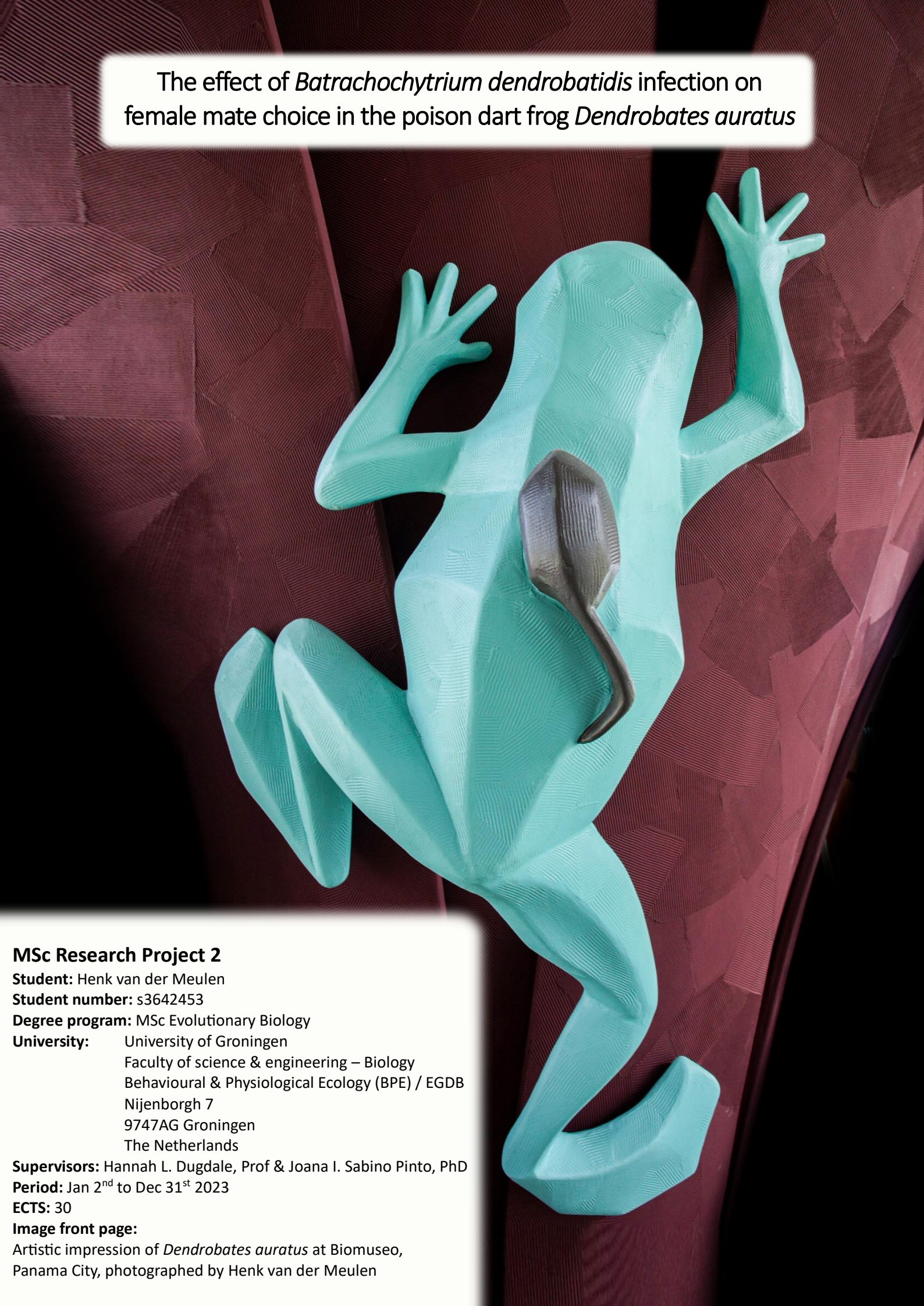
The effect of *Batrachochytrium dendrobatidis* infection on female mate choice in the poison dart frog *Dendrobates auratus*



MSc Research Project 2

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Artistic impression of *Dendrobates auratus* at Biomuseo, Panama City, photographed by Henk van der Meulen

Abstract

Batrachochytrium dendrobatidis (*Bd*) is a fungal pathogen that has currently infected over half of the studied species and is causing severe amphibian declines worldwide. As geographic boundaries preventing disease spread are disappearing due to increasing human mobility, it is crucial to better understand how species can evolve resistance against newly emerging diseases. To investigate this in the particularly urgent case of *Bd*, this study aimed to identify the effect of *Bd* infection on female mate choice, for which the poison dart frog *Dendrobates auratus* was utilized as a study system. Female mate preference was analyzed for specific males and in relation to the level of resistance to *Bd* the males possess to identify if a preference for resistance exists. To do so, 10 male and 8 female *D. auratus*, collected from Cerro Ancón in Panama City (Panama), were used in a mate choice experiment performed at the Smithsonian Tropical Research Institute, Panama. Females were exposed to pairs of males to choose from and the frogs were subjected to three different treatments: control (no infected frogs), male infection (all males infected), female infection (all females infected). 160 trials were performed for each treatment. Results show that female preference for specific males is altered by the presence of *Bd* infection, but to a minor extent. This is found in both the male and female infection treatment. This potentially provides support for the terminal investment hypothesis, and additionally suggests that males may also alter their reproductive effort based on female health. Additionally, this study finds evidence that sexual selection may function as a mechanism in the evolution of resistance against *Bd* in *D. auratus*, as females show significant preference for *Bd* resistance, but only when the males are infected and females are offered a resistant and non-resistant male to choose from. This provides evidence that selection for resistance can only occur when potential partners are infected with the disease, because only then will informative traits representing an individual's level of resistance likely become visible. The fact that this preference is not as strongly observed when all male pairs are included suggests that females are incapable of detecting minute differences in the degree of resistance, but rather identify the presence or absence of resistance. Furthermore, a general trend is observed for females to select for resistance, regardless of whether *Bd* is present, which calls for further research into the presence of selection for general pathogen resistance. Based on the outcomes of this research, there is an urgency to further study the presence of evolutionary mechanisms to acquire pathogen resistance across amphibian taxa and populations to direct conservational efforts towards the most vulnerable species.

Key words: *Bd*, amphibian, chytrid, chytridiomycosis, sexual selection, mate choice, pathogen resistance, emerging infectious diseases, Panama, Central America, terminal investment hypothesis.

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Introduction

The past decades have seen a multitude of infectious diseases emerging (Daszak et al., 2008; IOM, 2011). Global spread of pathogens is promoted by increasing human mobility, which has led to the eradication of previous geographic boundaries (Wilson, 1995). Moreover, changing environmental conditions, because of human interference, create new opportunities for pathogens to evolve (Fisher et al., 2012). Examples of such diseases are tuberculosis (Daszak et al., 2000), and more recently Covid-19 (Muralidar et al., 2020). However, newly emerging infectious diseases (EIDs) are not only seen in humans. Such diseases in wildlife are also common and increasing (Daszak et al., 2000; Fisher et al., 2012; Tompkins et al., 2015; Walker et al., 2008), mediated through human activities, such as global trade in animals and plants (Fisher & Garner, 2007; Mazzoni et al., 2003; Rush et al., 2021; Swift et al., 2007).

Potentially one of the most prominent emerging infectious diseases is chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) (Luedtke et al., 2023; Olsen & Ronnenberg, 2014; Scheele et al., 2019; Skerratt et al., 2007). *Bd* inhabits the keratinized skin cells of amphibians. When asexually reproducing in the host cells, *Bd* eventually causes apoptosis of the host cells to facilitate spread of its zoospores to other cells and hosts (Scott Fites et al., 2013). Studies suggest that *Bd* prevents a host immune response by suppressing lymphocyte activity, thereby rendering its host defenseless (Ellison et al., 2014a; Scott Fites et al., 2013). The cause of death by *Bd* infections is related to the dysregulation of skin functions. The skin surface is of particular importance to amphibians because it is strongly involved in respiration and regulation of water and electrolytes (Australian Government, 2013). Electrolyte levels are strongly decreased in infected individuals, which commonly leads to severe dehydration and death through cardiac arrest (Voyles et al., 2009).

Since 1980, the overall decline of amphibians – encompassing a total of 8,689 species – has increased to as much as 50% of the species being threatened and over 70 species being driven to extinction (Ellison et al., 2014a; Luedtke et al., 2023; Scheele et al., 2019). Therefore, amphibians are currently the most severely threatened class of vertebrates (Fisher et al., 2009). Amphibians are negatively impacted by many factors, such as habitat loss, climate change, and disease (Luedtke et al., 2023). Specifically, *Bd* is proposed to be one of the main drivers of the rapid global decline witnessed in amphibians (Walker et al., 2008). Discovered only in 1998, this pathogen has now been reported to be involved in the decline of at least 501 species of amphibians (Scheele et al., 2019). However, this number may be significantly higher as 1,062 out of 1,966 tested species (54%) were positive for *Bd* (Castro Monzon et al., 2020). The most impacted regions include Australia, and Central and South America (Berger et al., 1998; Lips et al., 2008). Tropical highlands are one of the most strongly affected areas as they house some of the greatest amphibian diversity (Ellison et al., 2014a) and provide an environment optimal for *Bd* growth (Becker & Zamudio, 2011). Contrastingly, regions such as West Africa so far seem to be much less susceptible to *Bd* outbreaks (Castro Monzon et al., 2020; Penner et al., 2013). The origin of *Bd* has been strongly debated and studies have pointed at various areas of the world (Bataille et al., 2013; Goka et al., 2009; Rodriguez et al., 2014; Talley et al., 2015; Weldon et al., 2004), but genetic analyses have recently suggested Southeast Asia as the location where Bd originated (O'Hanlon et al., 2018).

