uploaded the raw data we obtained in our experiment as well as the R-code we used for our analysis to Figshare (doi: 10.6084/m9.figshare.6792347).

In the current study, we created 15 different virtual model females with a gravid spot for Treatment 1 and identical 15 model females without a spot for Treatment 2. All model females had a virtual standard length (SL) of 50 mm. The relative gravid spot area was 4.7% of the total body surface (excluding fins; as measured with ImageJ v1.51j8) for all females in Treatment 1. Further, we created 30 different virtual stimulus males presented during mate-choice tests, allowing for 15 unique male stimuli pairings. Stimulus males had a virtual SL between 41-45 mm. We performed 15 trials for each treatment and the control for choice consistency. We tested a total number of 55 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. 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. The control for side bias in focal fish (protocol step 6.3) required that we further exclude 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. We compared the standard length (SL) of focal females across treatments and the control using a Kruskal Wallis rank sum test for independent data revealing that SL did not differ between treatments and the control (Kruskal Wallis rank sum test: n = 45, df = 2, χ^2 = 0.329, p = 0.848).

The most important parameter measured in an MCC experiment is the focal female's association time for each male (protocol step 6.1). Association time is an indirect measure of female mate preference in fish[63,64,65,66] and a well-established measure to determine mate choice in sailfin mollies when no direct contact is provided[12,48,61,67,68]. For each treatment and the control, we first used association time to analyze whether the choosing motivation differed between mate-choice tests. Choosing motivation is defined as the total time a focal female spent in both choice zones within a mate-choice test. However, a change in choosing motivation does not necessarily reflect a change in preference for either male. If choosing motivation is significantly different between the two mate-choice tests it is obligatory to use preference scores instead of absolute association time, for further analysis to ensure comparability within and between treatments (see protocol step 6.2). 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).

The most important determinant of whether MCC occurred is a significant increase in time spent/preference scores for the prior non-preferred male from the first to the second mate-choice test[22]. Transferred to a natural situation, an increase in time spent with the prior non-preferred male, consequently increases the probability that a female will mate with that male. Therefore, the main analysis compared either the absolute times or the preference scores for the prior non-preferred male between the two mate-choice tests. This analysis has to be done for each treatment and the control separately. Since in our study, choosing motivation differed in Treatment 2, we used preference scores for the initially non-preferred stimulus male, instead of absolute association time, to determine 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.

For this, we fit a linear mixed effect (LME) model with the lme function from the 'nlme' package[69] 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[70]. We set the contrasts of the model (1) 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 (2) 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"[71,72]. We used the R package 'phia'[73] for a *post hoc* analysis with Holm-Bonferroni correction of significant interaction terms. We inspected model assumptions (Q/Q-plots, residuals, residuals against fitted values) for all models visually[74]. 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 $p \leq 0.05$.

The results of this analysis are given in **Figure 7A** and **Tables 1** and **2**. 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 (χ^2-test: df = 1, χ^2 = 2.747, p = 0.097). Here, our results demonstrate that, as predicted for MCC, preference scores for the prior non-preferred virtual male significantly increased from M1 to M2 after focal females had been presented with the simulated mate choice of a virtual model female. We found this effect in both treatments but not in the control for choice consistency. Instead, in the control where no model female was present during the observation period, focal females were consistent in their mate choice for a male.

Factors	Lower	Estimate	Upper	SE	df	t-value	p-value
(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 1. 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.

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

Table 2. LME variance components for focal female ID. Variance and standard deviation for the random effect "focal female ID" and the residuals are given.

