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Pair-bonding influences affective state in a monogamous fish species

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In humans, affective states are a key component in pair-bonding, particularly in the early stage of a relationship. Pairing with a high-quality partner elicits positive affective states which, in turn, validate and reinforce the mate choice. Affective states thus strongly affect pair stability and future reproductive success. We propose generalizing the link between affective states and pair-bonding to encompass other monogamous species exhibiting biparental care, chiefly where the reproductive success of the pair critically depends on the coordination between partners. The convict cichlid *Amatitlania siquia* is a monogamous fish species that forms long-lasting pairs with strong cooperation between parents for parental care. In this species, we showed that females paired with their non-preferred male had lower reproductive success than those paired with their preferred male. We then transposed the judgement bias paradigm, previously used in other animal species, to assess objectively affective states in fishes. Females that were assigned their non-preferred partner exhibited pessimistic bias, which indicates a negative affective state. By contrast, females that were assigned their preferred partner did not exhibit changes in their affective state. Our results highlight that the influence of pair-bonding on affective states is not human-specific and can also be observed in non-human species.

1. Introduction

Affective states (which are also referred to as emotional states or mood [1–3]) result from positive or negative experiences and can be objectively measured by the means of physiological, neurological, behavioural and cognitive markers [2,4–6]. Affective states are characterized by their valence (positive or negative) and their intensity (low or high) [5,6]. Following this practical definition, affective states do not imply conscious feelings [7,8] and have been reported in not only humans [9,10] but also other animal species, including mammals [1,11–15], birds [16–18], fishes [19] and invertebrates [20–22].

Affective states are influenced by internal factors such as hunger state or hormonal level [5,23] and environmental factors such as food quality or quantity [8,21], habitat quality [7], presence of predators [21] or conspecifics [17,24]. In turn, these affective states bias decision-making [25–28]. Such bias is observed when individuals more easily remember negative events than positive events (memory bias [29]), or when they pay more attention to negative events (attention bias [24,30]). Affective states are associated with judgement bias [4,8] when individuals overestimate the probability of loss or gain. Let us consider a hypothetical example: individuals who are repeatedly exposed to predation threats are in a negative affective state, which leads them to overreact to uncertain or ambiguous information. Branch movements in a shrub owing to an inoffensive breeze are usually ignored. By contrast, when in a negative affective state, individuals may interpret this neutral information as indicating an imminent attack by a predator, leading them to over-invest in antipredator behaviours, which is a sensible strategy to maximize survival probability [6,31,32].

Similarly, in the sexual context, affective states may carry relevant information about the state of a relationship or the quality of the partner, and they are expected to influence sexual decisions. Indeed, the reproductive

success of the individuals strongly depends on the quality of the chosen partner which is challenging to accurately estimate and monitor. This quality is multi-dimensional and is defined by many factors such as the genetic or behavioural compatibility between partners, or partner's commitment in parental care [33,34]. The affective state could be a way to summarize such complex information. Repeated negative interactions with a partner (e.g. repeated absence or low investment in parental care, aggressive behaviours) may ultimately result in a negative affective state that would in turn increase the probability of pair separation. By contrast, pairing with a compatible partner is expected to induce a positive affective state which validates the mate choice and strengthens the current relationship. To date, this effect of the quality of the relationship between partners on their affective states has only been described in humans [35,36]. Psychological studies have reported that the presence of the partner stimulates a positive affective state, whereas its absence elicits a negative mood [37]. This mechanism favours commitment to the relationship and thus long-lasting pairing in humans [35,36]. We hypothesize that similar mechanisms also exist in other species exhibiting long-lasting pair bonds. For instance, in most birds and some fish species, the rearing of young requires long-lasting nest defence and parental care, which assumes stable pair-bonding [38–41].

The convict cichlid *Amatitlania siquia* is therefore a suitable candidate model species to test the influence of pair-bonding on affective states. In this monogamous fish species, the young are highly vulnerable to predation, and parents must coordinate their behaviours for several weeks, during which the young develop from eggs to independent fry [41–43]. Finding a suitable mate is thus decisive for long-lasting pair-bonding and reproductive success. We predicted that the presence of such a male induces a positive affective state in the female, whereas being separated from a suitable male and remaining with a less suitable male induce a negative affective state. To test this prediction, we first had to address two practical issues: (i) we had to identify the preferred male for a given female in a binary choice test. We then compared her reproductive success after random pairing with either the preferred or the non-preferred male; and (ii) we also had to objectively assess the females' affective states. We transposed the cognitive judgement bias test to a fish species [44,45]. This test is based on the animal's response in the face of an ambiguous signal that is intermediate between previously learned positive and negative signals. In this ambiguous situation, a behavioural response that becomes more similar to the responses to the negative signal operationally characterizes a pessimistic judgement bias, which reveals a negative affective state. Conversely, a response that becomes more similar to the responses to the positive signal characterizes an optimistic judgement bias and reveals a positive affective state [5,7,8,11]. Using this experimental paradigm, we predicted that females paired with their preferred male are in a positive affective state, whereas females paired with their non-preferred male are in a negative affective state.