Several factors have been identified that facilitate the spread of this fungal pathogen. Through human contact, *Bd* has been able to spread intercontinentally and cross climatically uninhabitable areas (Scheele et al., 2019). In multiple instances, *Bd* has been detected on amphibians in the international trade and the fungus has been found in zoo animals, implying transportation of infected animals by humans (Fisher & Garner, 2007; Mazzoni et al., 2003; Saare et al., 2021; Wombwell et al., 2016). Moreover, animals, such as birds and crayfish transmit *Bd* to new locations as spores can survive in water and moist substrates for a long time (Johnson & Speare, 2005; McMahon et al., 2013; Nordheim et al., 2021). *Bd* zoospores can even be transmitted through fog (Prado et al., 2023), and from host to host by mosquitoes (Reinhold et al., 2023). Climate change additionally allows *Bd* to populate areas that previously did not allow for *Bd* growth (Bachtlin, 2020; Rohr et al., 2008). Evolutionarily naïve amphibian populations are more susceptible to severe forms of chytridiomycosis

than populations that have previously been in contact with the fungus (Bosch et al., 2021; Skerratt et al., 2007). Even when populations are tolerant or resistant to *Bd* in their natural environment, they may be strongly affected by different lineages of *Bd* to which they are still naïve (Becker et al., 2017). In addition, hybridization can result in increased virulence in *Bd*, which yet again leads to increased mortality in amphibians (Greenspan et al., 2018).

In an evolutionary arms race between host and parasite, organisms continuously need to adapt against each other just to maintain their current relationship. This is described by the Red Queen Hypothesis (Clay & Kover, 1996; Ladle, 1992). Such relationships also occur between fungal pathogens and their hosts (Clay & Kover, 1996). However, when pathogens are abruptly introduced into the environment, as is currently happening through human activities, host organisms do not have the necessary tools to evolve resistance against those pathogens (Daszak et al., 2000). Additionally, pathogen resistance traits are costly to maintain and often have a negative impact on other traits, such as reproductive success (Kawecki, 2020; Lazzaro & Little, 2009; Sheldon & Verhulst, 1996), which results in reduced resistance in the absence of the pathogen (Viney et al., 2005). However, when a pathogen does emerge, evolution of resistance can be mediated through various mechanisms of natural selection, such as survival of the fittest (Jiao & Fefferman, 2021) and sexual selection (Joye & Kawecki, 2019), when sufficient genetic variation is present. Several studies have found evidence for the occurrence of immunogenetic adaptation to *Bd* at the major histocompatibility complex (MHC) (Bataille et al., 2015; Ellison et al., 2014b; Rosenblum et al., 2012; Savage & Zamudio, 2016; Savage & Zamudio, 2011). These studies have focused on survival of the fittest as being the main evolutionary driver. Therefore, the need arises to increase efforts in studying sexual selection as well to acquire a more complete view of the evolution of pathogen resistance, especially in a world where fungal pathogens have the potential to be rapidly spread to new areas and quickly adapt.

To approach this question in the specific case of *Bd*, poison dart frogs are a highly suitable group of study organisms, as they exhibit very elaborate mating behavior (Summers, 1992; Wells & Bard, 1988) and are strongly affected by chytridiomycosis (Nichols et al., 2001; Pessier et al., 1999). *Dendrobates auratus* (Figure 1) is one such species of which reproductive behavior have been recorded in detail (Wells & Bard, 1988). This species is found in Central America in wet to seasonally wet forests (Eaton, 1941). It inhabits the lower levels of the forest, often found in-between leaf litter and underneath larger objects, such as fallen tree trunks. In *D. auratus*, parental care is performed by males. Because paternal investment is larger than maternal investment, females compete with each other for mates (Wells & Bard, 1988). Despite the partial reversal of sexual roles, males call to attract females and females exhibit strong mate choice (Dunn, 1941; Wells & Bard, 1988). Using *D. auratus,* this study aims to investigate how the emergence of *Bd* affects female mate choice in an evolutionarily naïve population (*i.e.*, not historically infected with *Bd*). More specifically, this study will analyze whether a relationship is observed between female mate preference and male resistance against *Bd*. It is expected that females will alter their mate preferences in the presence of *Bd* infection, as reproductive efforts of individuals could change when they are infected with a pathogen (terminal investment hypothesis: (Williams, 1966). in the absence of *Bd*, it is likely that female preference is not biased towards more resistant males, but when *Bd* is present mate choice is expected to shift towards males with a higher *Bd* resistance level, potentially increasing offspring survivability. This is based on the idea that traits representing an individual's resistance level will only become informative to a choosing individual when potential partners are infected with the pathogen (Joye & Kawecki, 2019; Kelleher et al., 2021; Roy & Kirchner, 2000).

Figure 1. Adult *Dendrobates auratus*, Cerro Áncon morph. Credit: Henk van der Meulen.

Methods

Animal collection and selection

Twenty-six wild adult *Dendrobates auratus* were collected from Cerro Ancón reserve (8.960N, -79.551W) in Panama City, Panama, between January 4th and 6th, 2023. Frogs were handled with nitril gloves at all times. While in the field, the frogs were kept in 24oz (15x23 cm) Nasco Whirl-Pak bags (Whirl-Pak®, Fort Atkinson, WI). As much air as possible was retained in the bags and the frogs were transported to the laboratory (Smithsonian Tropical Research Institute (STRI), CTPA Ancon) within 1.5 hours at most.

To determine sex, upon collection, individuals had their dorsum photographed with a Canon EOS RP full frame mirrorless camera with a Canon EF 17-40mm F4.0 L USM lens and the following measurements were taken in ImageJ (Schneider et al., 2012): snout vent length (SVL), abdomen width, width of the toe pads of toes II, III, and IV of the right front legs, and base width of the right thumb, toe I (Figure 2A). These features provide an accurate way of determining sex in *D. auratus* (Blanchette, 2017). Each measurement was taken in triplicate by two independent scorers and the mean was used in the analysis. In the end, the width of toe pad III relative to the SVL was found to most clearly distinguish between two sexes, where males have the relatively wider toe pads.

A skin swab was taken from all frogs to check whether or not they were already infected with *Bd* in the wild. This was done in duplicate, by rubbing two rayon swabs (MW113; Medical Wire & Equipment, Corsham, UK) simultaneously back and forth over the belly of the frog ten times (Figure 2B; Sabino-Pinto et al., 2017). From all 26 individuals collected, eight females and 10 males were selected at random and kept in the laboratory for the purpose of this research. In addition, one extra male and one extra female were kept in case health issues would arise in any of the specimens throughout the study, but they were never used. The remaining six individuals were released at the same location where they were initially collected.

The collection of wild *D. auratus* was approved by the "Ministerio de Ambiente, Dirección de Áreas Protegidas y Biodiversidad, Departamento de Biodiversidad, Sección de Acceso a Recursos Genéticos y Biológicos (SARGEB)". The experiments were approved by the "STRI Animal Care and Use Committee (ACUC, code: SI-22044)" following the animal experimentation guidelines of the United States of America. The frogs that were kept for the experiment were donated to the STRI Punta Culebra Nature Center after the study was finished, where they are part of a frog exposition (SI 1) to, among others, raise awareness for the amphibian chytrid fungus.

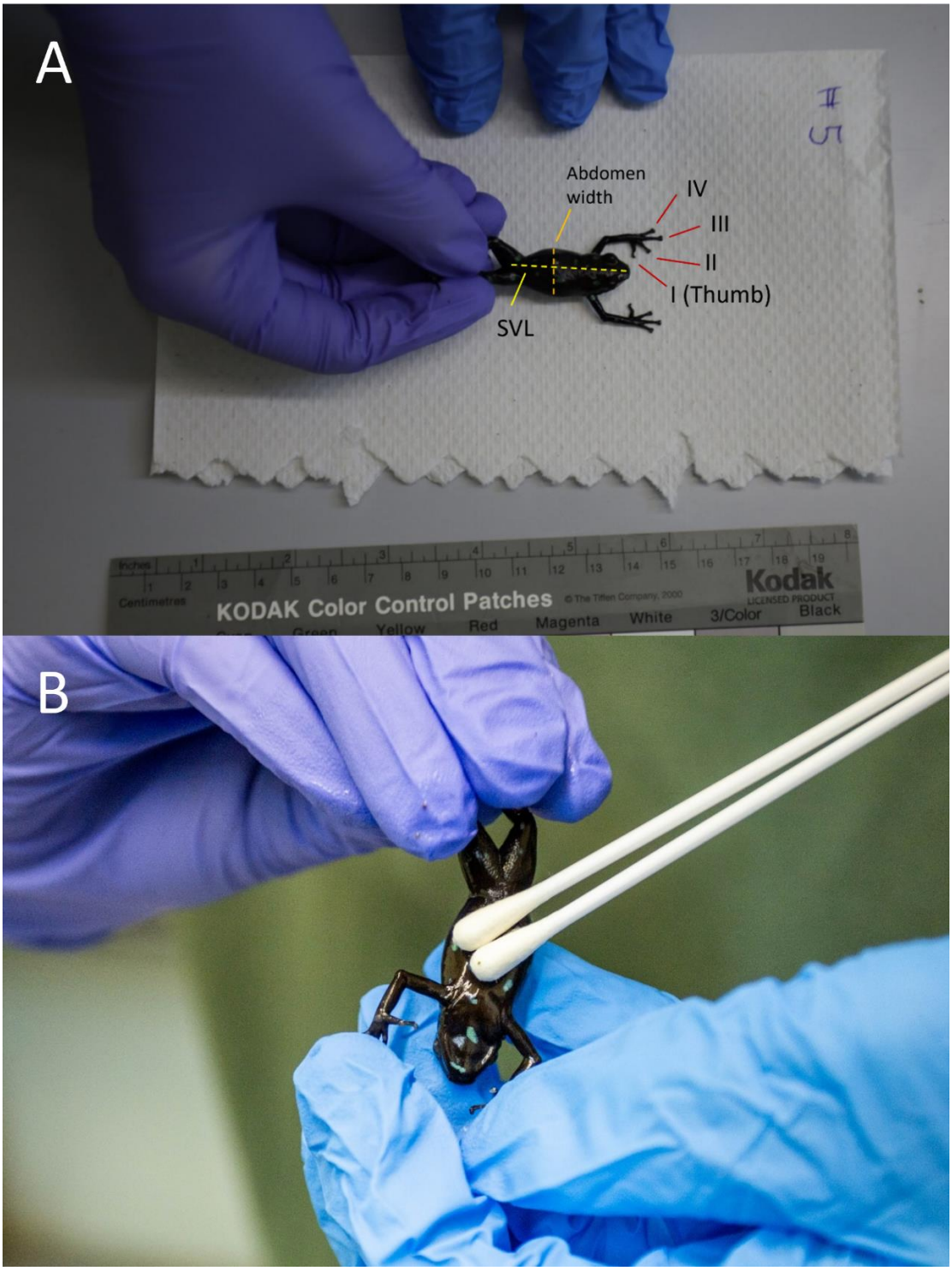

Figure 2. A: Dorsal image of *Dendrobates auratus*, used to take measurements in imageJ. Snout-vent-length (SVL), abdomen width, and toes (I – IV) are specified.
B: Skin swab of *Dendrobates auratus* taken in duplicate.

