Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58435R1
Full Title:	Using FishSim Animation Toolchain to investigate fish behavior: A case study on mate-choice copying in sailfin mollies
Keywords:	FishSim Animation Toolchain; computer animation; virtual fish; sailfin molly; Poecilia latipinna; mate-choice copying; mate choice; social learning; public information; gravid spot; behavioral experiment; fish behavior
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

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

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KEYWORDS:

FishSim Animation Toolchain, computer animation, virtual fish, sailfin molly, *Poecilia latipinna*, mate-choice copying, mate choice, social learning, public information, gravid spot, behavioral experiment, fish behavior

SHORT ABSTRACT:

Using the novel *FishSim* Animation Toolchain, we present a protocol for non-invasive visual manipulation of public information in the context of mate-choice copying in sailfin mollies. *FishSim* Animation Toolchain provides an easy-to-use framework for the design, animation and presentation of computer-animated fish stimuli for behavioral experiments with live test fish.

LONG 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.

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, 10-12]. 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[25-29] and invertebrates[30-32]. So far, MCC was predominantly studied in fish and is found both under laboratory conditions[33-38] and in the wild[39-42]. 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[44-47]. 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[44-46], or by her being a conspecific or a heterospecific[47]. In sailfin mollies that copy the mate choice of conspecifics[39, 48-51], 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[49-51, 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.

[Place **Figure 1** here]

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/).

1.1. **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 2**)!

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 2**).

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.

[Place **Figure 2** here]

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 3A**). Adjusting the scene has to be done only once.

[Place **Figure 3** here]

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 screwdriver 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 3B**).

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 3C**).

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 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).

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 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.

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 5**.

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.

[Place **Figure 4** and **Figure 5** here]

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 4** and **5**), 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 4**). 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 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**

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 6**).

[Place **Figure 6** here]

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**).

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 4**). 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 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.

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.

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

654 test for independent data revealing that SL did not differ between treatments and the control
655 (Kruskal Wallis rank sum test: n = 45, df = 2, χ^2 = 0.329, p = 0.848).
656
657 The most important parameter measured in an MCC experiment is the focal female's association
658 time for each male (protocol step 6.1). Association time is an indirect measure of female mate
659 preference in fish[63-66] and a well-established measure to determine mate choice in sailfin mollies
660 when no direct contact is provided[12, 48, 61, 67, 68]. For each treatment and the control, we first used
661 association time to analyze whether the choosing motivation differed between mate-choice
662 tests. Choosing motivation is defined as the total time a focal female spent in both choice zones
663 within a mate-choice test. However, a change in choosing motivation does not necessarily reflect
664 a change in preference for either male. If choosing motivation is significantly different between
665 the two mate-choice tests it is obligatory to use preference scores instead of absolute association
666 time, for further analysis to ensure comparability within and between treatments (see protocol
667 step 6.2). In our study, choosing motivation of focal females before and after observation of a
668 virtual model female sexually interacting with a male did not differ in Treatment 1 (Wilcoxon
669 signed rank test: n = 15, V = 44, p = 0.379) and in the control for choice consistency (Wilcoxon
670 signed rank test: n = 15, V = 42, p = 0.33). However, choosing motivation was significantly higher
671 after observation of a virtual model female without gravid spot sexually interacting with a male
672 in Treatment 2 (Wilcoxon signed rank test: n = 15, V = 22, p = 0.03).
673
674 The most important determinant of whether MCC occurred is a significant increase in time
675 spent/preference scores for the prior non-preferred male from the first to the second mate-
676 choice test[22]. Transferred to a natural situation, an increase in time spent with the prior non-
677 preferred male, consequently increases the probability that a female will mate with that male.
678 Therefore, the main analysis compared either the absolute times or the preference scores for the
679 prior non-preferred male between the two mate-choice tests. This analysis has to be done for
680 each treatment and the control separately. Since in our study, choosing motivation differed in
681 Treatment 2, we used preference scores for the initially non-preferred stimulus male, instead of
682 absolute association time, to determine whether these scores changed between the first and
683 second mate-choice test when public information was provided, compared to the control
684 treatment in which public information was absent.
685
686 For this, we fit a linear mixed effect (LME) model with the lme function from the 'nlme' package[69]
687 with preference score for the prior non-preferred male (pref_NP) as the dependent variable. We
688 included mate-choice test (Mtest: M1, M2) and treatment (treatment: spot, no spot, control) as
689 fixed factors as well as focal female's standard length (SL) as a covariate. To account for the
690 repeated measures design, focal female identity (ID) was included as a random factor. We were
691 especially interested in whether the effect of mate-choice test was different among treatments;
692 therefore, we included an interaction between mate-choice test and the treatment in our model.
693 We conducted two orthogonal comparisons for "treatment" using the function contrasts[70]. We
694 set the contrasts of the model (1) to compare the control against the mean of all treatments in
695 which any virtual model female was presented during observation [control | (spot, no spot)], and
696 (2) to compare the treatment showing a virtual model female with spot against that without a
697 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, $\chi^2 = 30.986$, $p < 0.001$) and Treatment 2 (no spot: df = 1, $\chi^2 = 19.957$, $p < 0.001$) but not for the control (χ^2-test: df = 1, $\chi^2 = 2.747$, 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.

[Place **Table 1** and **2** here]

[Place **Figure 7** here]

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.

[Place **Table 3** and 4 here]

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).

FIGURE AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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[87-89]. 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.

ACKNOWLEDGMENTS:

This work was supported by the Deutsche Forschungsgemeinschaft DFG (WI 1531/12-1 to KW and SG and KU 689/11-1 to KDK, KM and JMH). We sincerely thank the DAAD RISE Germany program for providing and organizing an undergraduate research internship between SG and DB (Funding-ID: 57346313). We are grateful to Mitacs for funding DB with a RISE-Globalink Research Internship Award (FR21213). We kindly thank Aaron Berard for inviting us to introduce *FishSim* to the JoVE readership and Alisha DSouza as well as three anonymous reviewers for their valuable comments on a previous version of the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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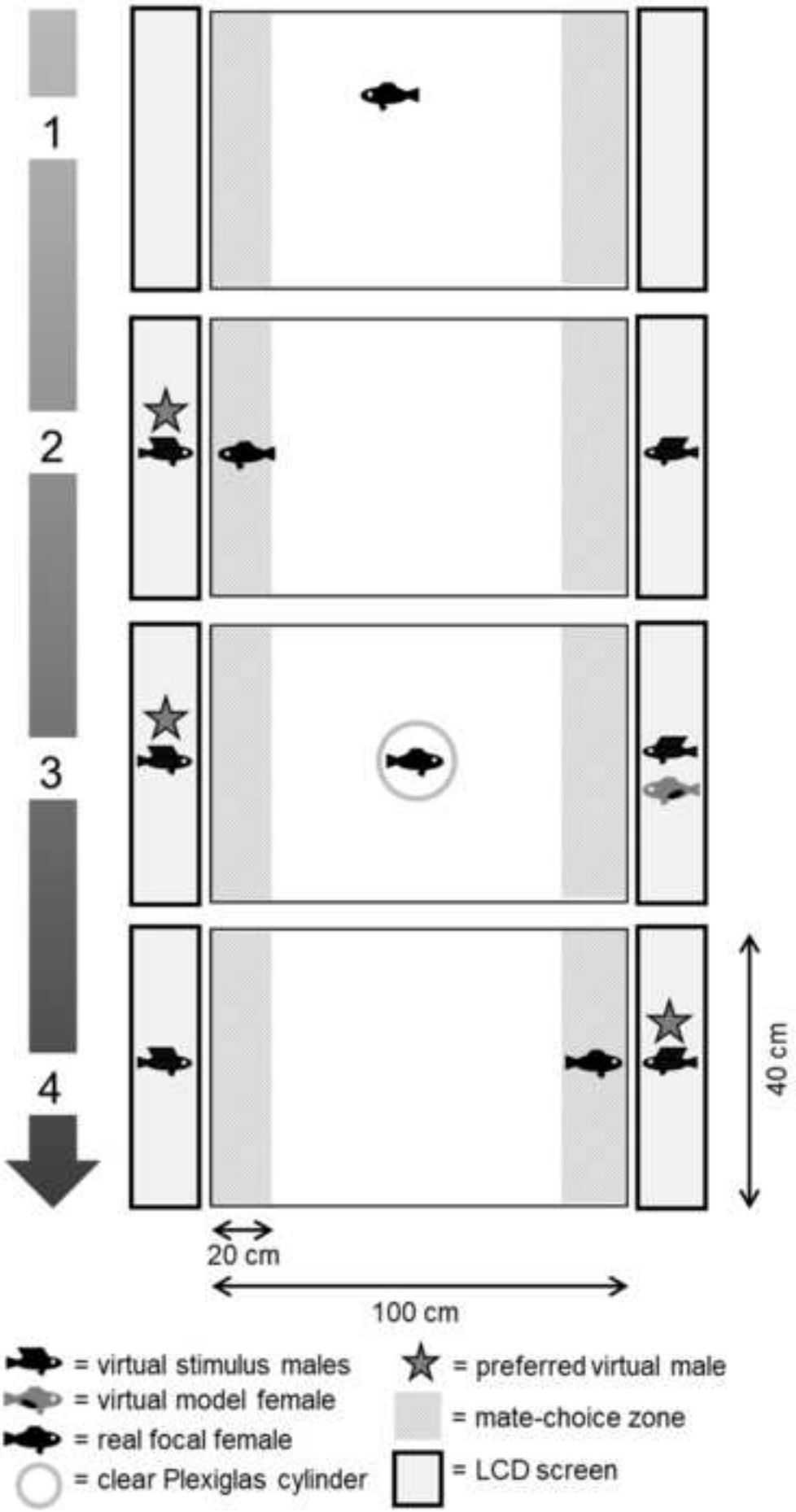

