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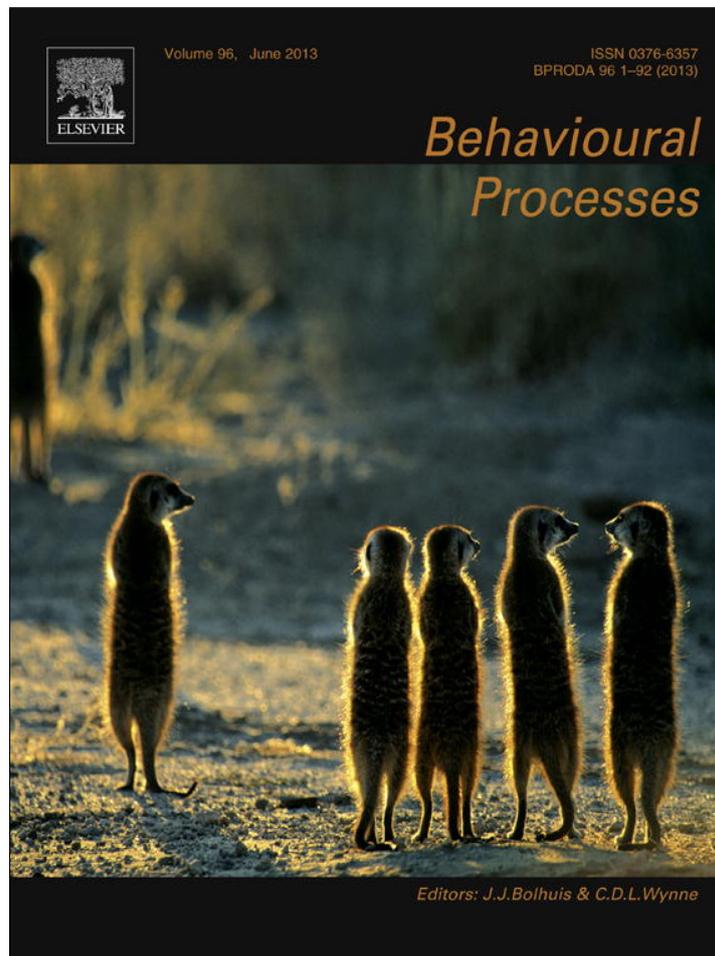
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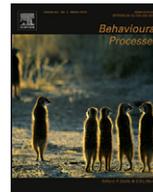
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Intra- and intersexual selection on male body size in the annual killifish *Austrolebias charrua*



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ABSTRACT

Since many traits are involved in both female mating decisions and male contest outcomes, female mate choice and male competition can act in concert to intensify sexual selection on male traits, or in opposition to weaken it. In the sexually dimorphic annual killifish, *Austrolebias charrua*, we evaluated the effect of male body size on female mate choice, male–male competition, and their interaction. We carried out an experiment with three consecutive stages: (i) female choice test between males of different size in a classic two-choice device, (ii) agonistic interactions between males used in the previous stage, and (iii) a second female choice test to evaluate preference consistency in females that either were allowed or were prevented from observing the male competition. Larger males were preferred by females and became socially dominant in agonistic interactions. Further, females were consistent in their choices, and this consistency was independent of whether they had observed or not the male contest. Our research shows that, in *A. charrua*, intrasexual competition and female mate choice act in concert with respect to male body size. The unique life-history of *Austrolebias* and the high repeatability of mate-choice assays make this system a promising candidate for studies of behavioural evolution.

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1. Introduction

Sexual selection results in differential reproductive success among individuals of the same sex (Darwin, 1871), and typically acts more strongly on males due to sex differences in search strategies and resource allocation among gametes (Kokko et al., 2006). Sexual selection can be subdivided into intrasexual selection (generally via male–male interference competition or sperm competition) and intersexual selection (usually via female mate choice). Although the evolution of certain male traits can be promoted exclusively by one of the two components of sexual selection, they often act simultaneously (in concert or in opposition) because many traits are involved in both female mating decisions and male contest outcomes (Andersson, 1994; Candolin, 2004; Moore and Moore, 1999; Qvarnström and Forsgren, 1998).