Captive animal care

Each frog was housed separately in an enclosure of 20x30x20cm (DxWxH; Figure 3). Each container was lined with moist paper towel and the lids of the enclosures were sealed off with cling film to prevent dehydration. Two film roll tubes with water inside were put in each cage to simulate egg laying sites. In addition, several large dry leaves and a stick were provided for shelter and environmental enrichment. The leaves were collected from the area around the laboratory and autoclaved before use to prevent potential introduction of *Bd* from the wild. The enclosures were sprayed with water every other day. At those times, the frogs were also fed approximately 15-20 flightless fruit flies dusted with calcium powder. In addition, each frog received two termites on a weekly basis for sufficient protein intake. During working hours (08:30 – 18:00), the enclosures were exposed to artificial fluorescent lights. Apart from that, the windows in the laboratory provided natural light to maintain the natural circadian rhythm of the frogs. Temperature control was mediated through the air conditioning of the building, which kept the temperature at approximately 25 ± 1°C. Daily health checks were performed to detect any abnormal feeding behavior, significant weight loss or any other irregular activity. After two weeks into the experiment, M5 was found to have one non-functional hindleg, but was otherwise healthy. Mainly F2, F6, and M6 have shown periods of low body weight, but never reached a point of concern or showed observable behavioral changes. Under normal conditions, the enclosures were cleaned once every once a week. This differed when frogs were going through a *Bd* curing or infection period (see *"Bd* infection and curing procedure*"*). When cleaning, paper towel and dry leaves and sticks were discarded and autoclaved. Then, the enclosure and film roll tubes were first dried and subsequently sprayed with 70% ethanol. The surfaces were then dried again and the bottom of the enclosure was lined with fresh paper towel, which was then moistened. Some water was added to the film roll tubes and freshly autoclaved dry leaves and a stick were finally put in the enclosure.

Figure 3. Laboratory setup of the enclosures.

Mucosome and *Bd* resistance analysis

Before the start of the mate choice experiment, the mucosome of each frog was collected. To do so, the frogs were bathed in water for one hour in Nasco Whirl-Pak bags. The volume of water used was standardized based on the surface area of each individual specifically (Woodhams et al., 2014). To calculate the surface area, the following formula was used: $cm^2 = 9.9 \times weight(g)^{0.56}$. 0.25mL of water per $1cm^2$ surface area was used. The water was then collected into 15mL screwcap tubes and stored at -20°C. Further analysis of the samples was performed at the University of Massachusetts. The water samples were lyophilized and then rehydrated. 50µL of 10^6 *Bd* zoospores/mL were then exposed to 50µL of the rehydrated mucosomes in triplicate. Zoospore viability was then measured using a SYBR 14 fluorescence assay according to the protocol by Woodhams et al. (2014). *Bd* growth relative to the control (*i.e.*, no exposure to mucosome) was then calculated. Decreased growth indicates inhibition of *Bd* growth by the *D. auratus* mucosome, whereas increased growth suggests enhancement of *Bd* growth. The data were visualized in R version 4.3.2 (R Core Team, 2023) using the package ggplot2 (Wickham, 2016). No statistical analyses were performed on this dataset, because it is not the main objective of this study and it is merely to show that variation in *Bd* resistance exists within this subset of individuals.

Bd infection and curing procedure

To infect *D. auratus* with *Bd*, individuals were bathed in Nasco Whirl-Pak bags for 60 minutes in 10 mL of water with *Bd* strain JEL 423 at a concentration of 6×10^5 zoospores per mL. This strain of *Bd* is part of the Global Panzootic Lineage (GPL) and was originally collected from *Hylomantis lemur* in Panama in 2004 (Direnzo et al., 2014; Lips et al., 2006). The frogs were then returned to their enclosures and the zoospore solution was released into the enclosures to maximize *Bd* exposure. The infection period of the frogs was considered to start on the day after the infection treatment took place. During the infection period, enclosures of infected individuals were not cleaned. The frogs were cured after day 5 of infection, because *D. auratus* shows minor symptoms within this period, but can be strongly affected by *Bd* after that (Nichols et al., 2001). During the period of infection, skin swabs to monitor infection status were taken on day 1, 3, and 5 (Figure 2B). To cure *D. auratus* from *Bd*, individuals were treated for 10 days with Itraconazole. Each day, the frogs were bathed in Nasco Whirl-Pak bags for 30 minutes in 30mL of 0.01% Itraconazole solution. During the curing treatment, the enclosures were cleaned on a daily basis. After finalization of the treatment, skin swabs were taken to confirm that the frogs were no longer infected with *Bd* (Figure 2B).

Bd qPCR infection analysis

The swabs that were taken during the experiments were stored at -20°C until further processing. At each sampling point, swabs were taken in duplicate and simultaneously. One of the duplicates was analyzed by STRI at the CTPA Acon laboratory. The other duplicate was processed at the University of Groningen following established protocols (Mantzana-Oikonomaki et al., 2021). To do so, the swabs were incubated for one hour at 37°C using a shaking incubator at speed 600 in 180µL of enzymatic lysis buffer (20mM Tris-HCl, 2.5mM EDTA, 1.2% Triton-X-100, with 20mg/mL lysozyme added briefly before use). The samples were then further using the DNeasy Blood and Tissue Kit (Qiagen, Cat No. 69504) or the NucleoSpin Tissue Kit (Bioké, Cat No. 740952). Depending on the kit, the samples were further incubated for 30 minutes at 70°C and speed 600 with 25uL Proteinase K, and 200µL buffer AL or 200µL buffer B3, respectively. Beyond this step, the protocols supplied by the kits were followed. After DNA extraction, a qPCR was performed with a total reaction volume of 15µL (2X iQ™ Supermix (BioRad, Cat No. 1708860), 0.9mM (*i.e*., 13.5nmol) each primer F and R, 0.15mM (*i.e.,* 2.25nmol) probe) with 5µL of DNA sample. Primers and probe are listed in Table 1. Each sample was run in duplicate in a Biorad thermocycler (Kriger et al., 2006). The efficiency across all qPCR runs was 85-100%. The threshold for a positive result was set at the detection of a single molecule. If the results from the duplicates were inconsistent (*i.e.,* one positive and one negative), the sample was repeated in duplicate. A duplex negative control was run in each plate, together with five gBlock standards (Standish et al., 2018; 0^0,

10^1, 10^2, 10^3, and 10^4 ITS copies) in duplicate. *Bd* qPCR results were included in the supplementary information (SI 2).

Table 1. Primers and probe used in the *Bd* qPCR infection analysis (Boyle et al., 2004).

Name	Sequence
F primer (ITS1-3 Chytr)	5'-CCTTGATATAATACAGTGTGCCATATGTC-3'
R primer (5.8S Chytr)	5'-AGCCAAGAGATCCGTTGTCAAA-3'
Minor groove binder probe	5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3'

Mate choice trials

In the setup (Figure 4), a box of the same type as of the frog enclosures was used on the side to function as the female container in the mate choice trials. The sides and back of the box were covered with duct tape to allow for as little visual distraction and stress from the environment as possible. Unfortunately, acoustic isolation from researchers was not possible in the available facilities. The front – originally the top – of the box was covered with cling film and holes were pinched in it so that potential male pheromones could enter the female enclosure. In front of the female container, two smaller enclosures of approximately 15x15x15cm held the males. Male enclosures were closed with cling film in the same way, again with pinched holes. All enclosures were lined with moist paper towel at the bottom. When introducing a female into a trial enclosure, this was done in a film roll tube from their own enclosure to provide a familiar environment. The film roll tube was placed and left in the middle back of the enclosure. A visual barrier was placed between the males to prevent them from interacting. Two trials were run next to each other simultaneously (Fig. 4), so a visual barrier was additionally used between these two trials. Above each of the two trial setups, a Raspberry Pi Rev. 13 camera was placed to record the trials. Using a custom Python script (https://github.com/marioasmira/Panama_cameras) run on a Raspberry Pi 4 the cameras were programmed to wait for 30 minutes at the start of a trial before recording. The time of acclimatization used in other amphibian mate choice experiments is not consistent, varying from 20 minutes (Maan & Cummings, 2008) to over one hour (Peignier et al., 2022). To fall within this range, 30 minutes should provide time for individuals to acclimatize and display more natural behavior during the mate choice trial. The cameras recorded for 30 minutes after the acclimatization period. The frogs were not interfered with during this period of one hour. On a day where mate choice trials were performed, eight rounds of two trials, thus 16 trials were performed. Males were clustered in two groups (M1-M5 and M6-M10). Within this groups, every possible combination of males was offered to the females (*e.g.,* M1 and M6 were never in the same trial) (Table 2.). Two pairs of males – one in each of the two setups run in parallel – were used each day. Each female was exposed to both male pairs, thus performing two subsequent trials on each trial day. The mate choice trials consisted of three different treatments: 1) a control with no infected frogs (*i.e.,* three uninfected frogs per trial); 2) a treatment where only males are infected (*i.e.,* two infected males and one uninfected female per trial); and 3) a treatment where only females are infected (*i.e.,* two uninfected males and one infected female per trial). In each treatment, two groups of five males were used and every possible combination of males within these groups was used. Every female was exposed to every male pair combination in each round of trials. This amounts to 160 trials for each round with an overall total of 480 trials done.