2. Material and methods

A total of 68 female convict cichlids were used in this study (for more details about the animals and rearing conditions, see the electronic supplementary material, appendix S1). The same set-

up was used in all experiments. Several days prior to the experiments, the focal female was housed in the central compartment of a 200 l tank separated into three identically dimensioned compartments by a plastic mesh partition (2 × 2 mm mesh squares) allowing visual, chemical and acoustic communication between the fish (figure 1a). In the front part of the focal compartment, a 12 cm wide opaque partition visually isolated the choice area that was used in experiment 2. The focal compartment was equipped with an artificial shelter (8 cm diameter PVC tube) and an artificial plant. The female remained in the same compartment for the complete duration of the experiments described below. The behavioural data were recorded in the absence of the experimenter using video cameras (Nikon D7000 and Sony HDR-PJ410).

These 68 females were randomly divided into two experimental groups. The first group (experiment 1, $n = 33$ females) was used to validate the method to identify her preferred and her non-preferred male in a binary choice test and predict the subsequent reproductive success of a pair. It was then possible to use this rapid test to investigate the influence of the preferred or non-preferred male on the female's affective state (experiment 2, $n = 35$ females).

(a) Experiment 1: female's preference and long-term reproductive success ($n = 33$)

Sexual preference was assessed using binary choice tests. The females were allowed to choose between two males, one in the left compartment and the other in the right compartment. Because body length is a major mate choice criterion used by females in this species [46], each male dyad were chosen in such way that the two males differed in body length (mean length difference = 1.59 cm, 95% confidence interval (CI) = (1.48, 1.71), Cohen's $d = 2.73$, 95% CI = (2.16; 3.61)) to facilitate the expression of a clear preference by the female. The experiment began after 30 min of acclimatization for the males. During three observation periods of 20 min (day 1: between 9.00 and 10.00 and between 16.00 and 17.00; day 2: between 9.00 and 10.00; see timeline, electronic supplementary material, figure S1), we recorded the total time spent by the female in front of each male, at a distance of less than one body length from the partition. The preference score was estimated from the relative time spent close to each male (total time spent on his side divided by total time spent in front of both males). The female was assumed to prefer a male when her preference score for this male was above 50%, which has been shown to be a reliable predictor of the reproductive preference in this species [47]. The strength of the preference was defined as the preference score for the preferred male.

Immediately after the third observation period of mate choice (see timeline, electronic supplementary material, figure S1), one of the two males was removed, and the other was introduced into the female's compartment by gently and temporarily removing the mesh partition. The female was randomly assigned either her preferred partner ($n = 16$) or her non-preferred one ($n = 17$), which were identified in the mate choice test. We estimated female investment, pair compatibility and reproductive success using four parameters: (i) spawning latency (time in days between pair formation and spawning) was measured by visually inspecting the tanks twice a day at 10.00 and 17.00; (ii) investment of the female in the spawn was assessed based on the proportion of time spent attending the eggs (ventilating and mouthing the eggs) during 10 min observation periods, the day following the spawning at 10.00; (iii) intra-pair conflicts were assessed by the frequency of agonistic behaviours between the partners. This measure was calculated as the number of lateral and frontal displays and biting attempts per minute, regardless of the identity of the initiator, during 10 min