Figure 2

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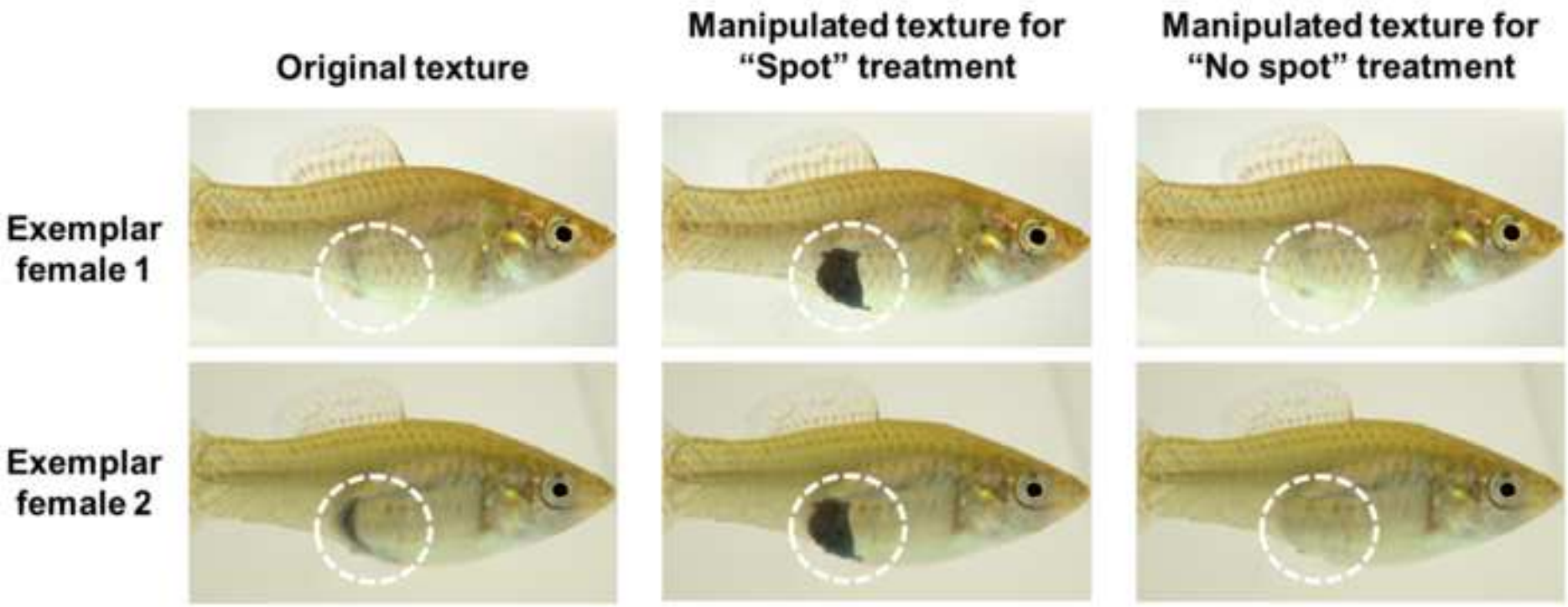

Figure 3

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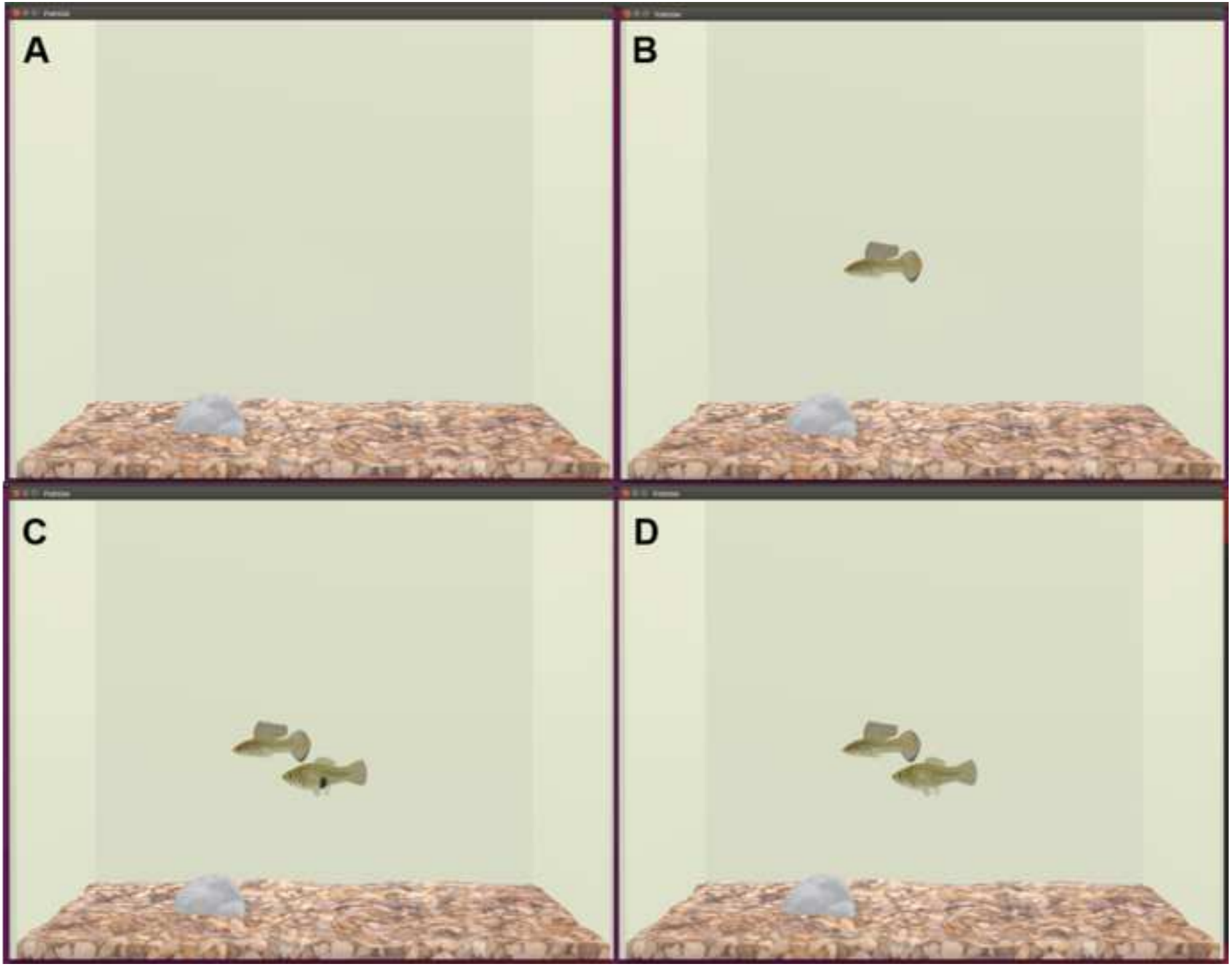

Figure 4

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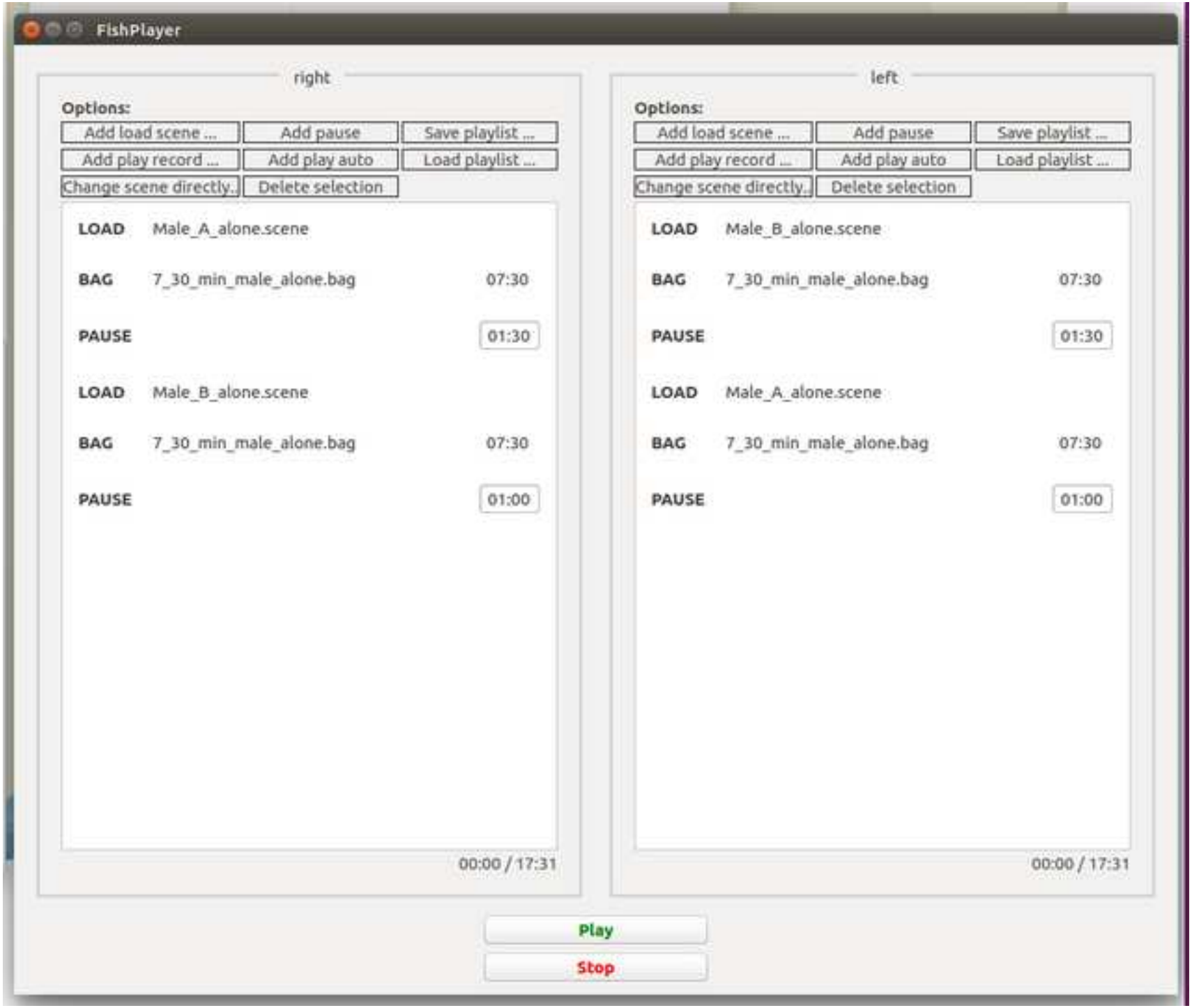