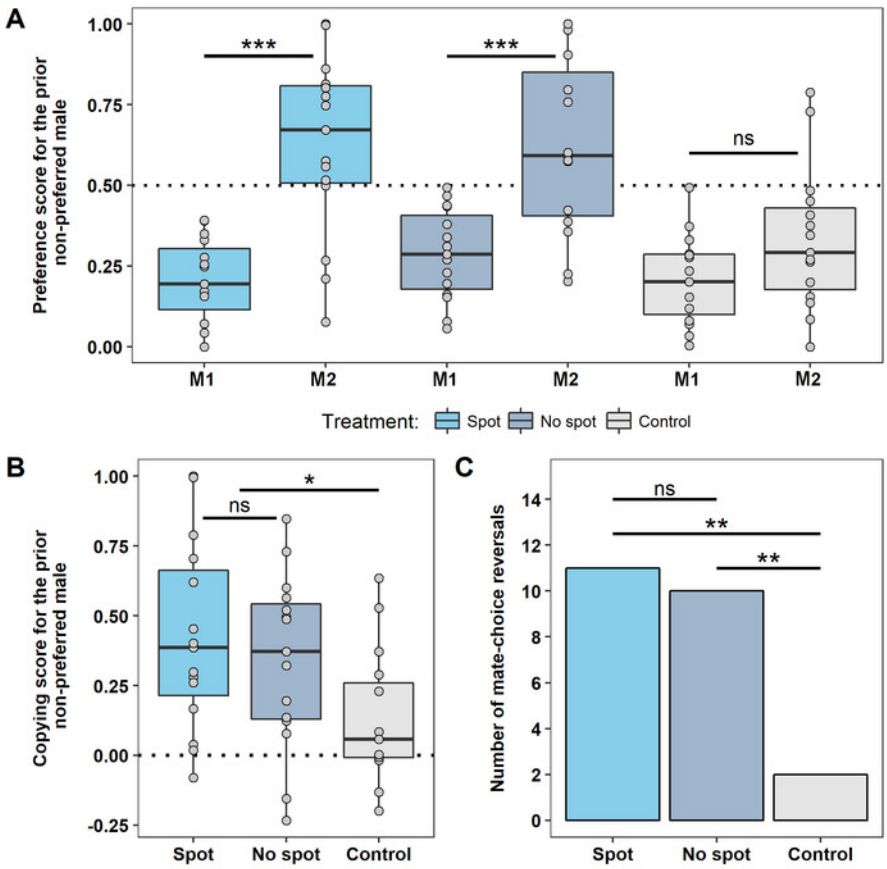

Figure 7: Results of the virtual MCC experiment manipulating model female quality by the visual absence or presence of a gravid spot. (A) Preference scores for the (prior) non-preferred virtual stimulus male in M1 and M2 for both treatments and the control. **(B)** Change of preference from M1 to M2 (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. Please click here to view a larger version of this figure.

To obtain additional information about whether a copying effect might be more or less strong depending on the respective treatment, a comparison between copying scores and the number of mate-choice reversals between the different treatments and the control was conducted. Therefore, 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 as a random factor. Here, we conducted the same two orthogonal comparisons for "treatment" as described above.

As we show in **Figure 7B** and Tables 3 and 4, 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.

Fixed factors	Lower	Estimate	Upper	SE	df	t-value	p-value
(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 3. 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.

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

Table 4. LME variance components for focal female spot area. Variance and standard deviation for the random effect "spot_area" and the residuals are given.

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'[75] with correction for multiple testing. As we show in **Figure 7C**, eleven out of 15 focal females reversed their mate choice in Treatment 1 and ten females reversed their mate choice in Treatment 2. On the other hand, only two reversals were observed in the control. Thereby, 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).

Discussion

The gravid spot in sailfin molly females was previously described to serve as a means of fertility advertisement towards conspecific males[59,60]. 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 to not 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 the female reproductive cycle, with the spot being largest prior to parturition and smallest or absent after giving birth[60], systematic visual observations of the development of gravid spots in individual females are still missing. Moreover, variation in gravid spot size can be high between individual females with spots also being completely absent in mature, gravid females[60]. Even though sailfin molly females are most receptive short after parturition[59,76], they are able to store sperm for several months[57]. Therefore, females should always be choosy for the best quality mate. With regards to our case study on MCC and the tested hypotheses, we conclude that a gravid spot may not be a valid indicator of model female quality to observing conspecific females. Information on the reproductive state of a model female that an observing female might possibly gain seems to not be important in the decision to copy her choice or not, at least among sailfin mollies.