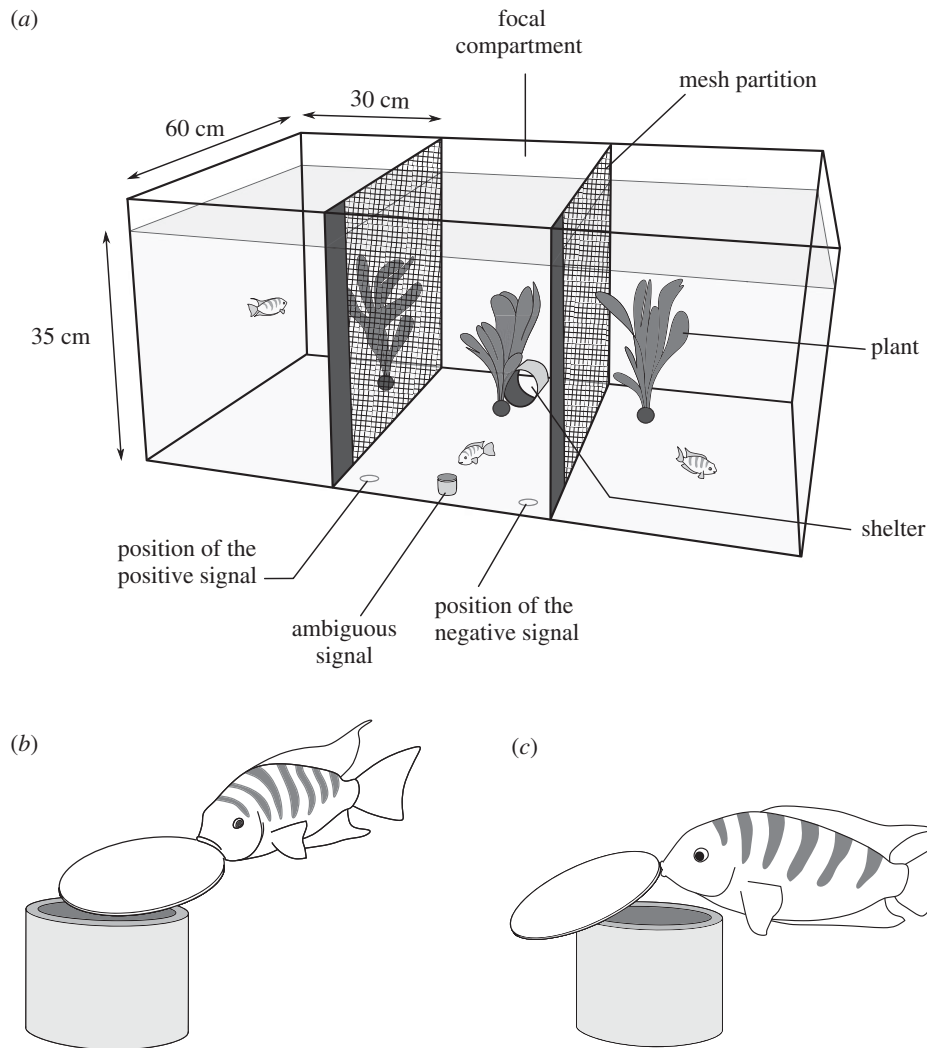


Figure 1. (a) The same set-up was used for experiments 1 and 2. The box in the front of the tank was only introduced in experiment 2. (b,c) Experiment 2: the female in the focal compartment was trained to remove the lid of a cylindrical box located in the front of her compartment, 2 cm from the glass. Each fish succeeded in performing this task either by pulling (b) or pushing (c) the lid (see example in the electronic supplementary material, video S1). The female was first trained to discriminate between sequentially presented positive (rewarded) and negative (unrewarded) signals based on a combination of randomly allocated spatial (left or right side of the compartment) and visual (black or white lid) cues. The judgement bias test evaluated her response to an ambiguous signal (intermediate position and colour).

observations at 10.00, 24 h after pairing (before spawning) and 24 h after spawning; and (iv) reproductive success was assessed from the number of live fry three weeks after spawning as a consequence of partner coordination in parental care [41].

(b) Experiment 2: influence of the male on an indicator of female's affective state ($n = 35$)

We used the cognitive judgement bias paradigm to objectively measure the change in the affective state of the female in the presence of her preferred or non-preferred male [5,44,48]. The rationale behind the judgement bias test is that the behavioural response to an ambiguous signal (intermediate between a positive signal and a negative signal) is a direct indicator of the positive/negative affective states of the individual [8,11,21,45]. We transposed this test to fish by training them to open boxes (figure 1b,c). These boxes were made of rigid polymer clay (Fimo, Staedtler, Germany). The base of the box was a cylinder with an external diameter of 28 mm and an external height of 20 mm (Fimo 8020-42), and the interior cavity of the boxes was in the shape of a hemisphere (25 mm in diameter) to avoid dead angle and facilitate the collection of food rewards. The lids were flat discs measuring 30 mm in diameter and 1 mm

thick (Fimo 8020-0 pure white, Fimo 8020-9 pure black or a grey mix of both colours).