Figure 5

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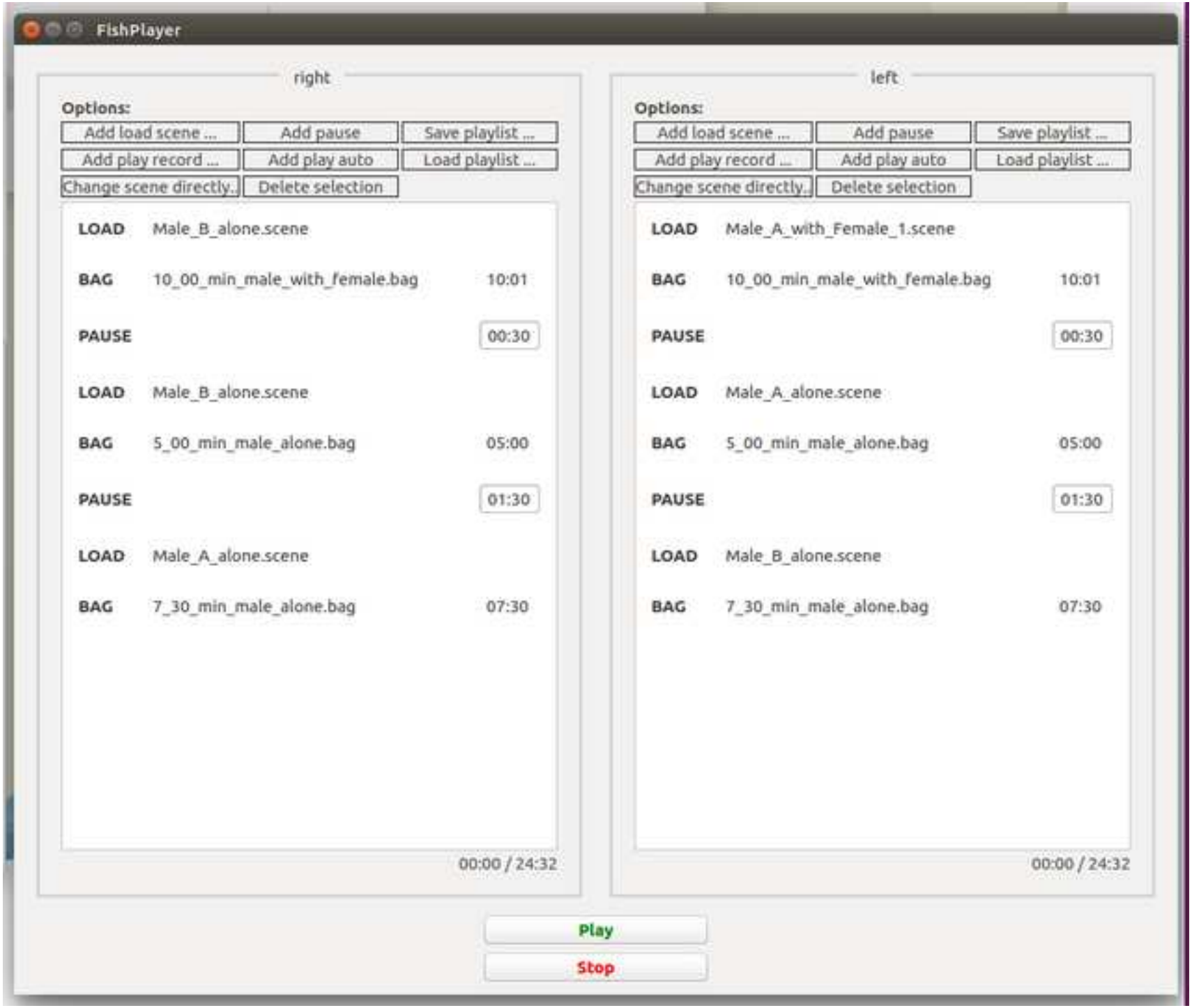

Figure 6

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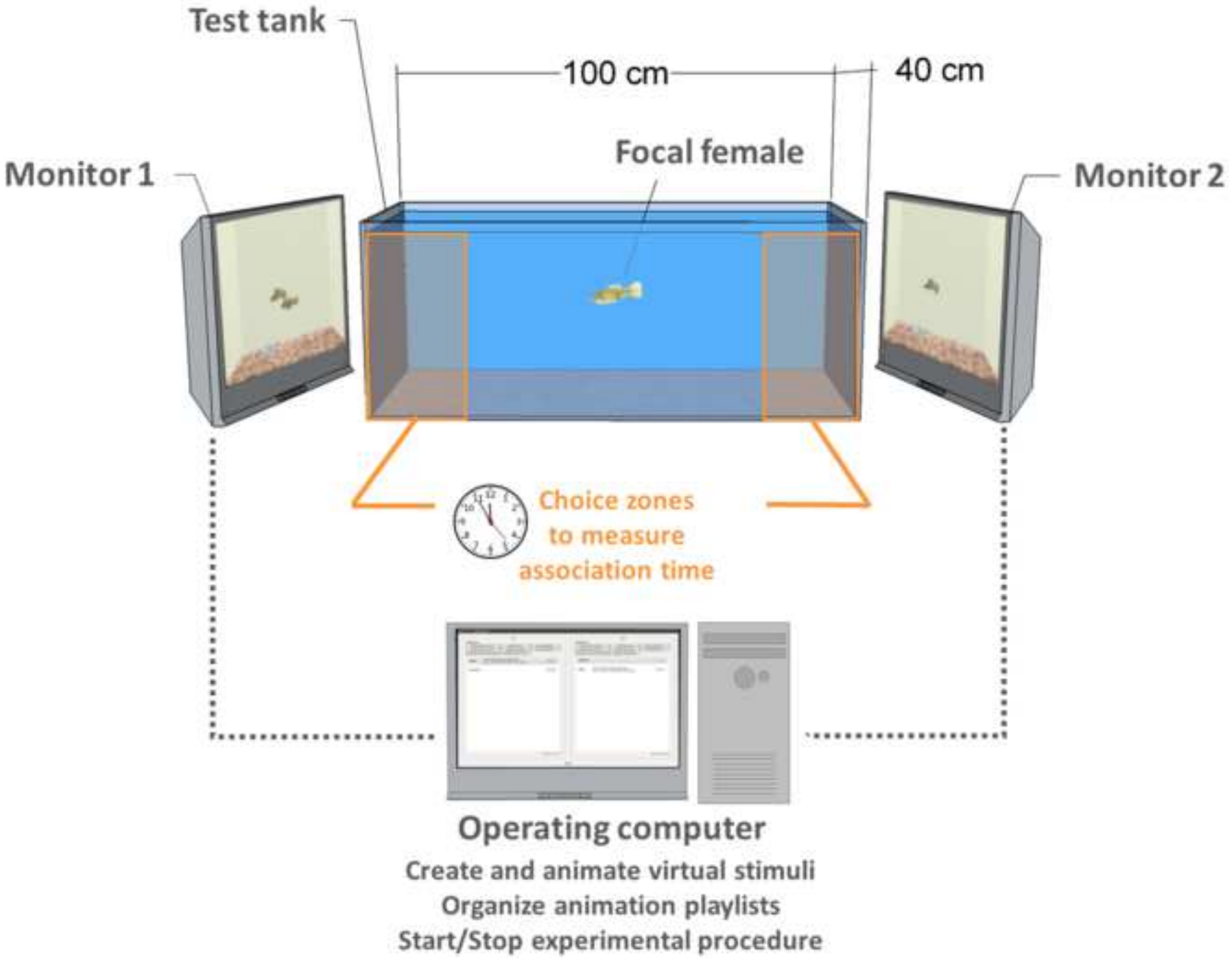