Empirical evidence suggests that sexual selection via intrasexual competition and sexual selection via mate choice could be in

opposition for some traits (reviewed in Arnqvist and Rowe, 2005) and the net result will reflect the balance between these forces. In many cases, however, intra- and intersexual selection have complementary effects, promoting the expression of the same traits in males (Benson and Basolo, 2006; Gagliardi-Seeley and Itzkowitz, 2006; reviewed in Wong and Candolin, 2005). Any traits favoured by intrasexual selection will therefore also be favoured by intersexual selection. In some cases, there is direct experimental evidence that witnessing the outcome of male–male interaction influences subsequent mate choice (Candolin, 1999; Doutrelant and McGregor, 2000). Females will even incite competition among their pretenders to increase the chances of mating with the dominant male (e.g. Cox and LeBoeuf, 1977; Montgomerie and Thornhill, 1989).

The annual fishes *Austrolebias* (Rivulidae, Cypinodontiformes) comprise 39 species distributed across south-eastern South America (Costa, 2006; Loureiro et al., 2011). *Austrolebias* shares with other rivulids and aplocheilids a unique mode of embryonic development that involves deposition of desiccation-resistant eggs in the substrate of the temporary ponds they inhabit (Simpson, 1979). These ponds have water only in winter, and sometimes go entirely dry for a year due to cyclical droughts. Embryos undergo up to three diapause periods (Berois et al., 2012; Wourms, 1972), allowing them to delay development until environmental conditions are

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favourable for fry to emerge. Individuals then hatch, mature, reproduce, and die within the span of a very short season (4–6 months). Males and females engage in courtship interactions before fully or partially burying themselves in the substrate to deposit and fertilize eggs (Belote and Costa, 2002; García et al., 2008; Vaz-Ferreira et al., 1964).

Austrolebias charrua belongs to the “*A. adloffii*” species complex endemic to the lowlands of the Patos-Merin drainage system (Costa and Cheffe, 2001; García et al., 2009; Loureiro and García, 2008). Like other species in the complex, *A. charrua* is highly sexually dimorphic: males are larger and have a pattern of dark vertical bands upon a light background on its body flanks with unpaired fins strongly pigmented, while females are relatively cryptic (Costa, 2006; García et al., 2009).

In this study, we evaluated sexual selection on male body size in *A. charrua* via female mate choice, male competition, and their interaction. First, we used simultaneous choice tests to measure female preferences for body size. Second, we used male–male competition assays to measure the effect of size on contest outcomes. Finally, we directly measured the effect of male competition on females’ mating preferences by allowing them to witness agonistic interactions and then allowing them to choose.

2. Methods

2.1. Collection and maintenance

Adult *A. charrua* were collected with a hand net from temporary ponds located in Rocha Department, Uruguay (Fig. 1), at the beginning of the reproductive season (August 2009). Test fishes were kept in the laboratory for at least 10 days under constant temperature (19 °C) and natural photoperiod conditions, and were fed daily with live *Tubifex* sp. Males were kept in individual tanks (20 cm × 9 cm × 15 cm, length × width × height) while females were kept in communal tanks (40 cm × 13 cm × 15 cm) in groups of up to 5 individuals. Aquariums were visually isolated from each other and their water was replaced weekly. Collection and experimental procedures were approved by the ethical committee of the Universidad de la República, Uruguay (Comisión de Ética en el uso de animales, Facultad de Ciencias, UdelaR, approval date 10-03-2009). Fish were returned to the same aquarium after experiments were completed, and retained as breeding stock.

2.2. Experimental aquarium

The experimental device consisted of aquariums (45 cm × 28 cm × 20 cm) divided lengthwise by a transparent glass into two compartments of equal size. For female preference trials, one of these compartments was halved by a black opaque glass (Fig. 2). Dividers were removable but were lined with silicone to prevent flow of olfactory cues between compartments. In order to resemble the natural substrate inhabited by annual fishes, the bottom of the aquarium was completely covered with a 2 cm deep layer of peat moss. To reduce any external disturbance and provide uniform background, black screens were placed covering both side walls and the rear wall of the aquarium. All experiments were performed between 8:00 and 14:00 local time.