The number of trials required was identified in an *a priori* power analysis using G-Power version 3.1 (Faul et al., 2007). A low effect size of 0.2 was assumed and a standard alpha and beta error of 0.05 was used. For the test family, the F test was chosen, with the parameter “ANOVA: Fixed Effects, omnibus, one way” and three sample groups. This results in a required number of trials per treatment of 130. Therefore, 160 trials should then provide sufficient power to find significant differences between the groups.

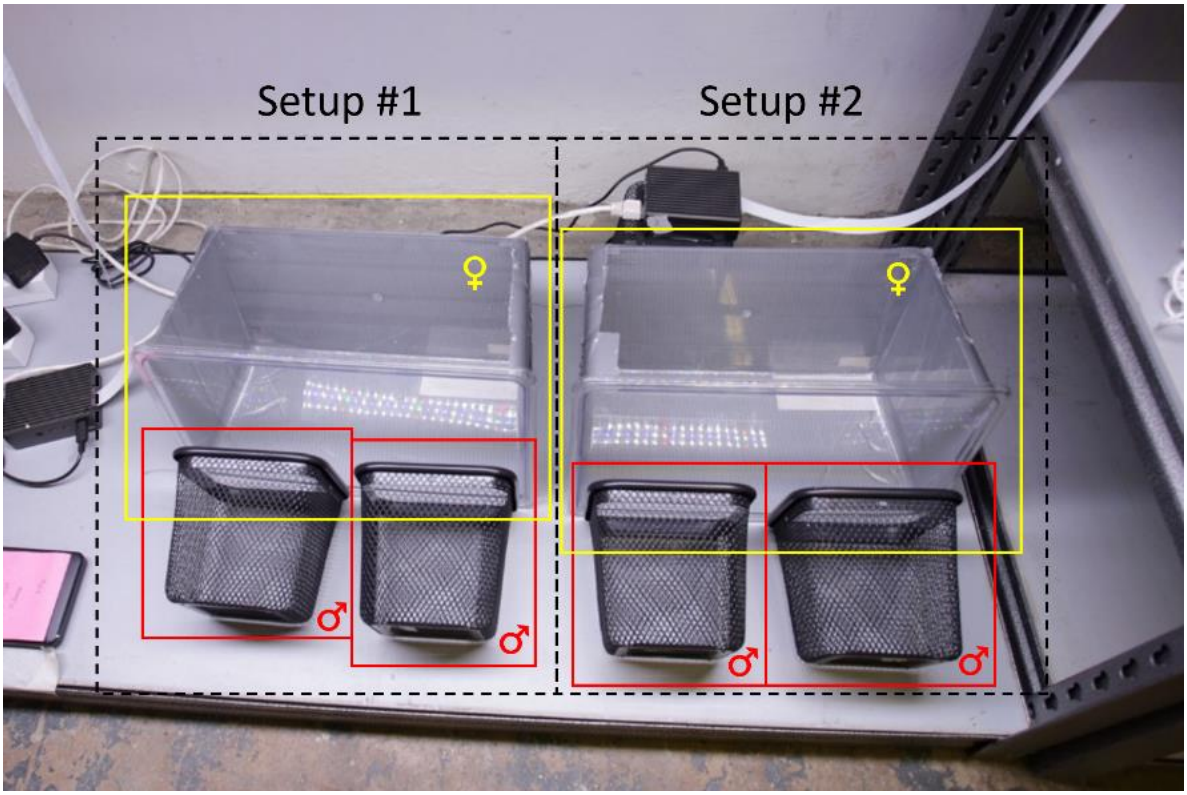

Figure 4. Setup of the mate choice trials. Two setups ran simultaneously (#1 and #2). Each female enclosure (yellow) has two male enclosures (red) on display.

Table 2. All combinations of males offered to each of the females during the mate choice trials.

	Male group 1		Male group 2	
1	M1	M2	M6	M7
2	M1	M3	M6	M8
3	M1	M4	M6	M9
4	M1	M5	M6	M10
5	M2	M3	M7	M8
6	M2	M4	M7	M9
7	M2	M5	M7	M10
8	M3	M4	M8	M9
9	M3	M5	M8	M10
10	M4	M5	M9	M10

The female's mate choice was scored based on the recorded videos. The cameras produced a top view as shown in Figure 5. The female enclosure was divided into multiple areas. The first 6cm away from the males was considered as the zone of interaction with the males (1LX and 2RX). This was determined based on a study on *Oophaga pumilio* by Maan & Cummings (2008), but adjusted to the larger body size of *D. auratus*. Within this area, some "escaping" behavior was witnessed during which the females would try to leave their container in the top corners of the enclosure (10-15% of the time across all treatments: SI 3). This specific location was separately scored as 1LE or 2RE to be able to analyze the results with and without this behavior included. The areas behind the interaction ("choice") zones, were considered "no choice" locations where no interaction between females and males took place (0LX and 0RX). The location of the female was scored every 20 seconds of the half hour recording by two independent scorers. The similarity between the two scorers was analyzed for every trial. When similarity fell below 80%, a trial was re-scored by both scorers until every trial was scored with at least 80% similarity between the two records.

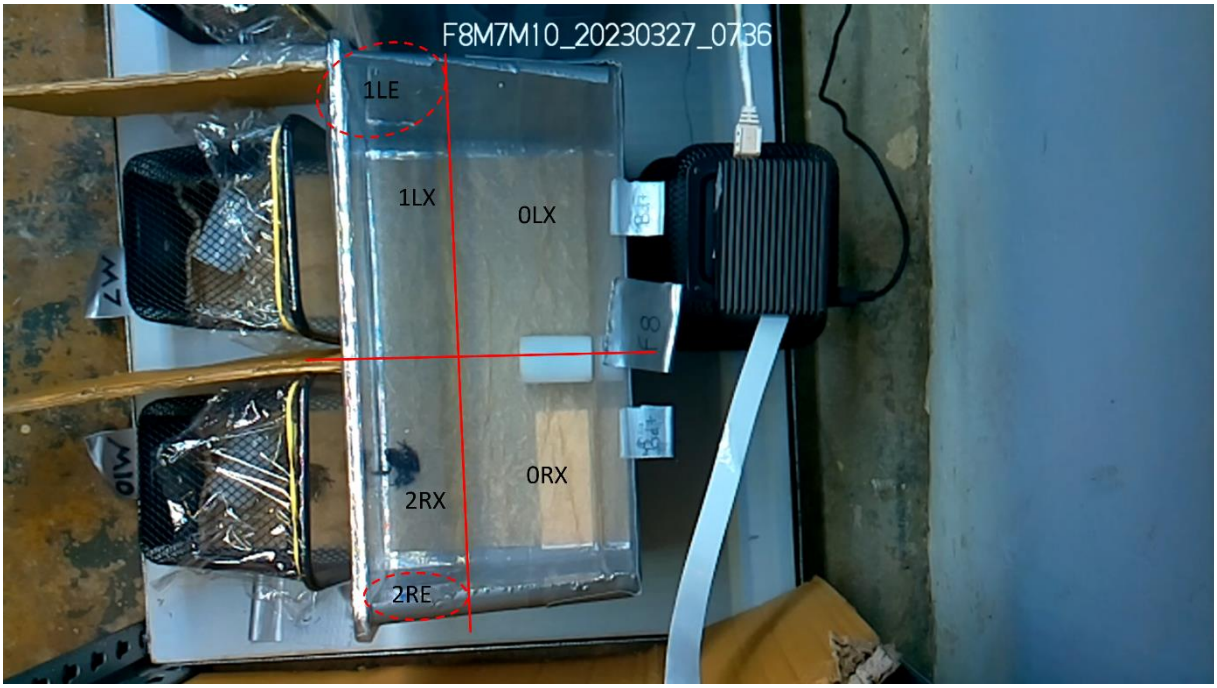

Figure 5. A frame from a mate choice trial recording. The lines represent the different areas in which a female could be located and each area has a code. 0LX and 0RX indicate "no choice" areas with no interaction between females and males. 1LX and 2RX represent "choice" areas with interaction between males and females. Within these areas, the subareas 1LE and 2RE were recorded as escape attempts.

Mate choice data analysis

Several preliminary analyses were done to determine the most suitable way to interpret the data, for which the models are listed in Table 4. For each analysis, multiple models were built to see which best fits the data, by selecting the model with the lowest AIC-value. Specifically, these preliminary analyses were performed both with and without including the escaping behavior as choice in order to identify the nature of this behavior and to see whether it should be included as choice in further analyses. Models A and B were used to analyze the effect of including escaping behavior and as a consequence, models C and D were built excluding escaping behavior (Table 4.). Firstly, the proportion of time a female spends in front of the enclosure with the males (*i.e.,* choice-zone) was analyzed in model A and C (Table 4. ; SI 3). Secondly, the proportion of time a female spends with the preferred male was analyzed in model B and D (Table 4. SI 4). Both datasets were visualized in R version 4.3.2 using the package ggplot2. Generalized linear mixed models were built in R using the package glmmTMB version 1.1.8 (Brooks et al., 2017) with proportion of time – spent choosing (Table 4. A+C), and spent with the preferred male (Table 4. B+D) – as the response variable and treatment as the predictor variable. Female ID was incorporated as a random effect. A logistic beta regression was used as distributional assumption for the data. Then, a Tukey post-hoc test was performed using the package multcomp version 1.4 (Hothorn et al., 2008) to infer Bonferroni-adjusted p-values for the difference between treatments as well as the inclusion of escaping behavior as choice. Even though including the escaping behavior as representing choice significantly increased the proportion of time a female spends choosing (Table 4. A; SI 3), this difference disappeared when identifying the strength of preference for one of the males (Table 4. B; SI 4). This indicates that the escaping behavior is randomly distributed over both sides of the enclosure in comparison to the time the females spend in zones 1LX and 2RX. Therefore, it was decided to exclude the escaping behavior in further analyses of female mate preference as there is no evidence it represents choice for the male on that side. No significant difference was found between the treatments in either of the two datasets (*i.e.,* the time spent choosing and the strength of preference for one of the two males: Table 4. C-D).