Figure 7

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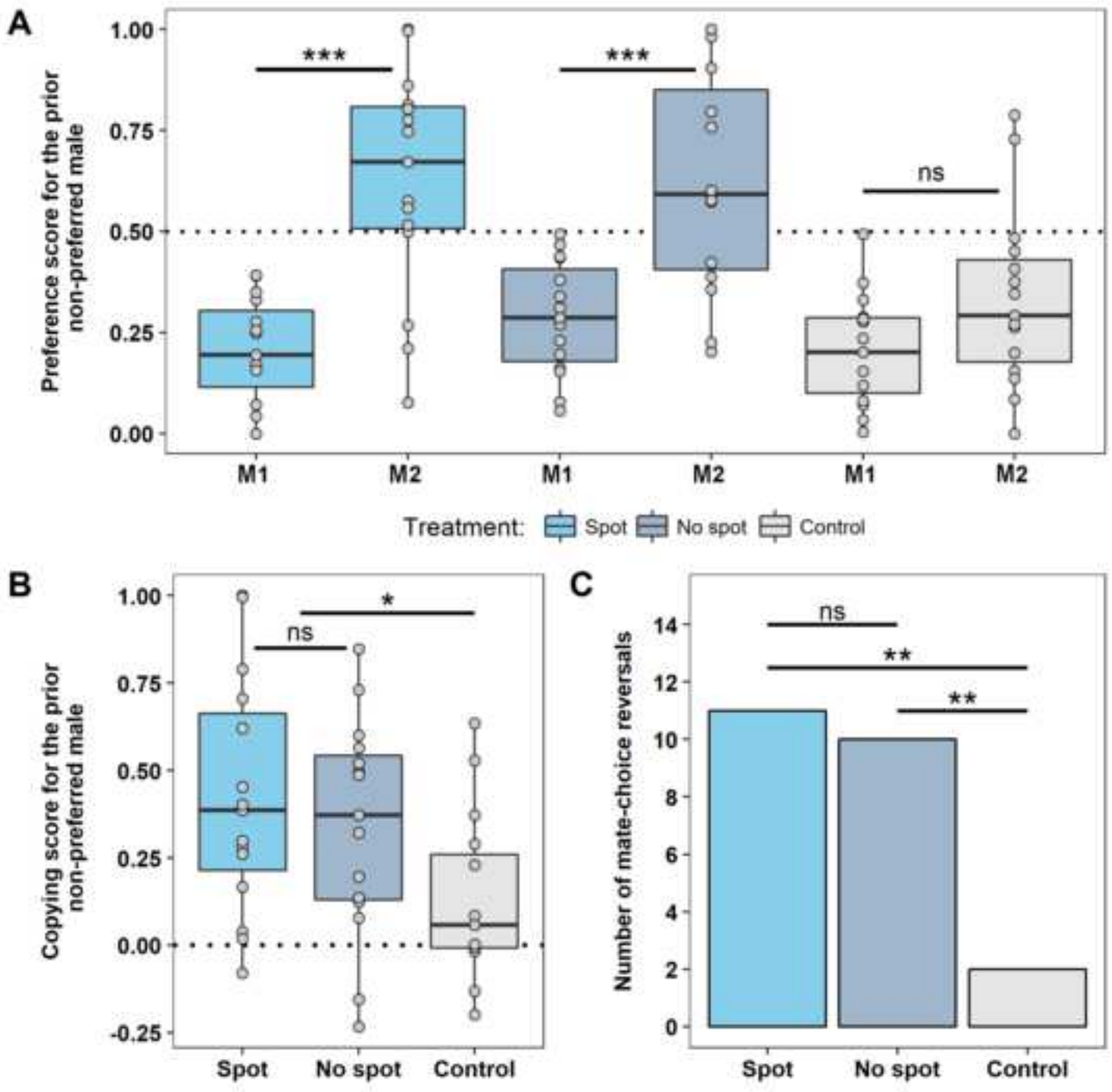

Table 1

Click here to download Table Table1_LME estimates for effects on preference scores_R1.xlsx

Factors	Lower	Estimate	Upper	SE	df
(Intercept)	0.046	0.339	0.632	0.145	42
M2	0.207	0.296	0.384	0.044	42
Control → (spot, no spot)	-0.041	-0.012	0.017	0.014	41
Spot → no spot	-0.093	-0.043	0.008	0.025	41
SL	-0.012	-0.003	0.006	0.004	41
M2 x [control → (spot, no spot)]	-0.148	-0.085	-0.023	0.031	42
M2 x (spot → no spot)	-0.067	0.042	0.15	0.054	42

t-value	p-value
2,336	**0.024**
6.750	**< 0.001**
-0.852	0.4
-1,715	0.094
-0.747	0.459
-2,743	**0.009**
0.777	0.441

Table 2

Click here to download Table Table2_LME variance components focal female ID_R1.xlsx

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

Table 3

Click here to download Table Table3_LME estimates for effects on copying scores_R1.xlsx

Fixed factors	Lower	Estimate	Upper	SE	df
(Intercept)	-0.889	-0.081	0.727	0.389	21
Control → (spot, no spot)	-0.153	-0.088	-0.023	0.031	20
Spot → no spot	-0.079	0.033	0.146	0.054	20
SL	-0.013	0.011	0.035	0.011	20

t-value	p-value
-0.208	0.837
-2,833	**0.01**
0.618	0.544
0.991	0.333

Table 4

Click here to download Table Table4_LME variance components spot area_R1.xlsx

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

Table of Materials

Click here to download Table of Materials Gierszewski et al_JoVE_Materials_R1.xls

Name of Material/ Equipment	Company	Catalog Number
Hardware		
2x 19" Belinea LCD displays	Belinea GmbH, Germany	Model 1970 S1-P
1x 24" Fujitsu LCD display	Fujitsu Technology Solutions GmbH, Germany	Model B24-8 TS Pro
Computer		
SONY Playstation 3 Wireless Controller	Sony Computer Entertainment Inc., Japan	Model No. CECHZC2E
Glass aquarium		
Plexiglass cylinder	custom-made	
Gravel		
2x OSRAM L58W/965	OSRAM GmbH, Germany	
2x Stopwatches		
Software		
ubuntu 16.04 LTS		
FishSim Animation Toolchain v.0.9		
GIMP Gnu Image Manipulation Program (version 2.8.22)		

	Comments/Description
	1280 x 1024 pixels resolution
	1920 x 1080 pixels resolution
	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 USB-cable for connection to computer
	100 cm x 40 cm x 40 cm (L x H x W) 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
	Illumination of the experimental setup
	Computer operating system; Download from: https://www.ubuntu.com/ Software download and user manual (PDF) from: https://bitbucket.org/EZLS/fish_animation_toolchain Download from: https://www.gimp.org/

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Author(s): Gierszewski S., Baker D., Müller K., Hürtwald J.M., Kuhnert K.D., Witte K.

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Article Title: In the "spotlight": do gravid spots predict model female quality in mate-choice copying? A study with fish and animation tool again in sailfin mollies

Signature: Stefanie Gierszewski Date: 7th May 2018

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Dear Alisha DSouza,

Thank you very much for giving us the opportunity to resubmit a revised version of our manuscript, now entitled “Using *FishSim* Animation Toolchain to investigate fish behavior: A case study on mate-choice copying in sailfin mollies” (JoVE58435_R1).

Below, we present our point-by-point response to the editorial comments as well as to the detailed comments of three reviewers. We hope that the changes made will be satisfactory and meet the JoVE requirements. Comments of the reviewers are marked with “Reviewer 1”, “Reviewer 2” and “Reviewer 3”, while our comments are marked with “Response”. Changes made in the manuscript (text from manuscript is given in italic writing) are given with the old (initial version) and new (revised version with track changes) line numbers, and words/passages that were added are underlined.

We hope that you will find our changes satisfying and that you will consider our manuscript for publication in JoVE.

Kind regards,

Stefanie Gierszewski

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response:
Derek Baker who is a native speaker and Co-Author of this manuscript, took the opportunity to thoroughly proofread the manuscript. We also edited the manuscript for conciseness and clarity, as suggested by Reviewer 3, and hope that we succeeded in improving the overall quality of the manuscript in our revised version.

2. Please revise lines 600-604 to avoid previously published text.

Response:
We revised new lines 932-936 (old lines 600-604) and shortened them to avoid previously published text as follows:
"Since in our study, choosing motivation differed in Treatment 2, we used ~~P~~preference scores for the initially~~prior~~ non-preferred stimulus male ~~were further used~~, instead of absolute association time~~,~~ to determine~~test~~ whether these scores changed between the first and second mate-choice~~preference~~ test when public information was provided, compared to the control treatment in which public information was absent."

3. Pleas revise the title to be less wordy and avoid puns.

Response:
We changed the title of the manuscript into "*~~In the "spot"light: dDo gravid spots predict model female quality in mate-choice copying? A study with FishSim Animation Toolchain in sailfin mollies~~Using FishSim Animation Toolchain to investigate fish behavior: A case study on mate-choice copying in sailfin mollies*" (new lines 2-4; old lines 2-3).

4. Please revise the Long Abstract to focus on the method being presented rather than the results of a specific experiment. Include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate. Please focus on the general types of results acquired.

Response:
To meet the requirements of JoVE, we deleted the previous version of the long abstract and completely revised it to focus on the method presented in our manuscript.

New lines 60-81 (old lines 37-57): "*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 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 alter virtual model females to copy the choice of presented to live focal females. 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."

5. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method

Response:
We give a clear statement of the overall goal of the development of our method in new lines 116-118: "*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."*

b) The rationale behind the development and/or use of this technique

Response:
In the beginning of the introduction, we give information on the rationale behind the use of computer animation in research in general:

New lines 87-95: *"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]."*

In new lines 110-115, we give information about the rationale behind the development of our technique in particular: *"Since creating computer animation may be difficult and time consuming, the need for software helping to facilitate and standardize the process of animation design arose. In this study, we ~~demonstrate the implementation of virtual fish stimuli presented on computer monitors to live focal females in a MCC experiment using~~ introduce the free and open-source FishSim Animation Toolchain[1644] (short: FishSim; https://bitbucket.org/EZLS/fish_animation_toolchain/), a multidisciplinary approach combining biology and computer science to address these needs."*

c) The advantages over alternative techniques with applicable references to previous studies

Response:
In new lines 87-95, we give examples highlighting the advantage of computer animation over conventional methods:
"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]."

We included the following references to our reference list (new lines 1326-1336):

3. *Basolo, A.L. Female preference for male sword length in the green swordtail, Xiphophorus helleri (Pisces: Poeciliidae). Anim Behav. 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. Proc Natl Acad Sci USA. 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. Proc R Soc B. 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 Ethol. 18 (3), 255–263 (2015).*

d) A description of the context of the technique in the wider body of literature

Response:
We restructured the introduction and now first start with a short introduction on the use of computer animations in research and typical approaches used to date.