2.3. Mating preferences

To determine whether females respond differently to males of different sizes, at day 1 the preference of 30 females was examined using 30 different pairs of males varying in their body size by 8.2 ± 0.5 mm (Table S1). Each individual was used only once. To avoid biased results due to female preferences for a sector of the aquarium, for each test, the focal female was placed in the choice

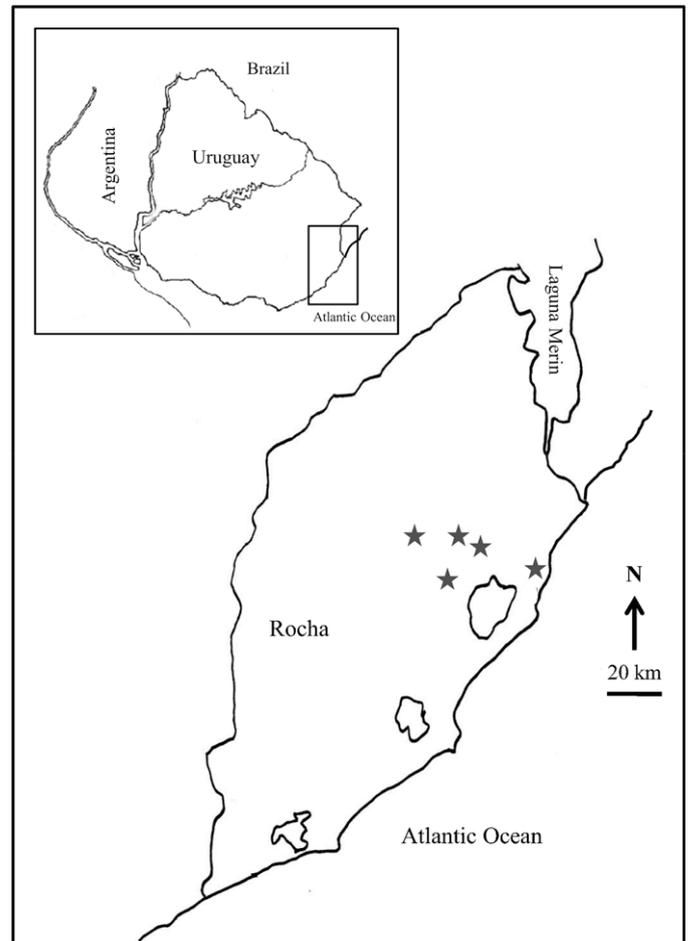


Fig. 1. Distribution of *A. charrua* and collection sites. The ponds are indicated by black stars.

compartment and both males were placed randomly one the right and the other one in the left compartment. The experimental design prevented males from seeing each other and therefore any effect of competition between them (Fig. 2a). During the first hour of acclimation to the experimental aquarium, a black screen visually isolated the female from the males. Later, the screen was removed and two 15 min periods separated by a 1 h interval video records were obtained. After the choice test, the screen was placed back again between the compartments of the female and both males, and individuals were visually isolated the rest of the day.

Supplementary material related to this article found, in the online version, at <http://dx.doi.org/10.1016/j.beproc.2013.01.008>.

2.4. Agonistic interaction

At day 2, the partition between the two males was removed, allowing them to interact freely. Preliminary observations were conducted of male–male encounters, to identify agonistic behavioural units, to establish contest resolution criteria and to determine the winner of the contest. Males exhibited two types of aggressive behaviour: attacks and fins display with body undulations (Table 1). The contest outcome was indicated by morphological and behavioural differences between the contenders. The subordinate male was characterized by colour loss, optional disappearance of vertical stripes and the folding of the dorsal fin, resembling a female. Also, the loser retreated to the periphery of aquarium and remained inactive most of the time, avoiding the other male. In contrast, the dominant fish had the dorsal