Each focal female was first trained to discriminate between a positive and a negative box. When opening the positive box, the fish were able to collect a food reward (one chironomid larva). Following recent studies [18,21,49,50], we used a box without a reward as a negative signal. Despite the absence of active punishment, opening this box is potentially associated with frustration when opening an empty box [49] and with several costs (energetic costs, time costs or exposure to predation threats) when the individual leaves its nest to approach the rear end of the tank. To maximize the discriminability of the positive and negative boxes, they differed according to two cues: their location (on the left or right side of the tank with a distance of 20 cm between these two positions, figure 1a) and the colour of their lid (black or white). For instance, for a given focal fish, the rewarded box was presented alone on the left side of the tank with a white lid (positive signal), and the unrewarded box was presented alone on the right side of the tank with a black lid (negative signal). The combination of spatial and colour cues was randomized between females. For more details on the training protocol, see the electronic supplementary material, appendix S2.

The judgement bias test consisted of evaluating a females' latency to open a box associated with an ambiguous signal (A).

This signal was an unrewarded box placed at an equal distance (10 cm, figure 1a) between the positions of the positive (P) and negative (N) signal. This box was covered by a grey lid with an intermediate brightness between the black and white lids used for the positive and negative signals (see the electronic supplementary material, figure S3 for details about grey scaling). The response latency to the ambiguous signal was an indicator of a female's affective state; the shorter the response latency was, the more positive the affective state (see example in the electronic supplementary material, video S1). An experimental session consisted of the sequential presentation of six boxes (one box at a time, with 5 min between two consecutive presentations) in the following order: P, N, A, P, N and P. The judgement bias of the focal fish was estimated from the third, fourth and fifth boxes (A, P and N). The first two boxes (P and N) were reminder trials [21], and the last box (P) was used to keep the fish motivated for the next session. Because the judgement bias of each female was repeatedly assessed in three sessions, we only used one ambiguous signal in each session to prevent her from learning that the ambiguous signal was not rewarded [12,45]. The fish was at the opposite end of the tank when each box was introduced, thus standardizing the distance between the individual and the box (see example in the electronic supplementary material, movie S1). The observation started immediately after the introduction of the box and lasted for 15 min, during which we recorded the latency time to the opening of the box. A maximum latency time of 901 s was recorded if the fish did not open the box, and it was taken into account as censored data in the Cox model analyses.

Three sessions of judgement bias test took place on three consecutive days (one session per day, see timeline in the electronic supplementary material, figure S1) and corresponded to three different contexts. (i) The first session ('before mate choice' context) was performed in a non-sexual context, with one female in each adjacent compartment to avoid social isolation. The adjacent females were then removed and replaced by two males (average difference in body length = 1.23 cm, 95% CI = (1.11, 1.35), Cohen's $d = 3.40$ and 95% CI = (2.91; 4.18)). Following the procedure validated in experiment 1, we assessed female preference for one of these two males (mean preference score: 66.3%, 95% CI (63.3%; 69.5%), repeatability $R = 0.38$, 95% CI (0.18; 0.58), $p = 0.0001$). We controlled for the initial absence of bias and lateralization in the females' preference. The left male was not more frequently preferred than the right one ($\chi^2_1 = 0.457$, $p = 0.499$). Female preference for one male was not biased by the position of the positive signal during the judgement bias test ($\chi^2_1 = 0.060$, $p = 0.81$). (ii) The second session of the judgement bias test ('during mate choice' context) took place in the presence of the two males. These first two sessions were used as controls to estimate the initial affective state of the female and the repeatability of this measure, which is rarely assessed in judgement bias studies [8]. We verified that the learned combination of spatial (left versus right side) and colour (black versus white) signals did not affect the female response latencies (electronic supplementary material, table S1). (iii) In the third session of the judgement bias test ('with one assigned male' context), the 35 females were randomly divided into two treatment groups: 18 females were assigned their non-preferred male (their preferred male was removed from his compartment); 17 females were assigned their preferred male (their non-preferred male was removed). The females from these two groups did not differ in their initial response latencies in the first two sessions, i.e. 'before mate choice' and 'during mate choice' contexts (electronic supplementary material, tables S2–S4). We assessed the influence of the partner on the female's affective state by comparing her response latency in the three sessions, the female being her own control.