New lines 84-108: "*Recently, ~~the use of~~utilizing modern techniques for the creation of artificial stimuli, such as computer -animations and virtual reality, has garnered ~~gained more and more~~ popularity in research[1~~4~~2].~~, since t~~These methods provide several advantages compared to classic experimental approaches with live stimulus animals[1~~4~~2, 2~~4~~3]. 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 more realistic and complex physical movement[6, 7, 10-12]. Even virtual reality designs that simulate a 3D environment where live test 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."

We included the following references to our reference list (new lines 1337-1347):

8. *Balzarini, V., Taborsky, M., Villa, F., Frommen, .JG. Computer animations of colour markings reveal the function of visual threat signals in Neolamprologus pulcher. Curr Zool. 63 (1),45–54 (2017).*
9. *Tedore, C., Johnsen, S. Visual mutual assessment of size in male Lyssomanes viridis jumping spider contests. Behav Ecol. 26 (2), 510–518 (2015).*
10. *Watanabe, S., Troje, N.F. Towards a "virtual pigeon": a new technique for investigating avian social perception. Anim Cogn. 9 (4),: 271–279 (2006).*
11. *Culumber, Z.W., Rosenthal, G.G., 2013. Mating preferences do not maintain the tailspot polymorphism in the platyfish Xiphophorus variatus. Behav Ecol. 24 (6), 1286–1291 (2013).*

And to new lines 1351-1353):

13. *Thurley, K., Ayaz, A. Virtual reality systems for rodents. Curr Zool. 63 (1),: 109–119 (2017).*
14. *Stowers, J. R. et al. Virtual reality for freely moving animals. Nature methods., 14 (10), 995 (2017).*

e) Information to help readers to determine whether the method is appropriate for their application

Response:
Since the general method of computer animation is very complex compared to the space given in the introduction (max 1500 words), we only give a brief description of the advantages of the method at the beginning of the introduction in new lines 87-108 (as already mentioned above). We specifically refer to two important recent studies that summarize all that is to know about the various techniques to create computer animations, advantages/disadvantages, pitfalls and things to consider when designing the experiment.

New lines 103-108: "*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.*"

We further give more information concerning our own software which hopefully helps the reader to determine whether it is appropriate:

New lines 116-118: "*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.*"

New lines 122-133: "*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 our 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).*"

6. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "FishSim" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Response:
We revised our manuscript and reduced the number of instances of "*FishSim*" wherever possible but kept it where we thought it was relevant. Instead, we referred to "*FishSim*" as "toolchain", "software", "tool", or "computer animation". We hope that our changes now meet JoVE policies.

7. Please revise the protocol so that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please revise 4.2, 4.5, 5.4, 6, 6.1-6.6 accordingly.

Response:
As requested, we revised steps 4.2, 4.5, 6, 6.1-6.6 as well as the whole protocol according to the editorial suggestions. All text is now written in the imperative tense. Whenever actions or functions needed further clarification we included additional "Notes".

8. 4.3: Please break up into sub-steps.

Response:
We split the procedure described in 4.3 into the new sub-steps 3.3.1, 3.3.2 and 3.3.3 (new lines 582-605; old lines 419-432).

"~~*4.3.*~~*3.3.* ***Specific playlist arrangement for the "spot" treatment***

~~*In each treatment and the control, playlists have to be arranged in two different orders.*~~ *For the first* *mate-choice*~~*preference*~~ *test in* *T*~~*t*~~*reatment 1, order the playlist* ~~*in FishPlayer*~~ *exactly as depicted in Fig. 4*~~*5*~~*.*

3.3.1. After the first mate-choice test and a break for calculating which virtual male was preferred (see step 5.9~~*11*~~ *below), 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. Arrange the playlist for observation and the following second mate-choice*~~*preference*~~ *test according to Fig. 5*~~*6*~~*.*

3.3.2. For the observation period, link the 10-minutes 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.*

3.3.3. For the ~~*other*~~ *playlist featuring the non-preferred male, link the 10-minutes record to the scene including the prior non-preferred male together with the model female.* ~~*The identical record will then be replayed but now the model female is visible.*~~ *Choose the scenes including a model female with a gravid spot (S) for this treatment.*

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

9. Please ensure that the highlighted steps (2.75 page limit for filmable content) identify the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
10. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
11. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response:
We revised the steps highlighted for filming. For better visibility, we deleted the old highlighting and only included the new and revised version (yellow markings). Since our protocol is very long, the highlighted steps now only concentrate on what we think are the most important steps to be visualized. We think it is important to visualize some aspects of the workflow with our software (steps 1.2., 1.3. and 2.2.) and, specifically, the complete running procedure of the MCC experiment (step 5). We highlighted all sub-steps we think are required to perform a given action.

12. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.

Response:
We thoroughly re-structured the representative results section (new lines 848-1016). Following the order given in the protocol, we briefly state how we used the method. We give advice on how to analyze the data obtained during experiments and illustrate this by presenting our own data analysis as an example. For this, we integrated most of the information that was earlier given in step 6 of the protocol (old lines 525-584) into the results section.

13. Discussion: Please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Response:
We revised the Discussion accordingly and added information on critical steps within the protocol and possible modifications and pitfalls (new lines 1184-1252):
"*Following the protocol, we identify several critical steps that need specific attention to ensure the correct handling of our toolchain and, further, 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 our 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 of 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 livebearing fishes in general. Apart from livebearing fishes, 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 with the functions of the Playstation 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 (fins etc.) 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 afterwards.

When running the experimental procedure (step 5) the timing is crucial for the success of the experiment. The times/durations we refered 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.).

We included additional references to the reference list in new lines 1522-1528:

78. *Hurlbert, S.H. Pseudoreplication and the design of ecological field experiments. Ecol Monogr.* ***54*** *(2), 187–211 (1984).*
79. *McGregor, P.K. Playback experiments: design and analysis. Acta Ethol.* ***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). Rev Biol Trop.* ***21*** *(2), 399–416 (1974).*

14. References: Please do not abbreviate journal titles.

Response:
We here followed the JoVE author's guidelines (revised version from February 2017) provided by the editor and as discussed via email, we kept the abbreviations of journal titles in our revised manuscript.

15. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Response:
We removed all trademark an registered symbols from the Table of Equipment and Materials and uploaded a new Excel file accompanying our revised manuscript.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript "In the "spot"light: do gravid spots predict model female quality in mate-choice copying? A study with FishSim Toolchain in sailfin mollies" provides a very nice description of a novel method to use animated fish stimuli in behavioural research. I have no doubt that the described detailed methods will help scientist to use this method in their future research.

Response:
We thank Reviewer 1 for his/her detailed comments on the previous version of our manuscript and we hope that we were able to address all concerns raised satisfactory.

Major Concerns:
Reviewer 1:
1) In LL 471-477 the authors explain in detail the protocol how to measure the association time after the cylinder is lifted. I think this part is difficult to reproduce because of the inaccurate information provided. How is the female released from the cylinder exactly?
If the cylinder is lifted outside of the water it will obviously produce some disturbance. Do the authors account for another acclimatisation time?
When exactly does the recording start?

Response:
Plexiglas cylinders or cubes to frequently fixate and release focal fish during experiments are widely used for these kinds of experiments in fish. Therefore, we are confident that scientists interested in our method are familiar with this approach. If not, it will of course be necessary to train the correct handling of the cylinder beforehand which, however, we think should go without saying in good experimental practice. Here it is critical to act slowly and gently to not disturb the fish. How easily a fish may get disturbed by this kind of procedure may depend greatly on the tested species. In our lab, these kinds of cylinders have been employed in various different experiments and, at least in our fish, we do not usually find critical changes in behavior. In the seldom case that fish are indeed severely disturbed, the experiment is terminated and the trial is excluded from analysis. General water disturbance, while lifting the

cylinder, can be lowered by drilling small holes into the lower part of the cylinder, which we did for our experiment. We included this information in the supplementary materials list.

When no automatic tracking system is used during experiments, which was the case in our study, it is recommended to already have a stopwatch in one hand while lifting the cylinder with the other hand. By this, it is possible to start measuring association time directly after the focal female is released from the cylinder. Again, this procedure has to be trained beforehand. Information on when recording should start, i.e. when to start measuring association time, is given in step 6.1 of the protocol (new lines 762-764, old lines 536-538):
*"**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."*

In general, the time needed for fish handling throughout the procedure needs to be determined prior to experiments during several test runs. Handling time mostly affects the duration of the "pause" which is set in *FishPlayer*. In our protocol, we suggest a duration of 1:30 minutes for the pause. This duration, however, depends on the specific test situation in each lab and may therefore differ from our protocol. It is possible to individually change the duration in our tool.

We added information on this issue in the protocol and now also refer to this point in the discussion:
New lines 671-679: "*5.4. Before the timer ~~in FishPlayer~~ reaches 02:30 minutes, 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 minutes.*

***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 prevent the fish from getting stressed. 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).*"

New lines 1232-1245: "*When running the experimental procedure (step 5) the timing is crucial for the success of the experiment. The times/durations we refer to in the protocol (step 5) derive from previous studies with sailfin mollies. They should be regarded as suggestions and are not obligate 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 pause duration by training of the experimental procedure during test runs. The pause should be at least as long as the time needed for catching the fish with the cylinder, as well as the timing when as walking back and forth 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.).*"

Reviewer 1:
The times given (e.g. when to lift the cylinder) are approximations but will obviously influence the preference time. How do the authors control for the obvious variation between trials depending on how fast the experimenter returns to the table and starts recording?
I think this might be crucial as maybe the first minute is the most important period for choosing a mate.