Consequently, the change in preference for specific males across the treatments was analyzed. The relative preference of a female for each male in a trial was calculated by subtracting the preference for that male in the control from the preference in the comparing trial, either the male or female infection treatment. A generalized mixed model with a logistic beta regression as distributional assumption was built in R using the package glmmTMB (Table 4. E). Relative preference was set as the response variable, and specific male ID and treatment was set as a combined predictor variable. Female ID was included as a random effect. In the next model, trial ID is included as a random effect as well, because two datapoints are included for each trial. Even though that is the case in this model as well, these datapoints are never combined in a comparison, for which reason trial ID was not included as a random effect in this model. In a Tukey post-hoc test performed using the package multcomp, each male*treatment was compared to 0, and Bonferroni-adjusted p-values were inferred from this. The data was visualized in R using the package ggplot2.

Lastly, the resistance of males against *Bd* was related to female preference. The preference of a female was calculated by dividing the time spent with a specific male by the total time spent with both males in that trial. The resistance was taken as a proportional value as well to account for differences in resistance between males, by dividing the resistance of a specific male by the resistance of both males in that trial combined. This way, values between 0.0 and 0.5 indicate males that are less resistant than their competitor and values between 0.5 and 1.0 indicate males that are more resistant. The further a datapoint is located away from 0.5, the stronger the difference in resistance is between the competing males, which would be expected to result in a stronger preference of females for one of the males. A generalized mixed model with a logistic beta regression as distributional assumption was built in R using the package glmmTMB (Table 4. F). Relative preference was set as the response variable, and relative resistance and treatment were set as predictor variables. Female ID was included as a random effect and so was the trial ID, to account for double datapoints for each trial (*i.e.,* one datapoint for each of the males of the same mate choice trial). As it was not possible to run a post-hoc Tukey test on a model with multiple fixed effects, the model was run multiple times with different datasets, each containing only one treatment. An Anova was run on each model to infer significance using the package car version 3.1 (Fox & Weisberg, 2019). The data was visualized in R using the package ggplot2.

Results

Resistance against *Bd*

The mucosome analysis provided insight into the degree of resistance of each frog used in the mate choice experiment (Figure 6). Even though several individuals show some degree of resistance against *Bd*, in most cases the growth of the fungus even seems to be enhanced on the skin of *D. auratus*. Males M4, M5, M8, and M9 show inhibition of *Bd* with a decrease in growth of up to approximately 55% in M5. In the females, only F7 showed a slight level of growth inhibition in contrast to the other females, who showed an increase in *Bd* growth as compared to normal growth conditions.

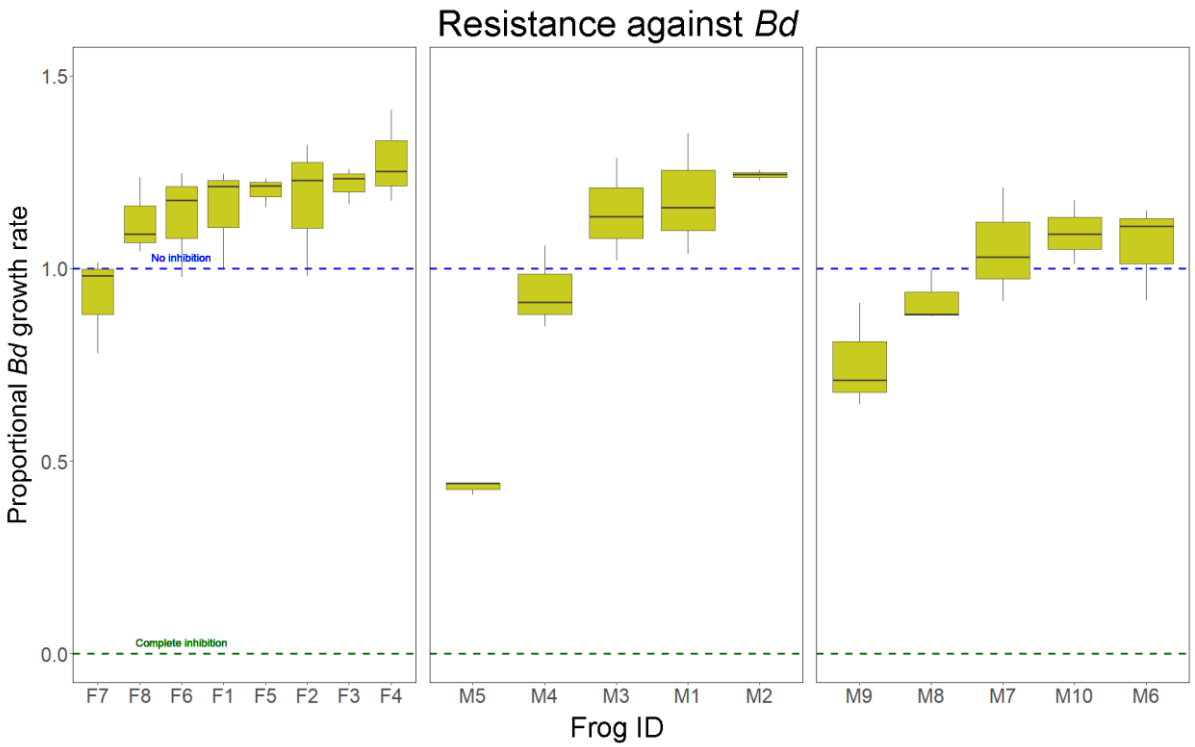

Figure 6. Resistance of the frogs against *Bd* based on the mucosome of the frogs used in the mate choice experiment. The individuals are divided between females (F1-F9) and the two groups of males (M1-M5 and M6-M10) ordered from the most to the least resistant individual. A *Bd* growth rate of 1.0 indicates no effect from the mucosome on the normal growth of *Bd*. A value above 1.0 indicate that the mucosome promotes the growth of *Bd* in comparison to normal growth, whereas a value below 1.0 indicates a certain degree of resistance against *Bd* up to 0.0, where *Bd* growth is completely inhibited.

Effect of *Bd* infection on mate preference

The change in preference of females for specific males was analyzed based on the data from the mate choice experiments (Table 4. E; Figure 7). In the first group of males (M1-M5), M2 seems to be slightly more preferred in both infection trials as compared to the control and so does M5 in the female infection treatment only (Figure 7). A slight decrease in preference for M1 is observed when the males were infected and M3 when the females were infected (Figure 7). However, no significant differences were found between either of the infection treatments in comparison to the control (Table 4. E), indicating that the male preference remained fairly stable across the different treatments. On the other hand, a stronger change in preference is observed for the second group of males (M6-M10) (Figure 7). M6 and M7 were significantly less preferred in comparison to the control during the male and female infection treatment, respectively (Table 4. E: $p = 0.0021$ & $p = 0.0016$). Also in the male infection treatment, a decreased preference was shown for M7 to some extent, but this was not significant (Figure 7; Table 4. E). In addition, M10 seems to receive a relatively high preference in both infection treatments, but this difference was also not significant (Figure 7; Table 4. E).

Table 4. Table containing the statistical models used in this study and the results they yielded. Several abbreviations are used: control treatment (C), female infection treatment (FI), male infection treatment (MI), model including escape (E), and model excluding escape (NE). "prop.Choice" refers to the proportion of time a female spends choosing in comparison to the time she spent in the no-choice areas of the enclosure. "choice.Strength" indicates the time a female spends with the preferred male relative to the other male in the competition. "prop.Preference" refers to the proportion of time a female spends with either of the males relative to the other male in the competition. Models C-F no longer include escaping behavior as choice, based on the results of models A and B. For models A-E results from the post-hoc Tukey test are shown. For model F, it was not possible to run a post-hoc test. Instead, the results from an Anova are shown for each treatment separately. "Extremes" refers to the dataset that only includes trials in which a resistant and non-resistant male were competing. In model F, trial is included as a random effect, as two datapoints – one for each male – are included for each trial. Therefore the number of included trials is also listed for this model.