(c) Statistical analysis

(i) Experiment 1

The repeatability (intraclass correlation coefficient, ICC) of the female preference score for the preferred male was calculated using R package 'rptR', with female identity as a random factor [51]. The spawning latencies of the females from the two treatment groups (preferred or non-preferred male) were compared using the Cox proportional hazard models, using the 'coxph' function from the R package 'survival' [52]. The proportions of time spent attending the eggs by the females from the two treatment groups were compared using Student's t -test after arcsine square root transformation. The numbers of intra-pair agonistic behaviours in pairs from the two treatment groups were compared using mixed-effects linear models with female identity as a random variable using the 'lmer' function from the R package 'lme4' [53]. The numbers of fry between treatment groups were compared using the Wilcoxon signed-rank test because the conditions for parametric tests were not met.

(ii) Experiment 2

The repeatability of the female's preference score and her response to the ambiguous signal were calculated using ICC, as in experiment 1. Following the recommendation for the statistical analysis of data from a go/no-go experiment [54], we used mixed-effect Cox models with the female's identity as random variable to take into account the repeated measures design. A distinctive feature of our study was that the responses of each female were measured sequentially in the three contexts (before mate choice, during mate choice and with one assigned male). Instead of comparing the response latency of females from two independent groups as commonly done in judgement tests, we followed the recommendation of Roelofs *et al.* [45] and Bateson [8] and assessed the change in response latency of the individual across contexts, the individual being its own control.

A prerequisite for the judgement bias test [5] is that the response latencies to the positive signal do not differ between treatments (preferred versus non-preferred male) or contexts (before mate choice, during mate choice and with the assigned male). The same condition must also be verified for the negative signal. To indicate a change in affective state, the response latency should only differ for the ambiguous signal. We thus analysed the response latencies for each signal (positive, ambiguous and negative) separately. Thus, we used mixed-effect Cox models with the 'coxme' function from the R package 'coxme' [55]. For each signal, the full model included the treatment (preferred versus non-preferred male), context (before mate choice, during mate choice and with the assigned male) and their interaction term as fixed variables, and the identity of the female as a random variable. We followed a backward stepwise model selection procedure to identify the best model. We first assessed the significance of an interaction term by comparing the models with and without the interaction using likelihood ratio tests. When the interaction was significant, we split the data as a function of the treatment (preferred versus non-preferred male), and we continued the model selection procedure by assessing the significance of the context (before mate choice, during mate choice and with the assigned male). When the interaction term was non-significant, the effect of each variable was assessed sequentially by removing one variable at a time.

The same procedure of backward model selection was used for each linear model presented herein. For every Cox model, the measure of effect size for the latencies was the hazard ratio (HR), estimated as the exponential of the regression coefficient. An absence of difference between the observed latency time and the control latency time corresponds to HR equalling 1, whereas an increase in latency time corresponds to HR of greater