Response:
Here, please also see our response to your previous question. When the experimental procedure is properly trained during prior test runs, which is standard procedure in our lab, the variation depending on how fast the experimenter releases the fish and returns to the table is actually very low. In case that something goes wrong and the timing cannot be met due to whatever reason, it is at the discretion of the experimenter to exclude the trial from analysis.
As we stated in your comment above, we now discuss the importance of timing in the discussion in new lines 1232-1245.

Reviewer 1:
Alternatively, the experiment could be started when the test fish enters a middle zone without the need from the observer to approach the experimental tank again. This would reduce any further disturbances.

Response:
As stated above, the use of Plexiglas cylinders or cubes to fixate and release focal fish during experiments is common in MCC experiments. The advantage of using a cylinder (or a similar device) is that the focal female may be fixated at a specific position in the test tank (here, the middle). By this, we ensure that the focal female maintains an equal distance to each presented stimulus for the whole time she is given to watch both stimuli. By restricting her to, first, just watch the stimuli we try to ensure that she is aware of both stimuli and not just only one. Particularly during the observation period, which is the crucial step of the MCC experiment, it is obligatory that the focal female is able to watch the simulated mate choice of a virtual model female for the prior non-preferred virtual male without being able to engage with either stimulus. During the observation period, we want to create a situation which favors the occurrence of MCC in focal females. This situation of the focal female observing a conspecific model female and her mate choice for a male reflects the definition of MCC per se. However, the use of cylinders to fixate individuals in a specific position might not be applicable for every fish species. With our study species, the sailfin molly, as well as with other Poeciliids kept in our lab, this procedure works very well.

Reviewer 1:
2) I was also wondering why the authors don't use any video recordings to analyse the choice of the test fish. This would improve the manuscript two-fold: First, it would reduce the variation in terms of how long it takes the observer to return to the table and the observation could be started at the same time. Second, it would also make the observer blind with respect to the treatments when analysing the videos. I think this aspect (blind analysis) should be considered more in the protocol.

Response:
We understand the concerns of Reviewer 1 concerning blind analysis but with respect to the experimental procedure described in our study, we have to state that blind observation and blind analysis are of no option. The individual choice of each focal female for a certain male stimulus cannot be known prior to the experiment but is only revealed after the first

preference test. Only then, when we know which male is preferred by the focal female, are we able to assign the position of the model female during the observation period. In our study, the virtual model female was always presented together with the prior non-preferred virtual male. After the first preference test, the experimenter needs to rearrange the playlist in *FishPlayer* according to the individual preference of each focal female. For this reason, the experimenter is not allowed to be blind with respect to the treatments and blind analysis is unfortunately not possible. However, we agree that the experimental procedure may be improved by the use of a tracking system to automatically measure association time in real-time. In this case the experimenter only needs to handle the fish and not the stopwatches and measured times might be more precise.

We included this information in our discussion on crucial steps within the protocol in new lines 1246-1252: "*The experimenter can improve the measuring of association time for each preference 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.)."*

Reviewer 1:
Along the same lines, In LL 789-791 the authors mention tracking software and I was wondering whether this could be incorporated in the FishSim software. It would improve the experimental protocol if automatic tracking could be used to analyse the time fish spent on each side. This would also allow to increase the time periods or enable to divide it into shorter periods.

Response:
Yes indeed, as we state in new lines 1286-1288 (old lines 791-795) it is possible to incorporate tracking software in *FishSim*. References 87-89 (old references 68 and 69) give information on the technical background of the tracking system we use. We further added a new reference of a recently accepted paper (new lines 1551-1552).

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).

Tracking systems as such could also be used to measure association time in real-time automatically to enhance experimental procedure. We revised this paragraph in the manuscript in new lines 1284-1295 (old lines 791-795):
"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[87-89]. 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. ~~which could already be successfully demonstrated in the science communication event "Molly knows best" ().~~ 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."

Timing and duration of created animation sequences are in general totally up to the experimenter and may be varied accordingly. We provide information on that in the discussion in new lines 1242-1245:
"*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.).*"

Minor Concerns:
Reviewer 1:
1) The protocol starts with the experimental setup and then continues to describe the production of the virtual stimuli. From a logical point of view, I would switch the order and describe first the production of the stimuli and then the experimental set-up.

Response:
We agree with Reviewer 1 and put the description of the experimental setup as new step 4 in front of step 5 "Running the MCC experiment" in new lines 628-642. We changed the order and numbering of figures in the text accordingly.

Reviewer 1:
2) L.153: The authors describe to use tap water. Is it really water that came straight from the tap or from another tank without any fish inside but already filtered etc?

Response:
We agree that this description may be too vague. Actually, we use fresh water from a reverse-osmosis system which is re-mineralized after filtering according to the specific needs of livebearing fishes. Here, we tried to give only general advice to leave room for scientists using other conditions in their labs or even other fish species.
We describe the setup prior to running the experiment and during experiments there should not be any additional devices present in the experimental tank (e.g. filter, heating, aerating stone) so the focal fish can completely focus on the presented stimuli and the view of both monitors is at no point obscured. Further, the test tank is not intended to serve as a permanent holding tank. Nevertheless, between experiments, it would be possible to include devices for filtering or aerating the water if it is not replaced in total for each trial.

We revised the manuscript in new lines 634-635 (old lines 153-154):
"4.2. Cover the tank bottom ~~of the tank~~ with a thin layer of gravel and fill it with water appropriate for live fish to the height of the LCD screens."

Reviewer 1:
3) L 168: Maybe the authors should explain a bit more what GIMP is and provide a link to the programme also in the main manuscript.

Response:
We agree with Reviewer 1 and added a note in the protocol stating the function of GIMP and providing a link to the website for download of the software.

New lines 279-280: "***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.*"

Reviewer 1:
4) LL. 334-336: Here the authors describe that the swimming path of the stimuli can be altered using a PlayStation controller. Although I really like the idea, would it also be an option to include a random swimming path determined by a random computer-generated process. I think this might reduce any bias which might exist when manually determining the swimming path.

Response:
Yes, this is in general possible with *FishSim* but requires sophisticated programming skills. Our intention was to create a toolchain which can more or less readily be used by scientists who do not have too much computer knowledge. However, the modularity of our toolchain as well as its open-source nature makes it possible that scientists, who indeed have this knowledge, may adapt and develop our toolchain to their own needs. In new lines 1286-1291 we describe that it is even possible to integrate a real-time tracking system which can be used to make the virtual stimuli "behaving" interactively. Meaning the tracking data of the live focal fish may actually be used to "command" the virtual fish to follow it along the monitor screen. Such swimming paths are then completely independent from any experimenter.

Reviewer #2:
This study not only presents a step-by-step guide to how to use computer animation to study mate-choice copying in fish (FishSim) but also interesting results from a study using this system in mollies as a case study. Their result is quite clear, and probably this result itself is interesting and worthy of publication although this is not the main focus of this paper. The paper is very well written and detailed. One main thing I would request from the author is that they should make their experimental data and R code available for reproducibility. This can be done by via the Open Science Framework (OSF; http://osf.io/) or Figshare (https://figshare.com/).

Response:
We thank Reviewer 2 for his/her comments on our manuscript. As requested, we uploaded our R-script (R-Code_FishSim_Analysis.R) as well as four tables including raw data ready to be processed in R (MCC_copy.txt, MCC_motivation.txt, MCC_pref.txt, MCC_SL.txt) into a repository at https://figshare.com. We included this information in our manuscript in new lines 861-863: "*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).*"

We set the files to be confidential for now (until publication of the manuscript) but it can be accessed via the following personal link: https://figshare.com/s/cdb0cd2fe73ff8a5a2d0

Reviewer 2:
Also, the authors probably want to present the variance component from mixed models. This paper tells you an important of presenting variance components, which relates to the repeatability of response for individual fish (probably such variance is interesting itself). Schielzeth, H & Nakagawa, S (2013) Nested by design: model fitting and interpretation in a mixed model era. Methods in Ecology and Evolution. 4: 14-24

Response:
Unfortunately, we are not quite sure what Reviewer 2 is expecting us to do. Regarding the suggested paper and the repeated measures design of our LME model, with individual focal female as random factor, we assume that we were asked to present random-effect variances.

We included an additional table (new Table 2) presenting the random effect and residual variance and standard deviation for focal female ID. We did the same for the random effect "spot area" in the analysis of copying scores (new Table 4).

New lines 1125-1126: *"**Table 2. LME variance components for focal female ID.** Variance and standard deviation for the random effect "ID" and the residuals are given."*

New lines 1137-1138: *"**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 now give additional information on lower and upper confidence intervals of model parameter estimates in Tables 1 and 2:

New lines 1116-1117 and 1130-1131: "*Given are estimates ± standard error and lower/upper confidence intervals, degrees of freedom, t-values and p-values for each fixed factor*."

Related to Reviewer 2 mentioning "*the repeatability of response for individual fish*" we would like to clarify that the repeated measures design found in our model reflects the fact that for each focal female, two values are used in the model, one for P1 and one for P2. But we used unique focal females in each treatment and the control, e.g. a focal female tested in Treatment 1 was not used in Treatment 2 and/or the control. We did this to prevent females from being "more experienced" in later subsequent treatments.
We revised the manuscript accordingly in new line 876: "*All focal females were mature adults and were only tested once*."

Reviewer #3:
There are many areas where the writing needs to be streamlined (edited for conciseness). I will highlight some below in detailed comments, but my list is not exhaustive. One general note is over-use of the passive vs. active voice. I know the journal states that they have in-house copy-editing, but I provide comments here that may be of use to the authors in general for honing their writing skills. Related to point 1, there are places where the word choice makes statements that are too broad, and the manuscript should be edited for clarity. Again, I will highlight some examples in the detailed comments.

Response:
We thank Reviewer 3 for his/her detailed comments on our manuscript. We thoroughly revised the whole manuscript and hope that we succeeded in making the text more clear and concise. We further tried to minimize the use of passive over active voice.