#	Model	AIC	Predictor	Levels	Comparison	Std. Error	z-value	p-value
A	prop.Choice ~ Treatment * Model + (1|femaleID)	-374.2	Trt. * Model	3	C - E vs. NE	0.124	-2.865	0.0042**
					FI - E vs. NE	0.117	-2.882	0.0040**
					MI - E vs. NE	0.131	-2.470	0.0135**
B	choice.Strength ~ Treatment * Model + (1|femaleID)	-985.1	Trt. * Model	3	C - E vs. NE	0.111	0.017	0.9860
					FI - E vs. NE	0.108	-0.646	0.5180
					MI - E vs. NE	0.096	0.675	0.5000
C	prop.Choice ~ Treatment + (1|femaleID)	-360.2	Treatment	3	FI - C	0.090	1.408	0.4770
					MI - C	0.090	1.781	0.2250
					MI - FI	0.090	0.367	1.000
D	choice.Strength ~ Treatment + (1|femaleID)	-487.0	Treatment	3	FI - C	0.109	1.066	0.8590
					MI - C	0.106	0.198	1.000
					MI - FI	0.106	-0.897	1.000
E	Prop.Preference ~ Treatment * maleID + (1|femaleID)	-9.8	Trt. * maleID	20	FI*M1 == 0	0.241	-0.120	1.000
					MI*M1 == 0	0.331	-1.922	0.5460
					FI*M2 == 0	0.341	0.009	1.000
					MI*M2 == 0	0.323	0.357	1.000
					FI*M3 == 0	0.349	-1.281	1.000
					MI*M3 == 0	0.341	0.134	1.000
					FI*M4 == 0	0.341	0.451	1.000
					MI*M4 == 0	0.328	1.338	1.000
					FI*M5 == 0	0.345	1.199	1.000
					MI*M5 == 0	0.328	0.524	1.000
					FI*M6 == 0	0.324	-1.300	1.000
					MI*M6 == 0	0.320	-3.703	0.0021**
					FI*M7 == 0	0.329	-3.773	0.0016**
					MI*M7 == 0	0.325	-2.384	0.1712
					FI*M8 == 0	0.339	-1.421	1.000
					MI*M8 == 0	0.335	-1.119	1.000
					FI*M9 == 0	0.325	-2.208	0.2724
					MI*M9 == 0	0.320	-0.573	1.000
					FI*M10 == 0	0.331	-0.673	0.1241
					MI*M10 == 0	0.227	2.500	1.000

#	Model	Predictor	Dataset	Ntrials	AIC	Std. Error	Chisq	p-value
F	Prop.Preferece ~ rel.Resistance + (1|femaleID) + (1|Trial)	Rel. *Bd* resistance	C	137	-7.900	0.893	1.197	0.2740
			FI	137	-26.400	0.955	0.622	0.4305
			MI	150	-20.200	0.867	3.198	0.0737.
			C - Extremes	82	-0.200	0.935	1.814	0.1780
			FI - Extremes	83	-5.800	0.989	1.346	0.2459
			MI - Extremes	93	-13.700	0.918	6.162	0.0131*

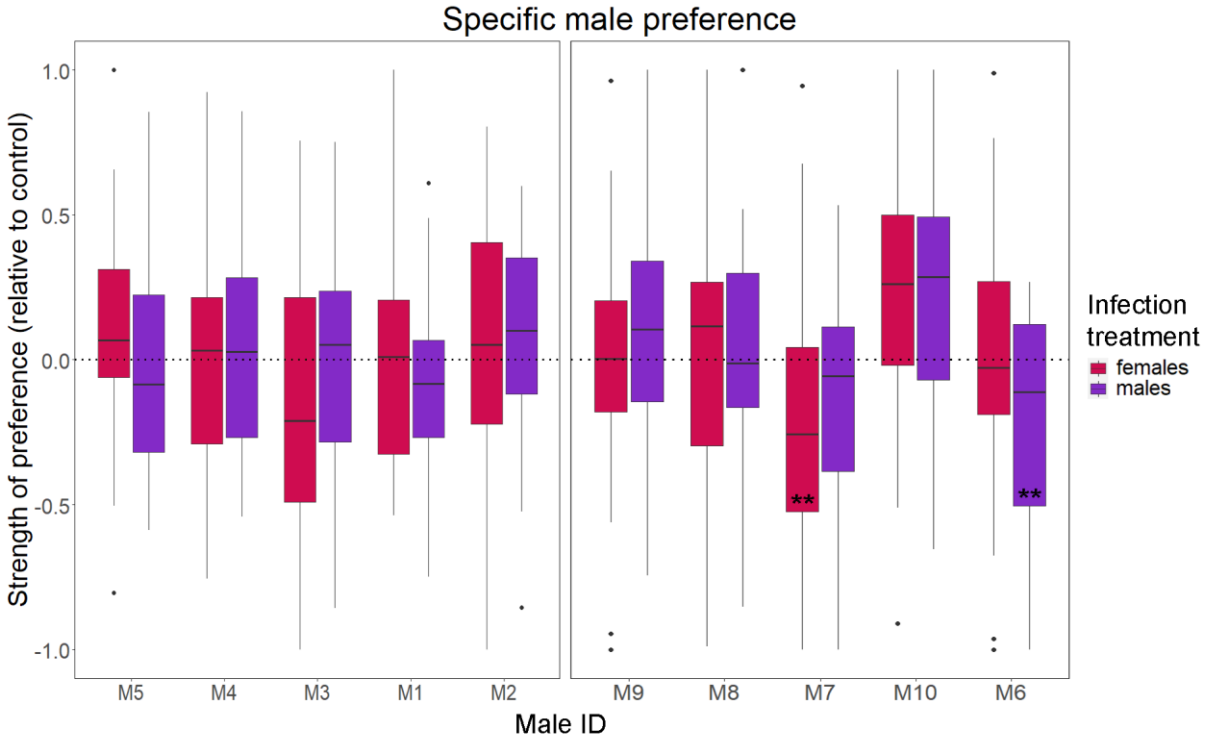

Figure 7. The preference that females showed for each male in both infection trials relative to the equivalent trial in the control. Yellow boxes represent the preference for the males in trials when the females were infected with *Bd*. Red boxes indicate trials in which the males were infected. The males are divided into two groups (M1-M5 and M6-M10) as they were during the trials. In both groups, the males have been ordered by their *Bd* resistance level, from most to least resistant to *Bd* (Figure 6).
** *boxes that are significantly different from 0.0 (i.e. the preference in the control trials) with p < 0.01.*

Preference for resistance against *Bd*

Finally, the relationship between the resistance of males against *Bd* and the mate preference of females was analyzed (Figure 8; Table 4. F). No significant effect was found of male resistance on female preference, even though there seems to be a tendency for females to choose a more resistant male across all treatments (Figure 8A; Table 4. F). This tendency is the weakest during the female infection treatment. When only trials were considered that have a male with no resistance competing against a male that does have a degree of resistance (Figure 8B), the female preference for *Bd* resistance was found significant when the males are infected with *Bd* (Table 4. F: p = 0.0131).

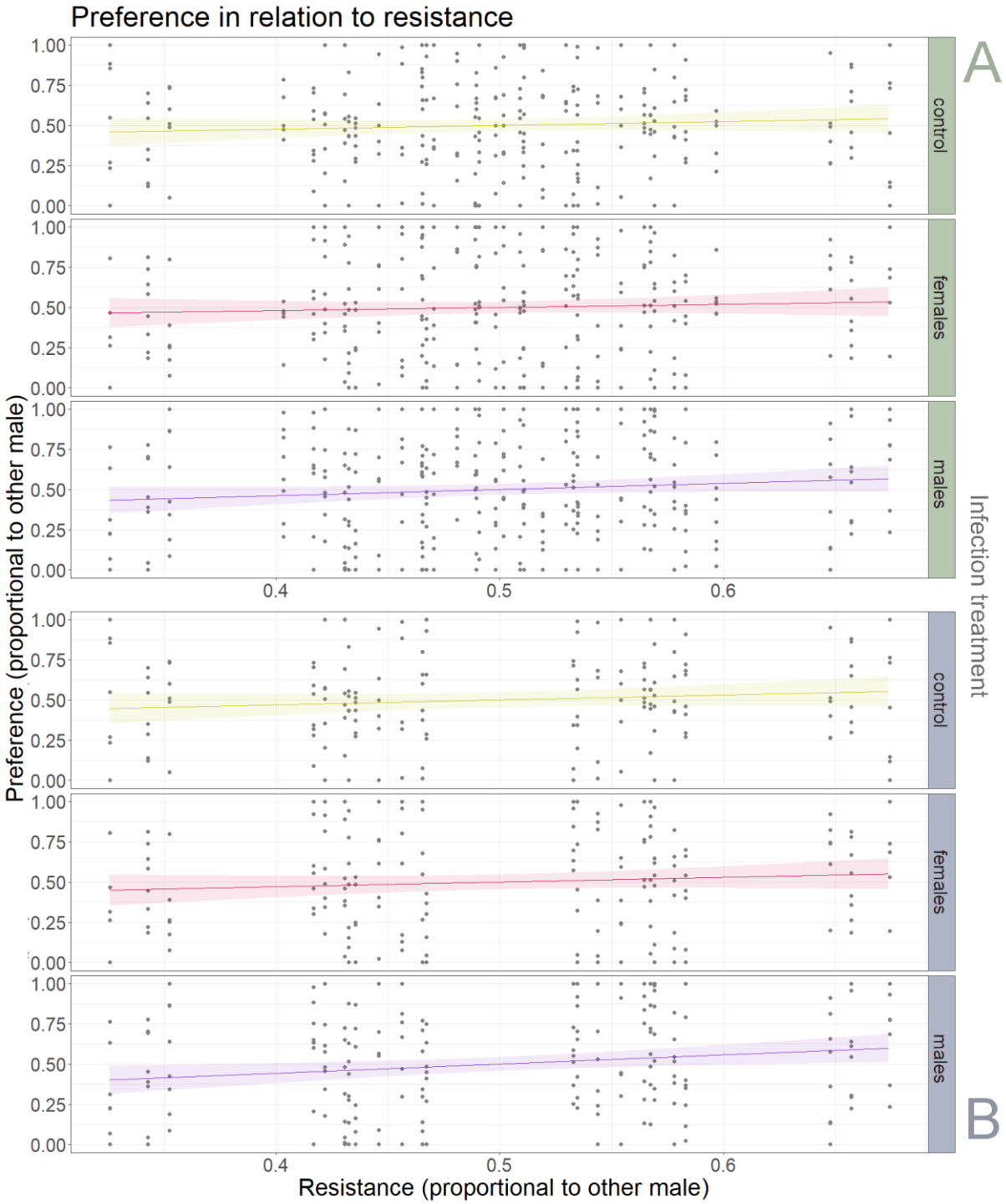

Figure 8. Preference for males based on their resistance, proportional to the other male in each trial. The graph provides visualization of two different sets of data: all trials (A), and only the instances where a resistant and non-resistant male were competing against each other in a trial (B). A proportional preference of 0.5 indicates that the male was not preferred over the other male. A value below 0.5 means that the male was less preferred than the other male, in contrast to a value above 0.5 indicating an increased preference over the other male. In the same way, a relative resistance of 0.5 indicates both males in the trial are equally resistant against *Bd*. A value below that suggests decreased resistance in comparison to the other male and a higher value means that the male is more resistant than its compatitor. The fitted lines include an upper and lower confidence estimation of 2SE.