Notably, our study demonstrates a highly standardized procedure for visual manipulation of public information provided in MCC experiments by using computer animated fish. In contrast to an earlier study by Benson[77], who injected live fish with tattoo ink to manipulate gravid spots, our method provides a completely non-invasive alternative for visual manipulation. We described in detail the procedure on how to create and animate virtual sailfin mollies in *FishSim*. We further showed how computer animation can be used to adopt the experimental procedure of a classic MCC experiment with virtual fish for the presentation towards live test fish in a binary choice situation.

Following the protocol, we identify several critical steps that need specific attention to ensure the correct handling of our toolchain and the success of the experiment. Since computer animations are created and presented using computers and display devices such as computer monitors, the technical equipment should always be good enough to ensure a smooth processing of the general workflow and, most importantly, the playback of the animation (steps 2, 3, and 5). When using two or more monitors for presentation of stimuli, their technical specifications should be identical. When using our software, the set monitor resolution should always be that of the presentation monitors (see step 1.2.1.). Setting the scene (step 1.2.) as well as the design (steps 1.3. and 1.4.) and animation (step 2) of virtual stimuli should always be done on a monitor later used for stimulus presentation during experiments to ensure the correct dimensions.

In this protocol, we concentrate on the necessary steps to create one set of fish stimuli (steps 1.3. and 1.4.) for the use in one trial of a treatment (step 5). Here, we would like to point out that it is important to create several different fish stimuli and/or animations to account for pseudoreplication[15,78,79] which affects the possible interpretation of the data obtained during experiments. With our toolchain, it is easy to create various fish stimuli offering possibilities to use a unique set of stimuli for each experimental trial. Overall the total number of stimuli needed depends on the intended sample size for each treatment (see "Representative results" section for information on our case study).

With our toolchain, we wanted to provide a fast and easy-to-create animation process by using a video game controller (step 2). Thereby, the general swimming behavior of the virtual fish is automatically generated, based on videos of swimming live sailfin mollies[80]. Swimming behavior (including movement of fins and gonopodium) is, therefore, tuned to the use with virtual stimuli of sailfin mollies in particular and live-bearing fish in general. Apart from live-bearing fish, an additional template for a three-spined stickleback provides additional functions for species-specific movement, such as raising/lowering of dorsal and ventral spines.

Animation functions currently provided by our toolchain might not be sufficient for every behavioral pattern and fish species. This, however, is up to the user and depends on the tested research question. Further, animation with *FishSteering* (step 2) needs a little practice beforehand to get accustomed to the functions of the gaming controller. Therefore, the animation process is probably the most time-consuming step of the protocol. A controller of a different brand may be used here but the functionality might not be that smooth and the button functions (as given in the user manual) may be different or completely absent. During the animation process, only one feature of a virtual stimulus (*e.g.,* position, fins, gonopodium) can be animated at a time. First, the swimming movement (position) and afterwards additional features (*e.g.,* fins) may be added independently. We recommend saving each step separately. This offers the advantage that the user has the possibility to come back to an earlier version of the animation to change a specific feature, for example keeping the swimming path constant but changing the dorsal fin movement compared to a previous version. Especially when animating more than one fish (step 2.2.), the order in which fish stimuli are animated is very important and needs to be determined beforehand. Here, it might be helpful to refer to the biology of the tested species. In our case study, we simulated the courtship behavior of sailfin mollies in which a male is generally following a female[81]. Hence, we first created the swimming path of the virtual female and added the path for the virtual male by following the female.

When running the experimental procedure (step 5) the timing is crucial for the success of the experiment. The times/durations we referred to in the protocol (step 5) derived from previous studies with sailfin mollies. They should be regarded as suggestions and are not obligated for the general success of the experimental but should, nevertheless, be tightly followed during the procedure. Acclimatization time may vary between fish species and even individuals and should generally be as long as the focal fish needs to explore the whole test tank and acclimate to its new surroundings. We determined the appropriate pause duration length in training runs of the experimental procedure. The pause should be at least as long as the time needed for catching the fish with the cylinder, as well as walking to and from the test tank and operational computer to release the fish from the cylinder. Here, times possibly vary depending on the specific experimental situation in each lab and the tested fish species. In any case, the experimenter may individually change times/durations either by setting a different time in *FishPlayer* (see step 3.1. 3.) or by creating animation sequences with a different length (see step 2.1).