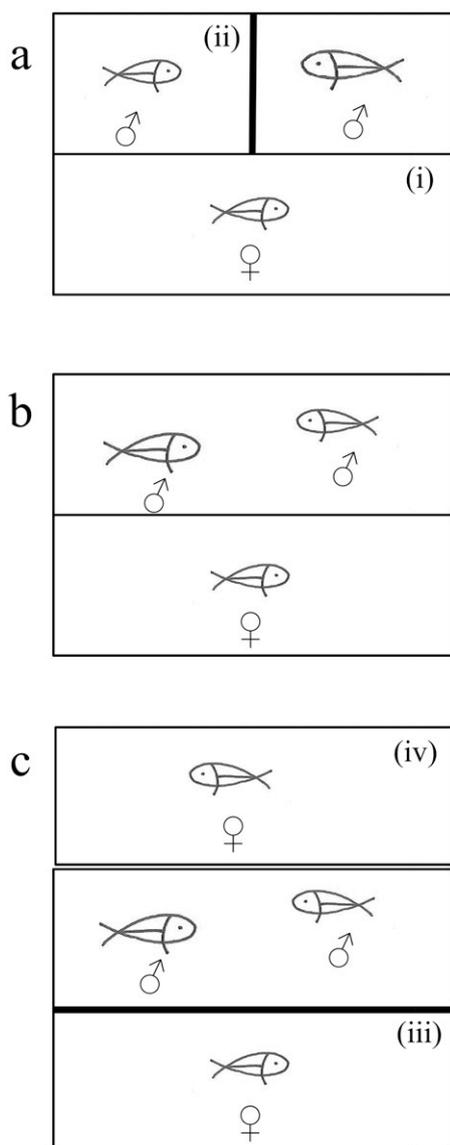


Fig. 2. Experimental setup. (a) During choice tests, the experimental aquarium was divided into three compartments. Transparent glass separated females from males (i) and black glass separated males from each other (ii). The separation between males was removed during agonistic interaction tests. In (b) females witnessed competitive interaction. In (c) females were prevented from visualizing male interaction by an opaque screen placed between female and male compartment (iii). To provide visual stimulation to males during the contest, another female was placed in an adjacent aquarium (iv).

fin deployed, darker colouration and moved freely around the aquarium displaying aggressive behaviour against the other male. Conflict resolution time was considered the period between the start of the encounter until one of the males showed submission (Table 1). Encounters were videotaped until 5 min after submission,

when males were separated by a black glass. Preliminary observations allowed us to determine that conflict resolution was established if the subordinate male does not display any aggressive behaviour for the next 5 min. Although male–male interactions are often aggressive, we separated males very quickly after determining a winner, and did not observe any injuries in any trials.

2.5. Mating preference consistency

After the agonistic interaction, individuals were visually isolated for 3 h, which provided sufficient time return to normal foraging and shoaling activities in aquaria (personal observation C. Passos). To evaluate the consistency of mate choice, we then carried out a second choice test between the same pairs of males. Two 15 min periods, separated by a one hour interval, were video-recorded. To investigate whether visualizing the male–male contest induces changes in the female mating preference, females were randomly separated in two groups. One female group (N = 15) had the opportunity to observe the previous male competition (Fig. 2b), while the other female group (N = 15) was prevented from witnessing the contest by the placement of a black screen between their compartment and the compartment of the males (Fig. 2c). When focal females were prevented from observing the contest, male encounters were observed by a second female who was otherwise not used in the study, to control for any audience effect on male–male interactions (Fig. 2c). The two experimental groups differed neither in female size nor in male size asymmetry (female standard length: two-sample t-test: $t_{28} = 1.721$, $N_1 = N_2 = 15$, $P = 0.097$; difference in males standard length: two-sample t-test: $t_{28} = -0.576$, $N_1 = N_2 = 15$, $P = 0.569$).

2.6. Variables and statistic analyses

After all behavioural trials were completed, the left flank of all fish was photographed in an aquarium of 15 cm × 10 cm × 12 cm, where they were gently immobilized between a sponge and the aquarium's wall. This procedure lasts less than 20 s and the manipulation had no detectable effect on subsequent behaviour. Digital images were used to measure standard length (anterior of head to distal margin of caudal peduncle). At the end of each stage, male appearance was scored along an index between 2 and 6. This score resulted from the sum of colour intensity value and the degree of dorsal fin deployment. The colour intensity is based in an ordinal scale: 1 – no vertical bars on flank, uniform light brown colour; 2 – faint vertical bars on flank, light-coloured sports on the fins faintly visible; 3 – well-defined, high-contrast dark vertical bars against an iridescent blue background, especially around the operculum; unpaired fins darken, intensifying the contrast with light-coloured spots. The ordinal scale of dorsal fin erection is: 1 – totally folded; 2 – partially folded; 3 – totally erect. The highest score (6) indicates the maximum of both colour intensity and fin deployment. In contrast, the lower score (2) indicates a total loss of colour and vertical stripes, and a complete folding of the dorsal fin.