Discussion

D. auratus is highly susceptible to *Bd* and suffers from fatal chytridiomycosis (Nichols et al., 2001). In this study, I show that some individuals possess a degree of resistance, but most do not or even enhance the growth of *Bd*, which coincides with the vulnerability of this species for the pathogen. Studies have suggested that possession of resistance is costly (Kawecki, 2020; Lazzaro & Little, 2009; Sheldon & Verhulst, 1996), therefore it is not surprising that no evolutionary driver of resistance against *Bd* previously existed in this population naïve to the pathogen. It was not possible to investigate this further within the given timeframe of this study, but the major histocompatibility complex (MHC) is established as a key element of disease resistance (Bernatchez & Landry, 2003). Based on the heterozygote advantage hypothesis, studies have suggested that heterozygous individuals have increased resistance through overdominance, maintaining high allelic diversity at MHC loci (Hughes & Nei, 1992; Penn et al., 2002; Stear et al., 2005). Even though evidence for this is not consistent (Ilmonen et al., 2007), heterozygosity could purely coincidentally lead to a degree of resistance against *Bd* as well.

After analyzing individual *Bd* resistance levels, the female mate choice was further looked into. Preliminary analyses showed that there is a weak tendency for females to spend more time with the preferred male when they are infected themselves (SI 4). On the other hand, much less difference was witnessed between the control and male infection treatment (SI 4). The pattern specifically observed in the female infection treatment could be driven by an increased urge to reproduce when a female becomes sick and may be nearing death, as described by the terminal investment hypothesis (Williams, 1966), which has been observed in amphibians before (Brannelly et al., 2016). On the other hand, literature shows that *Bd* infection might reduce the host's activity and movement as early as one to four days after infection (Nichols et al., 2001). An infected female might therefore stay in the same place for a longer time period and thus, purely by reduced stamina, spend more time in the choice area of a specific male. However, the amount of time that a female spends escaping is similar in the control and female infection treatment (SI 3), suggesting that female activity is not strongly reduced. To fully exclude this possibility, it would be interesting to track the mobility of the females and analyze whether it may be reduced when they are infected with *Bd*. This could provide insight into the effect of Bd on *D. auratus* as well as the effect of disease on female mating behavior.

In regard to female preference for specific males, M6 and M7 were preferred less during the male and female infection treatment, respectively (Figure 7). Additionally, a degree of increased preference is shown for M10 in both infection treatments compared to the control, but this difference was not significant. This means that the limited change that is observed occurs only in males that do not possess any resistance and that the preference for individuals with a degree of resistance remains relatively stable (Figure 6).

In the male infection treatment, it is possible that males experiencing increased sickness (*i.e.,* non-resistant males) will start to invest more in reproductive success, in line with the terminal investment hypothesis (Williams, 1966). This could explain why larger preferential change is observed among non-resistant individuals. However, preference for non-resistant males is not consistently increased or reduced, suggesting that *Bd* infection does not have the same effect on each individual. Many traits play a role in mate choice in poison frogs, such as coloration (Summers et al., 1999) and territory (Peignier et al., 2022). *Bd* is known to cause discoloration of the skin (Pessier et al., 1999), but could similarly have an impact on other traits affecting female mate preference, may it be positive or negative, that are not directly linked to individual resistance or health.

On the other hand, this could not explain why a change in preference arises during the female infection treatment, because the males were not affected by *Bd*. Courtship in dendrobatid frogs is known to be an interaction between males and females and not necessarily one-sided. Because there is a large paternal investment in these species, competition not only occurs between males, but also between females (Wells & Bard, 1988). Males might also change their interest in females when the

females are infected. In this way, it could be that M7 shows a stronger decrease in interest for infected females than other males do, thereby decreasing its relative reproductive effort towards the females. Conversely, M10 might have a much weaker decrease in interest, increasing its reproductive effort respective to other males.

Even though minor changes were observed, overall the preference of females did not change in the presence of *Bd* infection. The changes that are observed occur in the second group of males (M6-M10) and not in the first. If *Bd* resistance level would be the main driver of sexual selection in this species, one would expect the changes in mate preference to be more drastic in a group where the differences in resistance against *Bd* between males are more extreme. However, a larger difference in *Bd* resistance level is witnessed in the M1-M5 in comparison to M6-M10.

In the first model of preference for *Bd* resistance (Figure 8A), no significant preference for males with higher resistance against *Bd* is found. In this model, all datapoints from the mate choice trials are included. This means combinations of males exist for which both of them are either non-resistant or resistant to some degree. It could be that females are unable to detect the degree of resistance a male has, but rather observe the presence or absence of resistance.

This thought gave rise to the second model (Figure 8B), which solely includes data from male pairs in which one of the males has a degree of resistance and one does not. The trend increased in strength in this model, especially for the male infection treatment, where females show a significant preference for *Bd* resistance. This could be a direct consequence of reduced health in non-resistant males in comparison to males with a degree of resistance (Kelleher et al., 2021). Furthermore, traits representing resistance may only become visible to the female when potential partners are infected with the disease themselves (Joye & Kawecki, 2019; Kelleher et al., 2021; Roy & Kirchner, 2000), which would explain why the females show stronger preference when the males are infected. The fact that this trend is not as strongly observed when all trials are included does suggests that females are not fully capable of identifying the degree of resistance a male possesses and therefore overlook differences within resistant and non-resistant competitors. However, perhaps the visibility of such traits increases with the severity of symptoms. Then, it may become easier for females to detect resistance level as individuals start demonstrating stronger clinical signs.

No significant preference of females for males with higher resistance against *Bd* is observed in the first model (Figure 8A). It is known that pheromones are one of the key traits used in sexual selection across a wide range of animal taxa, including vertebrates (Buchinger & Li, 2023). In the current setup, it may have been difficult for pheromones to pass through both cling film barriers between the male and the female. Especially because the male enclosures were completely open on all other sides, pheromones might have had a much higher chance of escaping through those sides than through the side with two layers of cling film, albeit with small holes. This could have made it more difficult for females to detect a preferred male, might have prevented them from identifying resistance, or distinguishing between the two males because of pheromones mixing. Additionally, literature shows that chemical cues in amphibians are not always released into the environment, but sometimes directly transferred through physical contact (Thomas et al., 1993; Willaert et al., 2013), which would not be possible in this setup.

Regardless, a minor positive trend is still observed for females to choose more resistant males across all treatments, including the control, suggesting that sexual selection might also occur on general resistance. This trend could explain why no directional change is observed in the preference for specific males in relation to their resistance against *Bd* (Figure 7), because there may be an innate preference for resistance that is not kickstarted by the introduction of a pathogen, in this case *Bd.* Several traits contribute to an individual's resistance. A more well-studied component of pathogen resistance is the MHC gene complex (Bernatchez & Landry, 2003). Even though heterozygosity could increase the overall strength of resistance, a homozygous individual could randomly possess an allele that yields strong resistance against a specific pathogen, such as *Bd*, that a heterozygous individual may not. Alternatively, multiple heterozygotes may be equally preferred, whereas only part of them may possess an allele that yields resistance against *Bd*. Hence, if heterozygous mates are indeed preferred, that does not

necessarily mean that these individuals are all indeed resistant to *Bd*. Over generations, selection for heterozygosity can still lead to the acquisition of resistance against specific pathogens, but on a single generation time-scale, it would weaken the strength of selection for that. Such a mechanisms would in the long run be beneficial, because it prevents the absence of evolutionary defenses against emerging pathogens that were never encountered in the past and could otherwise lead to extinction (Hulse et al., 2023).

Evidence suggests that directional selection for MHC alleles that specifically convey *Bd* resistance can arise in populations where *Bd* exists (Savage & Zamudio, 2016). MHC was not specifically analyzed in this study, and it is important to mention that many other factors are likely involved in an individual's resistance as well. For example, skin microbial community is of importance to resistance against *Bd* (Nava-González et al., 2021; Walke & Belden, 2016). Regardless of the mechanism behind it, results show that females may indeed be able to select for *Bd* resistance. However, this is only observed when potential partners are infected and not when only the females are infected themselves. Even though a female may recognize that she is ill, she might not receive the required signals from the males to identify resistance when they are healthy (Joye & Kawecki, 2019; Kelleher et al., 2021; Roy & Kirchner, 2000). Thus, in a natural population consisting of infected as well as uninfected individuals, it is difficult to say if sexual selection will lead to an increased pathogen resistance level over generations. Nevertheless, studies suggest that amphibian populations can recover from and acquire resistance against *Bd* over multiple years (Voyles et al., 2018) although on the other hand, there are many instances where extinction is not avoided (Carvalho et al., 2017; Skerratt et al., 2007).

It is important to further investigate with the data from this study if sexual selection occurs for specific conformations of the MHC, such as heterozygosity, apart from resistance against *Bd* as a consequence of infection. It is possible that multiple MHC conformations are selected for, but do not all result in resistance against *Bd*, and vice versa. As research presents contrasting results as to whether species and populations can develop resistance and avoid extinction, this study should be repeated across different species and populations to acquire a more complete view of the occurrence of sexual selection on pathogen resistance – and MHC – in amphibians. This way, conservation efforts can be directed towards the more vulnerable species lacking such mechanisms to defend themselves. Similarly, it would be highly informative to study genetic adaptation and mate selection in a natural population, where the presence of infected as well as uninfected individuals may interfere with selection for pathogen resistance. One way of studying this in the laboratory instead of the field could be by repeating the mate choice experiment from this study, this time having an infected and uninfected male competing to identify changes in mate choice in regard to resistance, against *Bd* as well as in a general sense.