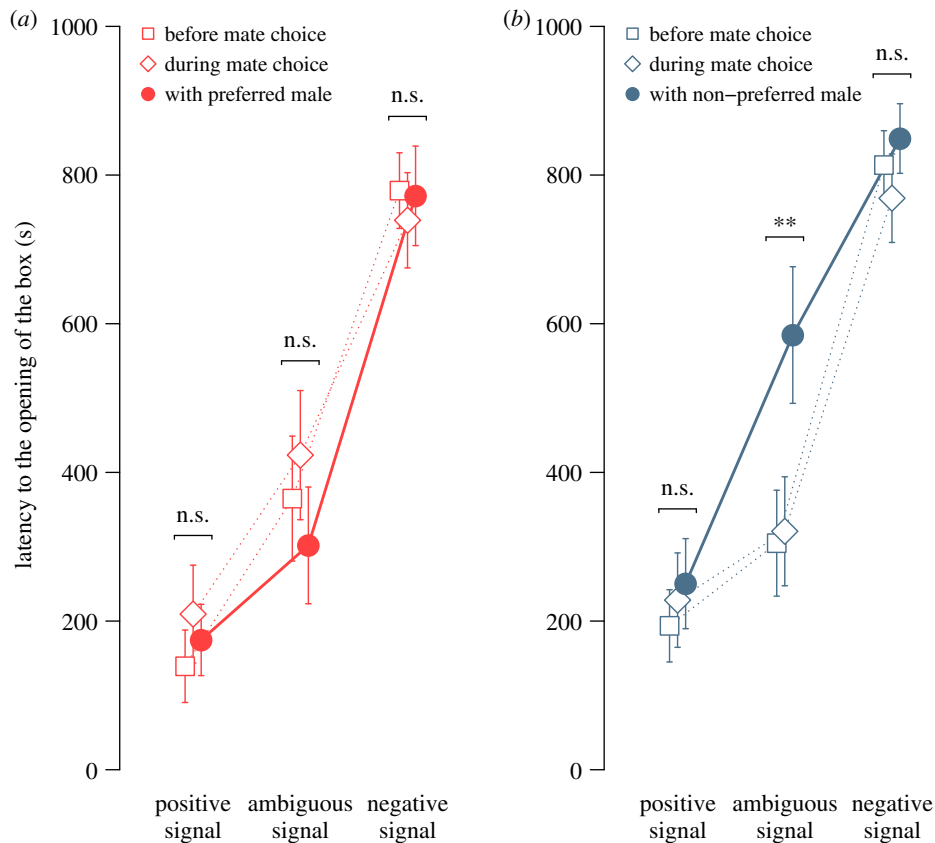


Figure 2. Evaluation of females' affective state based on the judgement bias paradigm (experiment 2). The latencies to the opening of the boxes associated with the positive, ambiguous and negative signals were measured repeatedly in three contexts: before mate choice, during mate choice and with one assigned male. Females were randomly divided into two treatment groups; they were assigned their preferred (a) or non-preferred (b) male ($n = 17$ and $n = 18$, respectively). The error bars denote the standard error. Asterisks indicate significant differences ($p < 0.01$) of the mixed-effects Cox regression. (Online version in colour.)

than 1 [56]. All statistical analyses were performed using R version 3.5.2 software [57].

3. Results

(a) Experiment 1: female's preference and long-term reproductive success

On average, the females spent 70.3% (95% CI (66.1%; 74.6%)) of their time in front of the preferred male, and this preference score was consistent across the three replicates of the binary choice test (repeatability: $R = 0.24$, 95% CI (0.029; 0.48), $p < 0.018$). The females were not lateralized; the left side was not more frequently preferred than the right side ($\chi^2_1 = 3.03$, $p = 0.082$). When paired with their preferred male, the females invested more in reproduction; they spawned earlier (Cox regression, $\chi^2_1 = 5.28$, $p = 0.022$, HR = 2.42, 95% CI = (1.14; 5.15); electronic supplementary material, figure S4a), and they spent more time attending their eggs (Student's t -test: $t_{22} = 2.65$, $p = 0.015$, Cohen's $d = 1.03$, 95% CI = (0.33; 1.98); electronic supplementary material, figure S4b) than did females paired with their non-preferred male. Pair compatibility was also higher for females paired with their preferred male than those paired with their non-preferred male, as fewer agonistic displays were observed between partners both before and after spawning ($\chi^2_1 = 7.73$, $p = 0.0054$; electronic supplementary material, table S7 and figure S4c). Finally, reproductive success was higher for females paired with their preferred male, as they had more fry three weeks after spawning

than did the females paired with their non-preferred male (Wilcoxon test: $W = 209$, $p = 0.0088$, Cohen's $d = 1.00$, 95% CI = (0.28; 2.10); electronic supplementary material, figure S4d).

(b) Experiment 2: influence of the male on an indicator of female's affective state

The female response latencies to the positive or the negative signal were not affected by the treatment (preferred versus non-preferred male), the context (before mate choice, during mate choice and with the assigned male) and their interaction (figure 2; electronic supplementary material, tables S2 and S3), which was a prerequisite for the judgement bias test. Between the two control contexts (before mate choice, and during mate choice), their responses were repeatable for the positive ($R = 0.37$, 95% CI = (0.09; 0.62)), the ambiguous ($R = 0.59$, 95% CI = (0.32; 0.78)) and the negative ($R = 0.41$, 95% CI = (0.14; 0.65)) signals.

In contrast with the positive and negative signals, there was a significant effect of the interaction between the treatment (preferred versus non-preferred male) and the context on the response latency to the ambiguous signal ($\chi^2_2 = 9.29$, $p = 0.0096$; electronic supplementary material, table S2). For the treatment group in which the females were assigned their preferred male, the response latency to the ambiguous signal did not change across contexts (figure 2a; $\chi^2_2 = 1.62$, $p = 0.45$; electronic supplementary material, table S4). The response latency also did not significantly decrease during the course of the experiment (comparison 'before mate

choice' versus 'with preferred male': HR = 0.76, 95% CI = (0.33; 1.74); comparison 'during mate choice' versus 'with preferred male': HR = 0.58, 95% CI = (0.25; 1.34)). For the treatment group in which the females were assigned their non-preferred male, the response latency to the ambiguous signal significantly differed as a function of the context (figure 2b; $\chi^2_2 = 9.66$, $p = 0.0080$; electronic supplementary material, table S4). The response latency was higher 'with the non-preferred male' than in the first control context (comparison 'before mate choice' versus 'with preferred male': HR = 3.74, 95% CI = (1.37; 10.24); comparison 'during mate choice' versus 'with preferred male': HR = 3.83, 95% CI = (1.43; 10.21)). In addition, this change in affective state was significantly affected by the strength of the female's preference; the stronger the preference was for the male, the higher the response latency to the ambiguous signal after his removal ($\chi^2_1 = 4.33$, $p = 0.037$, $r = 0.38$; electronic supplementary material, tables S5 and S6).