Table 1
Male–male agonistic behaviours.

Attack	Actor bites or attempts to bite recipient, often directed at the fins
Lateral display	Actor orients his flank towards recipient and maintains his position, with the body oscillating slightly and then fins fully extended
Lateral display with contact	Lateral display, above, with both males touching in a parallel or antiparallel position
Sigmoid display	Lateral display with body undulations
Submission	Rapid flight to sides or corner, accompanied by colour loss and folding of the dorsal fin

Female preference was measured as the time spent interacting with each male in a simultaneous choice assay (Basolo, 1990; Kingston et al., 2003; Lehtonen and Lindström, 2008; Walling et al., 2010). Interaction behaviour includes female orientation to the stimulus male, joint swimming, following acceptance and diving acceptance, as detailed in Belote and Costa (2004) and García et al. (2008). Video recordings were analyzed to quantify female interaction with males as well as the courtship of each male. Females interacted with both males in all trials, and 58/60 males displayed courtship behaviour. Male courtship consists of lateral displays with fin vibrations and sigmoid displays (Table 1), as well as invitation to follow and invitation to dive, as described in *A. reicherti* by García et al. (2008). We measured the strength of preference as the difference in the time females spent with each male, and examined its relationship with the male size difference (larger standard length – smaller standard length) and with the female size. Female size could affect not only the strength of her preference for a particular male, but also her general propensity to interact with males. Therefore, the potential relationship between the female size and the interaction time with both males was evaluated. Moreover, males have an active role in courtship (Belote and Costa, 2004), and female preference could be related with the intensity of male courtship. To evaluate the effect of male courtship on female choice, we compared the courtship activity (percentage of time spent courting when the female was in front of his compartment) and the appearance index of preferred and non-preferred males.

Agonistic encounters were divided in two stages: (1) pre-contest: from the start of the encounter (time 0) to the occurrence of the first attack; (2) contest: from the occurrence of the first attack to conflict resolution (resolution time). Conflict resolution was established as the moment in which one fish showed submission. Video recordings were analyzed to determine the pre-contest and contest durations and males' status based on the contest outcome. In addition, we recorded the frequency of aggressive behaviours (attacks, lateral displays, sigmoid displays, Table 1) and evaluated relationship between the resolution time and male size difference (dominant standard length – subordinate standard length).

The same criteria were used to determinate female preference during both, the first stage of the experiment and the stage of evaluation the consistency. A particular female was assessed as consistent when she preferred the same male at day 1 and day 2 (Wong, 2004). A particular male was considered preferred if the female interacted with him more than 50% of the total interaction time. The degree of the preference consistency and the change of the strength of preference between day 1 and day 2 (strength

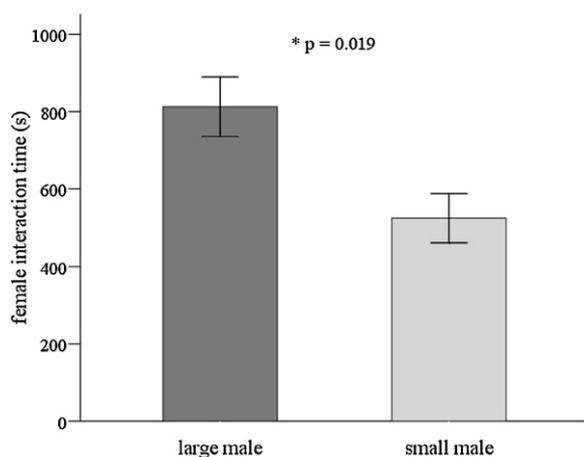


Fig. 3. Female interaction time (mean \pm SE) with stimulus males in a simultaneous choice experiment ($N=30$).

of preference day 2 – strength of preference day 1) was compared between females that witnessed the contest and females that did not. Due to technical problems one essay of each experimental group, was not registered during neither the agonistic encounter nor the evaluation of preference consistency. Data were checked for normality using Kolmogorov–Smirnov test. We applied standard parametric tests (t test, Pearson correlation) to the data, unless they violated the assumptions of normality. In such cases, we applied a corresponding nonparametric test (Wilcoxon signed-rank test, Spearman rank correlation). Binomial tests were used to test the frequency with which large or small males were dominants and to test mating preference consistency. Unless otherwise stated, reported values are mean \pm standard error.