The experimenter can improve the measuring of association time for each mate-choice test by implementing an automated tracking system, though it needs to be capable of tracking in real-time. Here, we also want to point out that there is no possibility of having a blind observer and, hence, blind analysis when following the procedure for testing MCC. Since the experimenter cannot know which virtual male stimulus will be preferred by the focal female prior to testing, he or she needs to be aware of the focal fish's choice to rearrange the order of animation sequences accordingly (see steps 3.2 and 5.10).

The protocol we describe here is specific to our study design on MCC in sailfin mollies. However, the toolchain can also be used in combination with other experimental designs with up to four monitors for presentation. In general, computer animation tools offer a wide variety of solutions to study various questions on fish behavior like mate choice, shoaling decisions or predator-prey detection, using artificial visual stimuli. General technical and conceptual considerations for the use of computer animation in animal behavior research should be carefully evaluated before using it in experiments[2,15]. Most important for the decision whether computer animation approaches can be implemented in research regards the visual capabilities of the tested fish species and whether it responds naturally towards virtual stimuli presented on monitor screens. Especially, when testing the effect of color aspects, it should be noted that monitor screens only depict colors as RGB values and that this might impede or limit research possibilities, although RGB color output may indeed be adjusted[82]. For some fish species, a limitation might certainly be that monitors do not emit UV wavelengths or that, on the other hand, certain monitor types are highly polarized which might be a limitation with fish being sensitive to polarized light for example in questions of mate choice[83]. Therefore, a validation for the effectiveness of presented stimuli as computer animations is necessary before testing any hypotheses[2,12,15,84,85].

In the future, new developments in animal tracking and action recognition might make it possible to create interactive virtual stimuli that react in real-time towards live fish and simulate corresponding behavior to massively increase realism for observing fish[86]. Thanks to the modularity of the underlying ROS, external devices such as cameras may be integrated into the toolchain, provided that the user has adequate programming skills. A first successful attempt showed that *FishSim* can generally be used to simulate interactive virtual fish stimuli by extension of a 3D real-time tracking system[87,88,89]. During the science communication event "Molly knows best" (https://virtualfishproject.wixsite.com/em2016-fisch-orakel), we were able to demonstrate that virtual fish can be programmed to follow live focal fish on screen and perform courtship behavior according to a predefined algorithm. Further, such real-time tracking systems could be used to measure association time automatically to enhance experimental procedure. This feature is not yet included in the current version of *FishSim* but is subject to future development.

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 live fish. Further, in line with the 3Rs-principle and similar guidelines for the use of animals in research and teaching[90,91], this technique bears the potential to 'reduce' and 'replace' live test animals as well as to 'refine' experimental procedures in research.

Disclosures

The authors have nothing to disclose.