Reviewer 3:
In the Introduction, specifically lines 63, and again 67 the authors refer to "genetic" or "non-genetic" mate choice strategies as though these are dichotomous to "not learned" or "learned". This is not the case, however. Behaviors are the result of expression of genotypes within the organism's environment (including the social environment that is relevant to this paper) and to what degree there is heritable variation in a behavior cannot be inferred from how much the behavior is influenced by social interactions.

Response:
We agree with Reviewer 3 that behavior is the result of expression of genotypes within the organism's environment. By "genetic" and "non-genetic" we more referred to mate choice

being independent (personal information), which may indeed be based on a genetically determined preference for a certain phenotypic trait, or non-independent (based on public/social information). We revised our manuscript accordingly.

New lines 137-148 (old lines 60-68): "*Mate choice~~Choosing a mate~~ is one of the most important decisions animals make in their life history.~~have to make.~~ Animals have evolved different strategies for finding~~choosing a mate and to find~~ the best mating partner. They may~~can~~ rely on personal information ~~and evaluate different~~when evaluating potential mating partners ~~by themselves~~ independently, possibly according to predetermined~~defined~~ genetic preferences for a certain phenotypic trait*[19, 20]*. However,~~But~~ they may~~can~~ also observe the mate choice of conspecifics ~~in their mate choice~~ and thereby utilize~~rely on~~ public ~~or social~~ information*[2,13]*. If the observer~~ing animal~~ then decides to choose the same mate (or the same phenotype) as the observed conspecific – the ~~so-called~~ "model" – chose previously, ~~we speak of~~this is termed mate-choice copying (hereafter abbreviated as MCC)*[2,22,4, 23,5]*. Mate-choice copying~~CC~~ is a form of social learning and, hence, a non-independent~~genetic~~ mate-~~-~~choice strategy*[2,46]*, which has been observed~~was found to occur across the whole animal kingdom~~ in both vertebrates*[2,57-29,11]* and invertebrates*[30,12-32,14]*."*

Reviewer 3:
In the Introduction I do not think the link between why a model female with a gravid spot/patch vs without is better to copy or not. This could be remedied by changing the end paragraph to have a hypothesis/prediction framework linked into the existing description of the approach.

Response:
We revised the introduction accordingly and tried to clarify the hypotheses tested in this study. New lines 188-198:
"*Considering the link between the gravid spot and a female's reproductive status, we ~~wanted to know whether~~predicted that the gravid spot serves as a ~~quality~~ sign ~~for good~~ of model female~~s~~ quality by providing information on her current reproductive state to observing focal females. ~~and whether focal females copy their mate choice or not~~. 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 spot). Here, focal females are more likely to copy the choice of a model with spot but not that of a model without 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.*"

Reviewer 3:
From the standpoint of the novelty / improvement of this application vs. others that have been used in previous work: an improvement would be to have an input for the observer to have the ability to record the times using the computer, so that all calculations of time variables would then be easily done by programming.

Resposne:
Yes, we agree with Reviewer 3 that this ability would be a great improvement to our software and the general experimental procedure. It is definitely possible to do this in *FishSim* and we actually already started developing this feature. We demonstrated the successful implementation of a tracking system during a science communication event and we have already published articles on the development of our tracking system (new references no. 87-

89). Unfortunately, this feature is currently not available in the software version that we offer for download but it is subject to future development. We give this information in the manuscript in new lines 1284-1295:
"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[87-8969]. 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. ~~which could already be successfully demonstrated in the science communication event "Molly knows best" ().~~ 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."

Detailed comments:

Reveiwer 3:
-Throughout: Does the style guide for the journal require the use of "mate-choice" rather than the more commonly used "mate choice" (no hyphen)?

Response:
Whenever we speak of "mate choice" we use no hyphen. If we speak of "mate-choice test", "mate-choice copying", "mate-choice strategy", "mate-choice reversal" and so on, we use a hyphen.

Reveiwer 3:
-Line 65: omit "the so-called" and just make "model" parenthetical

Response:
As suggested, we changed the sentence into: "[…] *as the observed conspecific – the ~~so-called~~ "model" – chose previously* […]" (new lines 144-145; old lines 65-66).

Reveiwer 3:
-Line 67: spell out abbreviations if they begin a sentence.

Response:
As suggested, we spelled out abbreviations beginning a sentence:

New line 146 (old line 67): "*Mate-choice copying~~CC~~ is a form of social learning and* […]"

New line 151-152 (old line 71): "*Mate-choice copying~~CC~~ is especially valuable* […]"

Reviewer 3:
-Line 69: How "widespread" is this in fish? Found in all families of fishes? Or some percentage of species? Be specific when using adjectives like that term.
-Line 69: Change "can be" to "is"

Response:
We agree that this term is misleading. We wanted to refer to the fact that most studies concentrated on fish, so far. We revised the sentence accordingly…

New lines 149-151 (old lines 69-70): "*~~Specifically in fish, MCC seems to be a widespread strategy in mate choice that~~So far, MCC was predominantly studied in fish and is~~can be~~ found both under laboratory condition[33-38] and in the wild[39-42].*"

Reviewer 3:
-Line 70: Omit sentence beginning with "Several different aspects..."

Response:
We deleted this sentence from our revised manuscript.

Reviewer 3:
-Line 70: What is a "correct" decision?

Response:
When we speak of "good/correct" or "bad/false" choices we mean with regard to potential fitness consequences for the individual (good = increased fitness; bad = reduced fitness) When we speak of good or bad model females, we mean this with regard to the reliability of that model for making a "good" choice, regarding fitness consequences for the copier ("good" model = more reliable to make a "good" choice; "bad" model = less reliable to make a "good" choice).

New lines 151-154 (old lines 71-72): "*Mate-choice copying~~CC~~ is especially valuable for an individual~~observer~~ if two or more potential mating partners are apparently~~very~~ similar in quality and a "good~~correct~~" mate choice~~decision~~ - in terms of maximizing fitness - is~~, therefore, quite~~ difficult to make[43~~25~~]".*

New lines 155-159: "*The quality of a model female herself can affect whether focal females copy her choice or not[44~~26~~-47~~28~~]. 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[44-46], or by her being a conspecific or a heterospecific[47]."*

Reviewer 3:
-Line 72-73: The sentence that starts with "Prior studies..." Is vague. Clarify or omit.

Response:
We deleted this sentence from our revised manuscript.

Reviewer 3:
-Line 74-76: edit for clarity and conciseness.

Response:
We revised the sentence accordingly.
New lines 159-161 (old lines74-76): "*In sailfin mollies, who ~~were previously shown to~~ copy the mate choice of conspecifics~~in several studies~~[39~~21~~, 48~~29~~-51~~32~~], it was found that focal females even copy the rejection of a male[52~~33~~]~~, if this male was prior rejected by an observed model female~~."*

Reviewer 3:
-Line 82: By ending the first introductory paragraph with a focus on sailfin mollies, the authors restrict the scope of the importance of the work to a single species.

Response:
Yes, we agree with Reviewer 3. Since we were asked to focus more on the method described in our manuscript (see editorial comments), we restructured the introduction accordingly. We now first give an overview of the method in general (new lines 84-108), followed by an introduction to our software and the rationale and goal behind our approach (new lines 110-133). We then state, that we use our study as an example on how to use our software (new lines 133-135): "*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."*

Afterwards, we provide some background on MCC, what is already known about MCC in sailfin mollies and what we want to test with the help of our software.
Indeed the use of our software is not restricted to sailfin mollies. Therefore, we hope that this new structure will make this fact more clear to the reader.

Additionally, we changed the title accordingly (new lines 3-4): "*Using FishSim Animation Toolchain to investigate fish behavior: A case study on mate-choice copying in sailfin mollies*"

Reviewer 3:
-Line 84": Omit the term "so-called"
-Line 85: Change "also found as" to "also known as"

Response:
As suggested, we changed the sentence in new line 171-172 (old line 85-86): "*A distinct visual feature in female sailfin mollies, and other Poeciliids, is the so-called gravid spot (also known found as 'anal spot', 'brood patch' or 'pregnancy spot').*"

Reviewer 3:
-Line 85-87: this is a sentence fragment. In addition, this seems to be generalizable across poeciliids, so omit the restriction to just Gambusia holbrooki.

Response:
We revised the sentence accordingly (new lines 172-174, old lines 85-87): "*This A darkly pigmented area in their anal region which, in the mosquito fish Gambusia holbrooki, derives from melanization of the tissue lining the ovarian sac*[55 36]*."*

Reviewer 3:
-Paragraph Lines 84-97: This can be shortened to just a sentence or two summarizing what is known about the gravid spot, and then combined with next paragraph.

Response:
As suggested by Reviewer 3, we shortened this paragraph and combined it with the following paragraph (new lines 171-188, old lines 84-97):
"A distinct visual feature in female sailfin mollies, and other Poeciliids, is the so-called gravid spot (also known found as 'anal spot', 'brood patch' or 'pregnancy spot'). This A darkly pigmented area in their anal region which, in the mosquito fish Gambusia holbrooki, derives from melanization of the tissue lining the ovarian sac[56 36]*. Size of the gravid spot can be highly variable across conspecific females or completely absent. It may further change in its extent with progression of the ovarian cycle The size and presence of the gravid spot are variable across conspecific females, and may further individually change during the*

progression of ovarian cycles[5636]*. In guppies, the gravid spot was found to be largest prior to parturition*[37]*. Gravid spots may serve to attract males and facilitate gonopodial orientation for internal inseminatio*[58] *or as a means of advertising fertility*[59, 60]*.In an early study by Peden38 using dummy fish, he could show that anal spots served to attract males and facilitated gonopodial orientation for internal insemination in Gambusia. In sailfin mollies, the gravid spot was discussed to serve as a means of fertility advertisement39, 40. Farr and Travis39 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. In a study by Sumner et al.40, the presence of a visible gravid spot was discussed as mainly a signal for a non receptive status in females. However, 33 % of the studied receptive females had a spot."*

Reviewer 3:
-Line 100: What is meant by "good"?