3. Results

3.1. Mating preferences

Females spent most of the time ($74.3 \pm 4.4\%$) oriented to and interacting with one of the males. They also spent significantly more time interacting with the larger male (paired t -test: $t_{29} = 2.475$, $P = 0.019$; Fig. 3). However, preferred and non-preferred males were similar in both time spent courting and in appearance (courtship: preferred male = $68.3 \pm 3.7\%$, non-preferred male = $64.1 \pm 4.9\%$; paired t -test: $t_{29} = 0.696$, $P = 0.492$; appearance index: median of preferred male = 5, median of non-preferred male = 4; Wilcoxon signed-rank test: $Z = -1.403$, $N = 30$, $P = 0.161$). Therefore, female choice was determined neither by the relative time spent in courtship displays nor by male appearance.

There was no relationship between strength of preference and male size difference (Pearson correlation: $r_{28} = -0.007$, $P = 0.971$). Female size was correlated neither with preference index (Pearson correlation: $r_{28} = 0.085$, $P = 0.655$) nor with total interaction time with males (Spearman rank correlation: $r_s = 0.143$, $N = 30$, $P = 0.452$).

3.2. Agonistic interaction

During the pre-contest, males exhibited lateral displays with and without contact (Table 1). The pre-contest lasted 55.3 ± 6.2 s and in 6/28 cases dominance was established during this stage. Otherwise, dominance was established in the contest stage, which lasted 332.0 ± 106.2 s. During the contest, contact displays alternated with attacks and threat displays (lateral and sigmoid displays, Table 1). In each case, one male established dominance over the other, and usually the larger male became the dominant one (24/28; binomial test: $P < 0.001$). During the contest stage, larger males were more aggressive (Wilcoxon signed-rank test: $Z = -4.057$, $N = 28$, $P < 0.001$). Larger males performed significantly more attacks and more lateral displays than smaller ones (Wilcoxon signed-rank test: attacks: $Z = -4.040$, $N = 28$, $P < 0.001$; lateral display: $Z = -4.081$, $N = 28$, $P < 0.001$), but showed no differences in the amount of sigmoid displays (Wilcoxon signed-rank test: $Z = -0.360$, $N = 28$, $P = 0.719$) (Fig. 4). Moreover, appearance index was higher for the larger male of the dyad (median of larger male = 6, median of smaller male = 3; Wilcoxon signed-rank test: $Z = -3.636$, $N = 28$, $P < 0.001$). A negative correlation was found between the resolution time and male size difference (Spearman rank correlation: $r_s = -0.606$, $N = 28$, $P = 0.001$; Fig. 5).

3.3. Mating preference consistency

Females were consistent in their mating preference on day 1 and 2. In 71.4% of the cases, they chose the same male in the two trials (20/28 consistencies; binomial test: $P = 0.036$), and there were no differences between females that had witnessed the male compe-

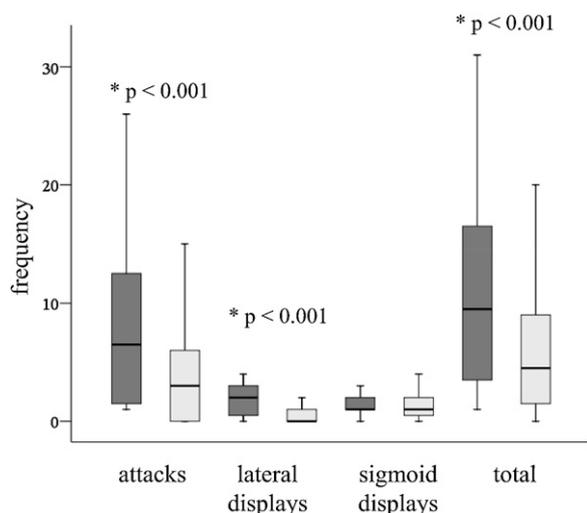


Fig. 4. Frequency of different units of aggressive behaviour identified during male–male competition. The dark grey and light grey bars represent larger and smaller males of the dyad, respectively.