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References

1. Witte, K., Gierszewski, S., Chouinard-Thuly, L. Virtual is the new reality. *Current Zoology.* **63** (1), 1-4 (2017).
2. Chouinard-Thuly, L. *et al.* Technical and conceptual considerations for using animated stimuli in studies of animal behavior. *Current Zoology.* **63** (1), 5-19 (2017).
3. Basolo, A. L. Female preference for male sword length in the green swordtail, *Xiphophorus helleri* (Pisces: Poeciliidae). *Animal Behaviour.* **40** (2), 332-338 (1990).
4. Rosenthal, G. G., Evans, C. S. Female preference for swords in *Xiphophorus helleri* reflects a bias for large apparent size. *Proceedings of the National Academy of Sciences of the United States of America.* **95** (8), 4431-4436 (1998).
5. Schlupp, I., Waschulewski, M., Ryan, M. J. Female preferences for naturally-occurring novel male traits. *Behaviour.* **136** (4), 519-527 (1999).
6. Campbell, M. W., Carter, J. D., Proctor, D., Eisenberg, M. L., de Waal, F. B. M. Computer animations stimulate contagious yawning in chimpanzees. *Proceedings of the Royal Society B: Biological Sciences.* **276** (1676), 4255-4259 (2009).
7. Woo, K. L., Rieucau, G. The importance of syntax in a dynamic visual signal: recognition of jacky dragon displays depends upon sequence. *Acta Ethologica.* **18** (3), 255-263 (2015).
8. Balzarini, V., Taborsky, M., Villa, F., Frommen, J. G. Computer animations of colour markings reveal the function of visual threat signals in *Neolamprologus pulcher. Current Zoology.* **63** (1),45-54 (2017).
9. Tedore, C., Johnsen, S. Visual mutual assessment of size in male *Lyssomanes viridis* jumping spider contests. *Behavioral Ecology.* **26** (2), 510-518 (2015).
10. Watanabe, S., Troje, N. F. Towards a “virtual pigeon”: a new technique for investigating avian social perception. *Animal Cognition.* **9** (4), 271-279 (2006).
11. Culumber, Z. W., Rosenthal, G. G. Mating preferences do not maintain the tailspot polymorphism in the platyfish *Xiphophorus variatus. Behavioral Ecology.* **24** (6), 1286-1291 (2013).
12. Gierszewski, S., Müller, K., Smielik, I., Hütwohl, J. -M., Kuhnert, K. -D., Witte, K. The virtual lover: variable and easily guided 3D fish animations as an innovative tool in mate-choice experiments with sailfin mollies - II. Validation. *Current Zoology.* **63** (1), 65-74 (2017).
13. Thurley, K., Ayaz, A. Virtual reality systems for rodents. *Current Zoology.* **63** (1), 109-119 (2017).
14. Stowers, J. R. *et al.* Virtual reality for freely moving animals. *Nature methods.* **14** (10), 995 (2017).
15. Powell, D. L., Rosenthal, G. G. What artifice can and cannot tell us about animal behavior. *Current Zoology.* **63** (1), 21-26 (2017).
16. Müller, K., Smielik, I., Hütwohl, J. -M., Gierszewski, S., Witte, K., Kuhnert, K. -D. The virtual lover: variable and easily guided 3D fish animations as an innovative tool in mate-choice experiments with sailfin mollies-I. Design and implementation. *Current Zoology.* **63** (1), 55-64 (2017).
17. Veen, T. *et al. anyFish*: an open- source software to generate animated fish models for behavioural studies. *Evolutionary Ecology Research.* **15** (3), 361-375 (2013).
18. Ingley, S. J. *et al. anyFish* 2. 0: An open-source software platform to generate and share animated fish models to study behavior. *SoftwareX.* **3**, 13-21 (2015).
19. Bakker, T. C. M., Pomiankowski, A. The genetic basis of female mate preferences. *The Journal of Evolutionary Biology.* **8** (2), 129-171 (1995).
20. Andersson, M., Simmons, L. W. Sexual selection and mate choice. *Trends in Ecology & Evolution.* **21** (6), 296-302 (2006).
21. Danchin, É., Giraldeau, L. -A., Valone, T. J., Wagner, R. H. Public information: From nosy neighbors to cultural evolution. *Science.* **305** (5683), 487-491 (2004).
22. Pruett-Jones, S. Independent *Versus* Nonindependent Mate Choice: Do Females Copy Each Other? *The American Naturalist.* **140** (6), 1000-1009 (1992).
23. Witte, K., Kniel, N., Kureck, I. M. Mate-choice copying: Status quo and where to go. *Current Zoology.* **61** (6), 1073-1081 (2015).
24. Witte, K., Nöbel, S. Learning and Mate Choice. In: *Fish Cognition and Behavior.* Brown, C., Laland, K. N., & Krause, J., eds., Blackwell Publishing Ltd., 81-107 (2011).
25. Waynforth, D. Mate Choice Copying in Humans. *Human nature.* **18** (3), 264-271 (2007).
26. Galef, B. G., White, D. J. Evidence of social effects on mate choice in vertebrates. *Behavioural Processes.* **51** (1-3), 167-175 (2000).
27. Kniel, N., Dürler, C., Hecht, I., Heinbach, V., Zimmermann, L., Witte, K. Novel mate preference through mate-choice copying in zebra finches: sexes differ. *Behavioral Ecology.* **26** (2), 647-655 (2015).
28. Kniel, N., Schmitz, J., Witte, K. Quality of public information matters in mate-choice copying in female zebra finches. *Frontiers in Zoology.* **12**, 26 (2015).
29. Kniel, N., Müller, K., Witte, K. The role of the model in mate-choice copying in female zebra finches. *Ethology.* **123** (6-7), 412-418 (2017).
30. Mery, F. *et al.* Public *Versus* Personal Information for Mate Copying in an Invertebrate. *Current Biology.* **19** (9), 730-734 (2009).
31. Dagaeff, A. -C., Pocheville, A., Nöbel, S., Loyau, A., Isabel, G., Danchin, E. Drosophila mate copying correlates with atmospheric pressure in a speed learning situation. *Animal Behaviour.* **121**, 163-174 (2016).
32. Monier, M., Nöbel, S., Isabel, G., Danchin, E. Effects of a sex ratio gradient on female mate-copying and choosiness in *Drosophila melanogaster. Current Zoology.* **64** (2), 251-258 (2018).
33. Dugatkin, L. A., Godin, J. -G. J. Reversal of female mate choice by copying in the guppy (*Poecilia reticulata*). *Proceedings of the Royal Society B: Biological Sciences.* **249**, 179-184 (1992).
34. Widemo, M. S. Male but not female pipefish copy mate choice. *Behavioral Ecology.* **17** (2), 255-259 (2006).
35. Heubel, K. U., Hornhardt, K., Ollmann, T., Parzefall, J., Ryan, M. J., Schlupp, I. Geographic variation in female mate-copying in the species complex of a unisexual fish, *Poecilia formosa. Behaviour.* **145** (8), 1041-1064 (2008).
36. Bierbach, D. *et al.* Male fish use prior knowledge about rivals to adjust their mate choice. *Biology Letters.* **7** (3), 349–351 (2011).
37. Munger, L., Cruz, A., Applebaum, S. Mate choice copying in female humpback limia (*Limia nigrofasciata*, family Poeciliidae). *Ethology.* **110** (7), 563-573 (2004).
38. Frommen, J. G., Rahn, A. K., Schroth, S. H., Waltschyk, N., Bakker, T. C. M. Mate-choice copying when both sexes face high costs of reproduction. *Evol Ecol.* **23** (3), 435-446 (2009).
39. Witte, K., Ryan, M. J. Mate choice copying in the sailfin molly, *Poecilia latipinna*, in the wild. *Animal Behaviour.* **63** (5), 943-949 (2002).