4. Discussion

Using the judgement bias test, we showed that the females which were assigned their non-preferred partner increased their response latency to the ambiguous signal, which characterizes a pessimistic bias [5,7,8,11]. This result thus provides evidence, in a non-human species, that affective state depends on the sexual partner.

The first step of this study was to develop an objective measure of the affective state in a fish species. Because homologous limbic and dopaminergic structures involved in emotional processes have been reported in fishes [58], the existence of an emotional response has been previously investigated using physiological and neuromolecular indicators [19]. However, such measures have some limitations; even if they reflect the intensity (low/high) of the emotional response, they generally provide poor information about its valence (positive/negative) [5,59]. For instance, an increase in heart rate can result from positive valence (arousal owing to the presence of a sexual partner), negative valence (fear owing to a threat) or even neutral valence (increased locomotor activity). By contrast, the judgement bias test enables an objective evaluation of the valence of cichlid affective states [5,8,11,48]. In contrast with most neurophysiological measures, the judgement bias test also has the advantage of being non-invasive. This cognitive test has thus been established as the 'gold standard' [13] for measuring affective states of a range of non-human species [21,44,45], but, to our knowledge, it had not been developed in fishes until now.

In humans, the influence of a partner on affective state is well established [36,60], but such a relationship has never been investigated in non-human species. In our study, we observed that in female convict cichlids, the separation from a preferred partner induced judgement bias compared with their initial responses to the ambiguous signal. Their response latency to the opening of an ambiguous box increased in the absence of their preferred male. One could object that such a response does not correspond to a change in affective state but to the female's lower motivation to approach the boxes owing to decreased locomotor activity or increased social stress caused by the non-preferred male. The females may also actively search for the 'missing' preferred male, which may have interfered with their attention to the judgement

bias task. These alternative explanations are unlikely because females did not increase their response latency to positive and negative boxes. A prerequisite to interpreting animal responses to the judgement bias test, as an indicator of affective state, was that their response to unambiguous signals remained unaffected [5], as reported herein.

In addition, the majority of studies using judgement bias tests compared the responses of individuals from different treatment groups, but few studies have directly compared changes in affective states within the same individuals [8,45,50]. One strength of our protocol was using the female as her own control. We first measured the initial affective state of each female and verified the repeatability of this measure during two control contexts. We then measured the female affective state in a third context (with one assigned male). The reported delayed response latency to the ambiguous signal indicated *within-individual* change in the affective state.

In addition to the relationship between the presence of a female's partner and her affective state, we observed that being paired with a non-preferred partner decreased compatibility between partners, the female's investment in reproduction and the reproductive success of the pair. Affective states may summarize multi-dimensional and uncertain information about the future outcomes of pairing with a given male. Negative affective states could inform individuals about unfavourable situations and encourage them to adjust their condition, while a positive affective state could be a sign that the situation is favourable and that they should avoid any immediate changes [61]. When sexual displays or parental care behaviours are well coordinated between partners, the resulting positive affective state may stabilize the pair and promote higher commitment towards reproduction.

This result raises a number of questions about the dynamics of affective state changes [50]. Does the effect on affective states caused by separation with the preferred male fade over time? How long does it take to return to the initial affective state? Is the initial affective state restored by the return of the preferred male? We predict that the change in affective state is neither highly volatile nor irreversible. Temporarily losing sight of the partner should not systematically elicit an immediate change in the female affective state because it would jeopardize pair stability. By contrast, divorce from a male should induce a negative affective state in the female, which would lead her to interrupt her own investment in the current reproductive event in favour of the expected future reproductive events with a different partner. However, this change should be reversible so that the female is receptive to subsequent reproduction. These open questions are particularly challenging to address because a large number of presentations of the ambiguous signal would be needed to test them, which would result in the signal losing its ambiguous nature after several repetitions [12,45].