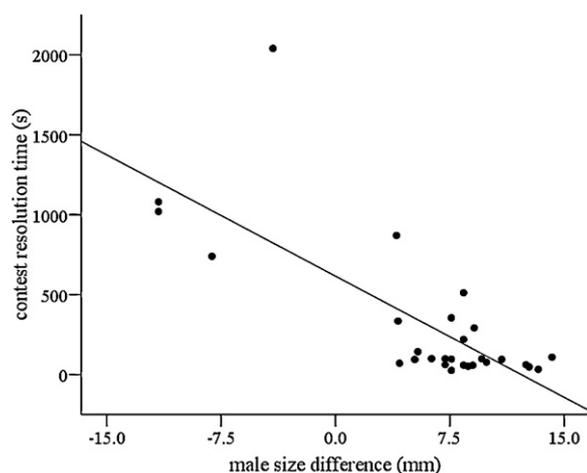


Fig. 5. Relationship between contest resolution time in male–male interaction tests and male size difference (dominant standard length–subordinate standard length).

tion and from those that had not (Fisher's exact test: $P=0.678$; Table 2).

The asymmetry in size between males was not significantly different in the trials where females changed or maintained their preference (consistent trials = 7.9 ± 0.6 mm, inconsistent trials = 9.5 ± 1.1 mm; two-sample t -test: $t_{26} = -1.252$, $N_1 = 20$, $N_2 = 8$, $P=0.222$). Moreover, changes in preference were not directed towards the dominant male (in females that had and had not witnessed the male competition 2/5 and 1/3 of the changes occurred towards the dominant male, respectively). Even though the choice on day 2 was consistent, the strength of preference with the first chosen male (initially preferred) decreased significantly (day

Table 2
Female choice consistency in mating preference tests conducted on two consecutive days, when females were and were not witnessed male's interaction. A female was valued as consistent when she preferred the same male both days (see text).

	Females witnessed male's interaction	Females not witnessed male's interaction	Total
Consistency	9	11	20
Inconsistency	5	3	8
Total	14	14	28

1 = 544.1 ± 75.7 s, day 2 = 242.6 ± 118.1 s; paired t -test: $t_{27} = 2.429$, $P=0.022$). Contrary to our prediction, the average change of the strength of preference with the initially preferred male, was not different between those females that had observed male–male competition from those that had not (females that observed the contest = -508.9 ± 164.9 s, females that did not observe contest = -94.1 ± 173.8 s; two-sample t -test: $t_{26} = -1.731$, $N_1 = N_2 = 14$, $P=0.095$). Subsequent male behaviour was not affected by contest resolution, since, during the preference test of the second day, neither the appearance index nor the courtship percentage were significantly different between dominant or subordinate males (appearance index: median of dominant males = 4, median of subordinate males = 4; Wilcoxon signed-rank test: $Z = -0.994$, $N = 28$, $P=0.320$; courtship: dominant males = $49.7 \pm 6.5\%$, subordinate males = $43.7 \pm 5.0\%$; paired t -test: $t_{27} = 1.606$, $P=0.120$).

4. Discussion

This study demonstrates that both intra- and intersexual selection favour larger males in *A. charrua*. Females interacted more with the larger male in a classic two choice paradigm. Also, male body size affected contest outcome with the larger male almost always becoming socially dominant.

The proportion of time that females remained oriented to and interacting with males (approx. 75%) and that males allocated to courtship activity (approx. 65%) in choice tests, reflects reproductive motivation and validates the use of *Austrolebias* in an experimental design widely used for fish mate choice evaluation (e.g. Berglund and Rosenqvist, 2001; Kangas and Lindström, 2001; Walling et al., 2010; Wong, 2004). Preference exhibited by *A. charrua* females towards larger males is consistent with female's choice based on male body size, documented already in several taxa (Andersson, 1994), especially in fishes (Rosenthal and Evans, 1998; Ryan and Wagner, 1987; Ryan et al., 1990).