40. Goulet, D., Goulet, T. L. Nonindependent mating in a coral reef damselfish: evidence of mate choice copying in the wild. *Behavioral Ecology.* **17** (6), 998-1003 (2006).
41. Alonzo, S. H. Female mate choice copying affects sexual selection in wild populations of the ocellated wrasse. *Animal Behaviour.* **75** (5), 1715-1723 (2008).
42. Godin, J. -G. J., Hair, K. P. E. Mate-choice copying in free-ranging Trinidadian guppies (*Poecilia reticulata*). *Behaviour.* **146**, 1443-1461 (2009).
43. Nordell, S. E., Valone, T. J. Mate choice copying as public information. *Ecology Letters.* **1** (2), 74-76 (1998).
44. Vukomanovic, J., Rodd, F. H. Size-Dependent Female Mate Copying in the Guppy (*Poecilia reticulata*): Large Females are Role Models but Small Ones are not. *Ethology.* **113** (6), 579-586 (2007).
45. Dugatkin, L. A., Godin, J. -G. J. Female mate copying in the guppy (*Poecilia reticulata*): age-dependent effects. *Behavioral Ecology.* **4**, 289-292 (1993).
46. Amlacher, J., Dugatkin, L. A. Preference for older over younger models during mate-choice copying in young guppies. *Ethology Ecology & Evolution.* **17** (2), 161-169 (2005).
47. Hill, S. E., Ryan, M. J. The role of model female quality in the mate choice copying behaviour of sailfin mollies. *Biology Letters.* **2** (2), 203-205 (2006).
48. Gierszewski, S., Keil, M., Witte, K. Mate-choice copying in sailfin molly females: public information use from long-distance interactions. *Behavioral Ecology and Sociobiology.* **72** (2), 26 (2018).
49. Schlupp, I., Marler, C., Ryan, M. J. Benefit to male sailfin mollies of mating with heterospecific females. *Science.* **263** (5145), 373-374 (1994).
50. Schlupp, I., Ryan, M. J. Male sailfin mollies (*Poecilia latipinna*) copy the mate choice of other males. *Behavioral Ecology.* **8** (1), 104-107 (1997).
51. Witte, K., Ryan, M. J. Male body length influences mate-choice copying in the sailfin molly *Poecilia latipinna. Behavioral Ecology.* **9** (5), 534-539 (1998).
52. Witte, K., Ueding, K. Sailfin molly females (*Poecilia latipinna*) copy the rejection of a male. *Behavioral Ecology.* **14** (3), 389-395 (2003).
53. Verzijden, M. N., ten Cate, C., Servedio, M. R., Kozak, G. M., Boughman, J. W., Svensson, E. I. The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution.* **27** (9), 511-519 (2012).
54. Varela, S. A. M., Matos, M., Schlupp, I. The role of mate-choice copying in speciation and hybridization. *Biological Reviews.* **93** (2), 1304-1322 (2018).
55. Nöbel, S., Danchin, E., Isabel, G. Mate-copying for a costly variant in *Drosophila melanogaster* females. *Behavioral Ecology.* ary095 (2018).
56. Norazmi-Lokman, N. H., Purser, G. J., Patil, J. G. Gravid Spot Predicts Developmental Progress and Reproductive Output in a Livebearing Fish, *Gambusia holbrooki. PLoS One.* **11** (1), e0147711 (2016).
57. Constantz, G. D. Reproductive biology of poeciliid fishes. In: *Ecology and Evolution of livebearing fishes (Poeciliidae).* Meffe, G. K., & Snelson, F. F., eds, Prentice Hall, 33-50 (1989).
58. Peden, A. E. Variation in Anal Spot Expression of Gambusiin Females and Its Effect on Male Courtship. *Copeia.* **1973** (2), 250-263 (1973).
59. Farr, J. A., Travis, J. Fertility Advertisement by Female Sailfin Mollies, *Poecilia latipinna* (Pisces: Poeciliidae). *Copeia.* **1986** (2), 467-472 (1986).
60. Sumner, I. T., Travis, J., Johnson, C. D. Methods of Female Fertility Advertisement and Variation among Males in Responsiveness in the Sailfin Molly (*Poecilia latipinna*). *Copeia.* **1994** (1), 27-34 (1994).