To conclude, this study has shown that the introduction of *Bd* into a naïve population of *D. auratus* does cause difference in female mate choice, but to a minor extent. On the other hand, females are shown to select for *Bd* resistance when choosing between a – to some degree – resistant and non-resistant individual. This species may therefore be equipped to accelerate evolution of resistance to specific pathogens through sexual selection. However, results suggest that potential partners need to be infected in order for females to select for resistance. Therefore, in a natural population where infected and uninfected individuals exist, it is uncertain whether sexual selection can result in the evolution of pathogen resistance. Nevertheless, results imply that selection may also occur on general resistance, regardless of pathogen infection, and further research the MHC may provide insight into this. Based on the outcomes of this research, there is an urgency to further study the presence of evolutionary mechanisms to acquire pathogen resistance across amphibian taxa and populations to direct conservational efforts towards species that, in the long run, do not possess the capability to survive *Bd* on their own.

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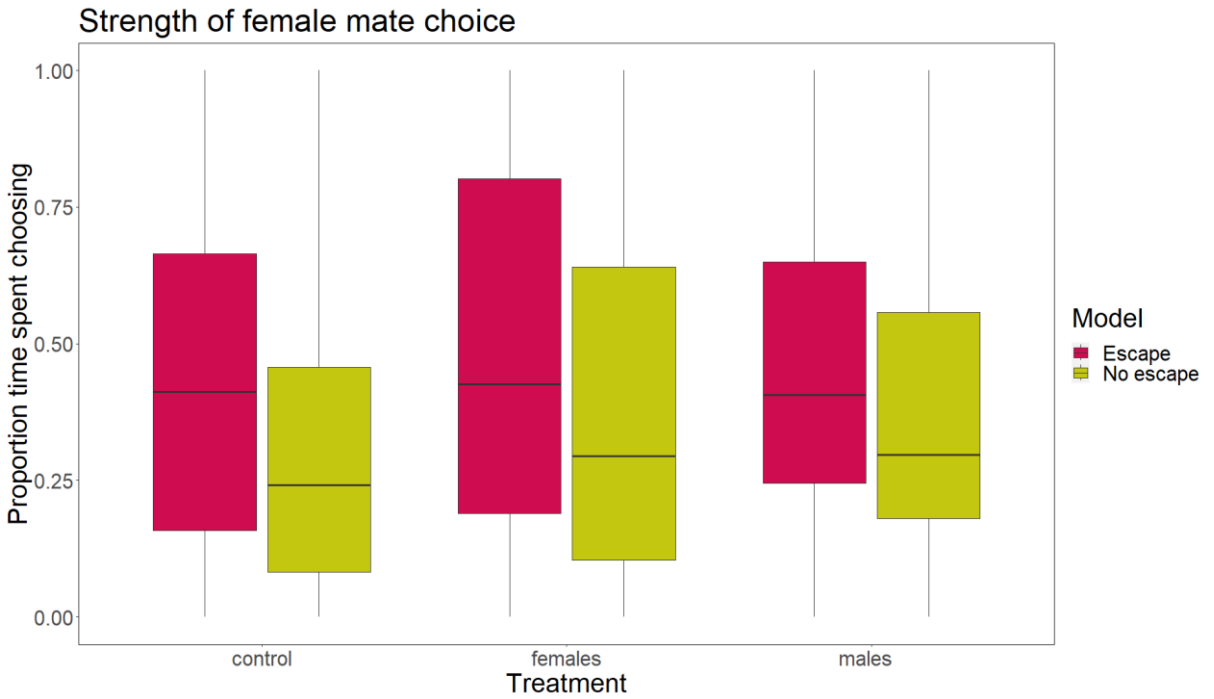
Support information

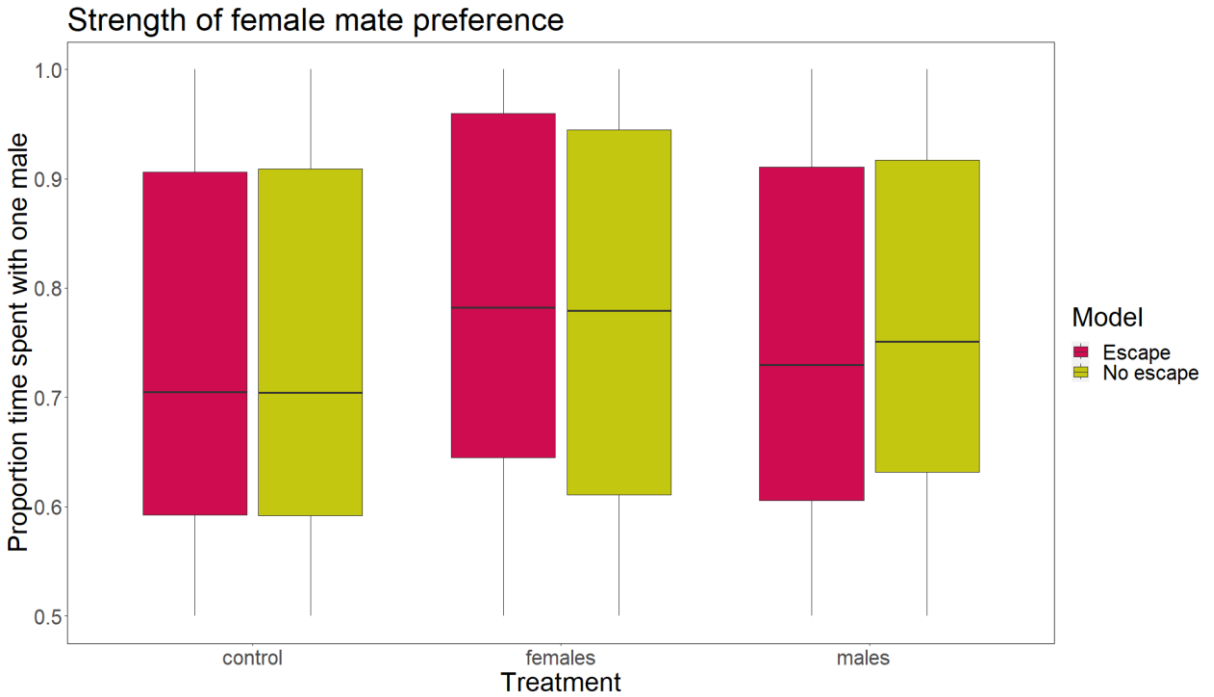
SI 1. An impression of the amphibian exposition at STRI Punta Culebra Nature Center

SI 2. *Bd* qPCR results. A value of 0.0 indicates a negative result (*i.e.*, no Bd infection detected). Values above 1.0 indicate a positive result and thus, detection of infection. During the male infection (MI) and female infection (FI) treatment, skin swabs were taken at three time points (day 1, day 3, and day 5). Individuals were regarded as infected when at least one of three provided a positive result. Unfortunately, an issue seemed to have arisen in the Bd extraction for the swabs of the FI1 infection treatment as many were found to be negative. In those instances, the result from the duplicate swab analyzed by STRI was included in the table (orange). From their independent analysis, STRI confirmed all individuals were infected when they were supposed to be and were uninfected after receiving curing treatment.

Frog ID	start	treated	MI1.1	MI1.2	MI1.3	T1	FI1.1	FI1.2	FI1.3	T2	FI2.1	FI2.2	FI2.3	T3
F1	0.0	0.0	-	-	-	0.0	27.0	0.0	0.0	0.0	26.9	14.4	1.4	0.0
F2	0.0	0.0	-	-	-	0.0	1.8	1.5	0.0	0.0	0.0	4.5	2.7	0.0
F3	0.0	0.0	-	-	-	0.0	44.0	0.0	0.0	0.0	97.6	1.5	4.2	0.0
F4	0.0	0.0	-	-	-	0.0	23.0	0.0	0.0	0.0	20.0	5.6	20.2	0.0
F5	0.0	0.0	-	-	-	0.0	23.5	0.0	0.0	0.0	0.0	11.1	0.0	0.0
F6	0.0	0.0	-	-	-	0.0	14.0	0.0	0.0	0.0	1.1	0.0	3.4	0.0
F7	0.0	0.0	-	-	-	0.0	8.1	0.0	0.0	0.0	2.9	1.9	0.0	0.0
F8	0.0	0.0	-	-	-	0.0	11.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0
F9	0.0	0.0	-	-	-	0.0	-	-	-	0.0	-	-	-	0.0
M1	0.0	0.0	14.6	0.0	0.0	0.0	-	-	-	-	-	-	-	0.0
M2	0.0	0.0	23.2	11.8	4.7	0.0	-	-	-	-	-	-	-	0.0
M3	0.0	0.0	39.1	0.0	0.0	0.0	-	-	-	-	-	-	-	0.0
M4	0.0	0.0	4.9	0.0	1.4	0.0	-	-	-	0.0	-	-	-	0.0
M5	0.0	0.0	56.0	3.2	14.8	0.0	-	-	-	0.0	-	-	-	0.0
M6	0.0	0.0	61.3	2.7	1.2	0.0	-	-	-	-	-	-	-	0.0
M7	0.0	0.0	27.7	0.0	0.0	0.0	-	-	-	-	-	-	-	0.0
M8	0.0	0.0	23.0	5.2	1.8	0.0	-	-	-	-	-	-	-	0.0
M9	0.0	0.0	25.0	0.0	4.2	0.0	-	-	-	-	-	-	-	0.0
M10	0.0	0.0	36.0	3.6	2.2	0.0	-	-	-	-	-	-	-	0.0
M11	0.0	0.0	-	-	-	0.0	-	-	-	-	-	-	-	0.0



SI 3. The proportion of time spent in the choice area of the enclosure by the females over the three different treatments. For each treatment, a box is included with (red) and without (yellow) escaping behavior included.



SI 4. The proportion of time that females spent with its preferred male from the total time spent choosing between the males. For each treatment, a box is included with (red) and without (yellow) escaping behavior included.