Female preference for larger males may have several causes. First, females may obtain direct benefits from mating with larger males because body size is correlated with dominance status (Wong and Candolin, 2005). Potential direct benefits of mating with dominant males could include spawning sites and refuges, both limiting factors for reproduction and viability of eggs. Second, larger males could generate great visual stimulation and therefore elicit sensory bias underlying female preferences (Rosenthal and Evans, 1998). Third, female preference could also evolve via indirect selection on male traits (Andersson, 1994; Fisher, 1930; Zahavi, 1975). Our results indicate that female preference for large male was not affected by the size difference between males. Possibly the variation in the males' size was too narrow because in most of dyads the big male was between 1.1 and 1.3 times greater than the small male.

Several studies have shown mating preference in favour of males with higher intensity of courtship display or more colours (see Andersson, 1994; Kokko et al., 2003). In the present study, neither the courtship time nor the appearance index differed between preferred and non-preferred males. In this experiment, female preference was evaluated against a pair of males with size asymmetry, previously selected according to body size. Although this approach increases our ability to detect preference for male size, it unavoidably reduces our power to detect the relative contribution of other traits.

Body size also affected the outcome of intrasexual competition, with larger males more dominant in male–male interactions. Larger males also have a competitive advantage in many fish species (Beaugrand et al., 1996; Benson and Basolo, 2006; Moretz, 2003). As found in numerous other studies (Arnott and Elwood, 2009; Briffa and Sneddon, 2010), conflict length decreased with increas-

ing difference in body size differences of contenders. The larger male, usually the winner, performed a greater number of agonistic displays towards his opponent.

Lateral and sigmoid displays are behaviours performed by males at courtship and aggressive contests, and similar behaviours are found in numerous other fishes (e.g. Enquist and Jakobsson, 1986; Morris et al., 1995). The lateral display, in which one individual exposes his flank to the opponent with fin extension and oscillation, likely provides vibrational, visual, and tactile cues that are correlated with body size. Sigmoid displays instead, in which an individual performs high speed undulating body movements, may be predictors of body condition, motivation and locomotor skills of the sender. Consistent with this interpretation, during agonistic encounters, larger males performed more lateral displays and more attacks than shorter ones, while the frequency of sigmoid displays was not related to body size.

Finally, the influence of witnessing the competitive interaction between males in female mating preference of *A. charrua* was evaluated. Females were consistent in their choice independent of whether they had observed or not the male contest. However, the overall strength of preference decreased on day 2, and the decrease was independent of whether the preferred male was dominant or subordinate in the competitive interaction, suggesting that competition did not increase the attractiveness of the dominant male over the subordinate. However, given our inability to distinguish between the effects of two highly correlated traits, we cannot conclude that female perception of male dominance is irrelevant in female choice. Larger males both have an advantage in agonistic interactions and are preferred by females. This pattern is consistent with the dual utility model (Berglund et al., 1996), in which important intrasexual competition traits are expected to be used by females as reliable signals for mate choice. Alternatively, female preference could simply be a function of sensory stimulation, with larger males eliciting more of a response from the visual periphery (Rosenthal and Evans, 1998). Since both female preference and male–male competition favour larger size, additional studies are required to assess the relative importance of intra- and intersexual selection in nature.

The fitness effects for females of mating with certain males may vary in magnitude and direction as environmental conditions or individual phenotypes change (Borg et al., 2006; Forsgren et al., 2004; Jennions and Petrie, 1997; Reichard et al., 2008). Annual killifish represent an interesting and novel model to study seasonal variation in sexual selection. The habitat of these fishes is extremely fragile; it consists of temporary ponds that dry up in summer and that vary hugely across several environmental axes throughout the season. This context, combined with substantial changes in sex ratio over the course of the short breeding season (unpublished data C. Passos), offers an excellent opportunity to investigate the causes and consequences of variation in the strength and direction of sexual selection over space and time (Forsgren et al., 2004).

In conclusion, our research shows that, in the annual fish *A. charrua*, intrasexual competition and mate choice act in concert with respect to male body size. This promising model in evolutionary ecology and developmental biology is amenable to powerful experimental studies of sexual selection and social interaction.

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