Response:
When we speak of "good/correct" or "bad/false" choices we mean with regard to potential fitness consequences for the individual (good = increased fitness; bad = reduced fitness) When we speak of good or bad model females, we mean this with regard to the reliability of that model for making a "good" choice, regarding fitness consequences for the copier ("good" model = more reliable to make a "good" choice; "bad" model = less reliable to make a "good" choice). We made several changes to the manuscript to clarify this:

New lines 151-154 (old lines 71-72): "*Mate-choice copying~~CC~~ is especially valuable for an individual~~observer~~ if two or more potential mating partners are apparently~~very~~ similar in quality and a "good~~correct~~" choice~~decision~~ - in terms of maximizing fitness - is~~, therefore, quite~~ difficult to make*[25]*".*

New lines 157-159: "*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*[44-46]*, or by her being a conspecific or a heterospecific*[47]*."*

Here, and in the reference list, we included a new reference to an earlier study on MCC (new lines 1425-1426):
47. *Hill, S.E., Ryan, M.J. The role of model female quality in the mate choice copying behaviour of sailfin mollies. Biol Lett.* ***2*** *(2), 203–205 (2006).*

New lines 163-164 (old line 78): "[…] *the consequences of copying a "false" choice may be tremendous in reducing the fitness of the copier*[55]*."*

Here, and in the reference list, we included a new reference (new lines 1442-1443):
55. *Nöbel, S., Danchin, E., Isabel, G. Mate-copying for a costly variant in Drosophila melanogaster females. Behav Ecol. ary095. In press (2018).*

New lines 165-167 (old lines 79-81): "[…] *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."*

In new lines 188-198 (old lines 100 and following), we revised the whole paragraph to clarify what a "good" model means related to the underlying hypotheses of our study:

"*Considering the link between the gravid spot and a female's reproductive status, we ~~wanted to know whether~~predicted that the gravid spot serves as a ~~quality~~ sign ~~for good~~ of model female~~s~~ quality by providing information on her current reproductive state to observing focal females. ~~and whether focal females copy their mate choice or not~~. 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 spot). Here, focal females are more likely to copy the choice of a model with spot but not that of a model without 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.*"

Reviewer 3:
-Paragraph Lines 99-106: could likely be omitted, or just make the statement about using non-invasive techniques in combination with the next paragraph on using computer animation.

Response:
We revised this paragraph and deleted the reference to the study by Benson (2007), since we also refer to it in the discussion (new lines 1176-1178, old lines 757-759) where it seems more appropriate.

Reviewer 3:
-Line 109: Add "has" after "reality" and delete "and more"

Response:
We changed the sentence as follows:
New lines 84-86 (old lines 109-110): "*Recently, ~~the use of~~utilizing modern techniques for the creation of artificial stimuli, such as computer-animations and virtual reality has garnered ~~gained more and more~~ popularity in research[1].*"

Reviewer 3:
-Line 111: For example?.... maybe here is where you can say it is non-invasive

Response:
As already stated above, we restructured the introduction and included additional information about the use of computer animations in research.

New lines 86-95: "*~~since t~~These methods provide several advantages compared to classic experimental approaches with live stimulus animals[1, 2]. Computer animation enables thorough 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].*"

Reviewer 3:
-Line 120: Add "of" after "instead"

Response:
As suggested, we changed the sentence as follows:
New lines 217-218 (old lines 120-121): "*We used FishSim to perform a MCC experiment by presenting virtual stimulus males and virtual model females~~ish~~ on computer monitors instead of using live stimulus and model fish* […]"

Reviewer 3:
-Line 121: Add "testing hypotheses about" after "for"

Response:
As suggested, we changed the sentence as follows:
New lines 219-221 (old lines 122-124): "*The general usability of our software~~FishSim~~ has previously been validated for testing hypotheses about ~~the use in~~ mate choice ~~experiments with~~ in sailfin mollies*[12]."

Reviewer 3:
-Protocol section: I am assuming the style of this journal is such that the traditional "methods" section is instead just written like a list / protocol that could be followed? That said, I did not edit this section.

Response:
Yes, Reviewer 3 is correct. The style of the journal requires the method to be written in form of a protocol or step-by-step description of how to perform the experiment.

Reviewer 3:
I did have a really hard time determining if the "Representative Results" section was based on a repeated measures design, and if so, how the treatment order was handled by the statistical model. This could be fixed by moving the material from lines 633-639 earlier.

Response:
As stated in the editorial comments above, we thoroughly re-structured the representative results section (new lines 848-1016). Following the order given in the protocol, we briefly state how we used our method. We give advice on how to analyze the data obtained during experiments and illustrate this by presenting our own data analysis as an example. For this, we integrated most of the information that was earlier given in protocol step 6 into the results section.

We would like to clarify that the repeated measures design found in our model reflects the fact that for each focal female, two values are used in the model, one for P1 and one for P2. But we used unique focal females in each treatment and the control, e.g. a focal female tested in Treatment 1 was not used in Treatment 2 and/or the control. We did this to prevent females from being "more experienced" in later subsequent treatments.
We revised the manuscript accordingly in new line 876: "*All focal females were mature adults and were only tested once*."

Additionally, per request of Reviewer 2, we uploaded our R-script (R-Code_FishSim_Analysis.R) as well as four tables including raw data ready to be processed in R (MCC_copy.txt, MCC_motivation.txt, MCC_pref.txt, MCC_SL.txt) into a repository at https://figshare.com. We included this information in our manuscript in new lines 861-863. We set the files to be confidential for now (until publication of the manuscript) but it can be accessed via the following personal link: https://figshare.com/s/cdb0cd2fe73ff8a5a2d0.

Reviewer 3:
-Line 750-753: Re-word for conciseness (use active not passive voice).

Response:
We revised our text as follows (new lines 1164-1166; old lines 750-753): "*Even though sailfin molly females are ~~reported to be~~ most receptive short after parturition[59~~39~~, 76~~61~~], they are ~~known to be~~ able to store sperm for several months[57~~37~~] ~~for up to nine months (Gierszewski, personal observation)~~.*"

Reviewer 3:
-Line 752-753: this is well known in literature, so there is no need for a personal observation reference.

Response:
We revised the sentence accordingly (new line 1165-1166, old lines 752-753): "[…] *they are known to be able to store sperm for several months[57~~37~~] ~~for up to nine months (Gierszewski, personal observation)~~."*

Reviewer 3:
-Line 753: You cannot make this conclusion based on non-significant results.

Response:
We are not completely sure to which conclusion and non-significant result Reviewer 3 is referring to in his/her comment. The conclusions we draw from our results are generally in relation to our case study on MCC in sailfin mollies and the hypotheses we investigated. We revised the first two paragraphs of the discussion to clarify our argumentation (new lines 1141-1172, old lines 736-753):
"The gravid spot in sailfin molly females was previously discussed 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 has never been tested so far. In our~~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. ~~We could~~ Our study shows that the gravid spot seems ~~not~~ 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 ~~in a MCC experiment.~~ 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.. ~~There was further~~ We found no difference in ~~the change of preference (~~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. ~~for a prior non-preferred virtual male showing~~ This supports that the observed change of preference within treatments can be explained by the presence of the virtual model female per se 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 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 reported to be most receptive short after

parturition[59, 76], they are ~~known to be~~ able to store sperm for several months[57~~37~~] ~~for up to nine months (Gierszewski, personal observation).~~ Therefore, females should always be choosy for the best quality mate. ~~It seems that the gravid spot is not a good indicator for model female quality~~With regard to our case study on MCC and our 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 seem to not be important in the decision to copy her choice or not, at least in sailfin mollies."

We disagree with Reviewer 3, since we draw our conclusion based on the results of our study as a whole. We found that preference scores significantly increase from the first to the second mate-choice test irrespective of whether the presented virtual model female had a spot or not (Treatments 1 and 2). This means that we found MCC in both treatments but that the presence or absence of a gravid spot did not alter the expression of MCC shown by the focal females. This is further supported by our analysis of copying scores and the number of mate-choice reversals.
We did not find this increase of preference scores in the control for choice consistency, so here we had no significant results. This non-significant result, however, is the prerequisite for us to even state that focal females showed MCC in our experiment. With the control, we want to show that focal females do not change (increase or decrease) their prior preference for a male from the first to the second mate-choice test, when we do not present a model female with either male. The rationale behind our analysis and interpretation of the data we obtained in our experiment is in line with other studies investigating MCC in fish and other animals.

Reviewer 3:
-Line 755-757: How is this new / different than other uses of animation to test fish behavior?

Response:
Originally, we wanted to refer to our own software *FishSim* as a new research tool. However, as requested by JoVE, we now describe the whole method in a more general way throughout the manuscript. We revised the sentence in new lines 1174-1176 (old lines 755-757):
"*~~With our exemplary study, we demonstrated~~Notably, our study demonstrates a ~~new and~~ highly standardized procedure for visual manipulation of public information provided in MCC experiments by using computer animated fish*."

Reviewer 3:
-Line 785: Change "e.g.", to "for example"

Response:
We changed the sentence accordingly. New line 1278 (old line 785): " […] *sensitive for polarized light for example~~e.g.~~ in questions of mate choice[64]*.

Reviewer 3:
-Line 786: Change "obligatory" to "necessary"

Response:
We changed the sentence accordingly. New lines 1279-1280 (old line 786-787): *"*[…] *the effectiveness of presented stimuli as computer animations is necessary~~obligatory~~ before testing hypotheses[2, 12, 15, 84, 85]."*