61. Witte, K., Noltemeier, B. The role of information in mate-choice copying in female sailfin mollies (*Poecilia latipinna*). *Behavioral Ecology and Sociobiology.* **52** (3), 194-202 (2002).
62. R Development Core Team. *R: a language and environment for statistical computing.* http://www. r-project. org/ (2015).
63. Bischoff, R. J., Gould, J. L., Rubenstein, D. I. Tail size and female choice in the guppy (*Poecilia reticulata*). *Behavioral Ecology and Sociobiology.* **17** (3), 253-255 (1985).
64. Forsgren, E. Predation Risk Affects Mate Choice in a Gobiid Fish. *The American Naturalist.* **140** (6), 1041-1049 (1992).
65. Berglund, A. Risky sex: male pipefishes mate at random in the presence of a predator. *Animal Behaviour.* **46** (1), 169-175 (1993).
66. Kodric-Brown, A. Female choice of multiple male criteria in guppies: interacting effects of dominance, coloration and courtship. *Behavioral Ecology and Sociobiology.* **32** (6), 415-420 (1993).
67. Witte, K., Klink, K. B. No pre-existing bias in sailfin molly females, *Poecilia latipinna*, for a sword in males. *Behaviour.* **142** (3), 283-303 (2005).
68. Nöbel, S., Witte, K. Public Information Influences Sperm Transfer to Females in Sailfin Molly Males. *PLoS One.* **8** (1), e53865 (2013).
69. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Team, R. C. *nlme: Linear and Nonlinear Mixed Effects Models.* at http://cran. r-project. org/package=nlme (2015).
70. Crawley, M. J. *The R Book.* John Wiley & Sons, Ltd. (2007).
71. Pinheiro, J. C., Bates, D. M. *Mixed-Effects Models in S and S-PLUS.* Springer-Verlag. New York. (2000).
72. Zuur, A., Ieno, E. N., Walker, N., Saveliev, A. A., Smith, G. M. *Mixed Effects Models and Extensions in Ecology with R.* Springer. New York. (2009).
73. De Rosario-Martinez, H. *phia: Post-Hoc Interaction Analysis.* http://cran. r-project. org/package=phia (2015).
74. Korner-Nievergelt, F., Roth, T., von Felten, S., Guélat, J., Almasi, B., Korner-Nievergelt, P. *Bayesian Data Analysis in Ecology Using Linear Models with R, BUGS, and Stan.* Elsevier Science. (2015).
75. Hervé, M. *RVAideMemoire: Testing and Plotting Procedures for Biostatistics.* https://cran. r-project. org/package=RVAideMemoire%0A (2017).
76. Travis, J. Ecological genetics of life history traits in poeciliid fishes. In: *Ecology and Evolution of livebearing fishes (Poeciliidae).* Meffe, G. K., & Snelson, F. F., eds, Prentice Hall, 185-200 (1989).
77. Benson, K. E. Enhanced Female Brood Patch Size Stimulates Male Courtship in Xiphophorus helleri. *Copeia.* **2007** (1), 212-217 (2007).
78. Hurlbert, S. H. Pseudoreplication and the design of ecological field experiments. *Ecological Monographs.* **54** (2), 187-211 (1984).
79. McGregor, P. K. Playback experiments: design and analysis. *Acta Ethologica.* **3** (1), 3-8 (2000).
80. Smielik, I., Müller, K., Kuhnert, K. D. Fish motion simulation. *ESM 2015—European Simulation and Modelling (EUROSIS) Conference Proc.* 392-396 (2015).
81. Baird, R. C. Aspects of social behavior in *Poecilia latipinna* (Lesueur). *Revista de Biología Tropical.* **21** (2), 399-416 (1974).
82. Tedore, C., Johnsen, S. Using RGB displays to portray color realistic imagery to animal eyes. *Current Zoology.* **63** (1), 27- 34 (2017).
83. Calabrese, G. M., Brady, P. C., Gruev, V., Cummings, M. E. Polarization signaling in swordtails alters female mate preference. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (37), 13397-13402 (2014).

84. Qin, M., Wong, A., Seguin, D., Gerlai, R. Induction of social behavior in zebrafish: live *versus* computer animated fish as stimuli. *Zebrafish.* **11** (3), 185-197 (2014).
85. Scherer, U., Godin, J. -G. J., Schuett, W. Validation of 2D-animated pictures as an investigative tool in the behavioural sciences : A case study with a West African cichlid fish, *Pelvicachromis pulcher. Ethology.* **123** (8), 560-570 (2017).
86. Butkowski, T., Yan, W., Gray, A. M., Cui, R., Verzijden, M. N., Rosenthal, G. G. *The Journal of Visualized Experiments.* **48**, 2374 (2011).
87. Müller, K., Gierszewski, S., Witte, K., Kuhnert, K. -D. Where is my mate? Real-time 3-D fish tracking for interactive mate-choice experiments. *ICPR 2016—23rd International Conference for Pattern Recognition; VAIB 2016, Proc.* 1-5 (2016).
88. Müller, K., Schlemper, J., Kuhnert, L., Kuhnert, K. -D. Calibration and 3D ground truth data generation with orthogonal camera-setup and refraction compensation for aquaria in real-time. *IEEE 2014 International Conference on Computer Vision Theory and Applications (VISAPP).* **3**, 626-634 (2014).
89. Müller, K., Hütwohl, J. M., Gierszewski, S., Witte, K., Kuhnert, K. D. Fish Motion Capture with Refraction Synthesis. *Journal of WSCG.* In press (2018).
90. ASAB Guidelines for the treatment of animals in behavioural research and teaching. *Animal Behaviour.* **135**, I-X (2018).
91. Russell, W. M. S., Burch, R. L. *The Principles of Humane Experimental Technique.* Methuen and Co. Ltd. London. (1959).