5. Conclusion

Our results suggest that the relationship between affective state and pair-bonding has evolved not only in humans but also in at least one other monogamous species, the convict cichlid. There is increasing evidence that non-human animals experience similar emotions to humans [62–66]. Even if human relationships are particularly complex and refined, there is no reason to deny *a priori* the existence of emotional

attachment to a partner in non-human species. The ecological pressures experienced by monogamous species may have shaped affective state responses to pair-bonding to stabilize reproductive pairs. If confirmed in phylogenetically distant species, then this affective state response would indicate evolutionary convergence; thus, we recommend that affective state response to pair-bonding be experimentally assessed in a wide variety of species, particularly those characterized by long-lasting biparental care.

Ethics. Animal care and all of the experimental procedures described were approved by Ethics Committee of the universit  de Bourgogne.

Data accessibility. Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.m7v5t98> [67].

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Electronic supplementary material for

Pair-bonding influences affective states in a monogamous fish species

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Appendix S1. Details on study animals and housing conditions.

Appendix S2. Details on the training protocol for the judgment bias test (Exp. 2)

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Table S5. Response latency a function of the preference score and the treatment in the third context ('with one assigned male') and separately for each signal (Exp. 2)

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Supplementary references

Appendix S1. Details on study animals and housing conditions

Individuals were from our laboratory breeding stock, which originally came from local commercial distributors. At the beginning of the experiments, the fish were approximately one year old and were sexually mature. They were housed in tanks (450 L, 150×50×60 cm) equipped with artificial plants, artificial shelter, rocks and gravel at $25 \pm 1^\circ\text{C}$ under a 12:12 light:dark cycle until the beginning of the training. The fish were housed in same-sex tanks (40-50 fish per tank) to ensure sexual receptivity and to avoid reproduction before the experiments. The fish were fed daily with Cichlid XL flakes (Tetra®, Germany). All of the experiments were performed between March and November 2017. The experiment was carried out with females for two main reasons. First, the cues used by the females to choose a partner (body length) have been described in previous studies (Dechaume-Moncharmont et al. 2011, 2013), which allowed for the formation of dyads of contrasting males (i.e., differing in body length) for the binary choice test. Second, the protocol for quantifying female preference for a male (time spent in front of the preferred male) and her investment in reproduction (spawning latency, time spent attending the eggs) has been validated (Dechaume-Moncharmont et al. 2011, 2013; Laubu et al. 2016). Similar information is scarce for males.

Appendix S2. Details on the training protocol for the judgement bias test (Experiment 2)

Before Experiment 2, each female was housed with two females, one in each adjacent compartment (Fig. 1), to limit social isolation. She was trained first to find a reward (one chironomid larva) in an open box (see main text for a description of the box), then in a half-open box, and finally in a box completely covered by a lid. These boxes were always located in the same place (either on the left or right side of the compartment) and were associated with the same colour lid (either black or white), which both characterized the positive signal in the remainder of the experiment. Once a female had learned to open the box (Fig. 1b-c), she was trained to discriminate between the rewarded box (positive signal) and an unrewarded box (negative signal). These two boxes differed according to a random combination of spatial (box on the left or right side of the tank with a distance of 20 cm between these two positions, Fig. 1a) and visual (black or white lid) cues. They were presented sequentially.

The training consisted of successive sessions, with only one session per day (between 2:00 and 4:00 p.m.) to avoid overfeeding and maintain motivation for the test. Each session consisted of the presentation of three positive boxes and three negative boxes, in a random order, with one box at a time, and with 5 minutes between two consecutive presentations. After training, the females promptly opened the positive box and refrained from opening the negative box (electronic supplementary material, Fig. S2). A female was considered to have successfully learned the signals when (i) her average latency to opening the positive box was shorter than that to opening the negative one and (ii) the latencies to opening the three positive boxes

were all shorter than the latencies to opening the three negative boxes the day prior to the first judgement bias test. On average, 3.04 ± 2.21 sessions (mean \pm sd) were sufficient for a female to learn the task.

In preliminary tests, we also verified that the fish did not respond to potential chemical cues from the chironomid larva. Naive fish (which had not learned to associate the signal with the reward) did not spontaneously inspect a covered box containing a larva. Similarly, experienced females, which had learned to avoid the negative box, were not attracted by this box when a larva was added. We also verified the absence of innate bias for side or colour in the training sessions; the latency was not affected by either the spatial position (positive signal: $\chi^2_1 = 0.289$, $p = 0.591$; negative signal: $\chi^2_1 = 0.381$, $p = 0.537$) or the colour (positive signal: $\chi^2_1 = 0.871$, $p = 0.351$; negative signal: $\chi^2_1 = 0.630$, $p = 0.427$) of the signal.

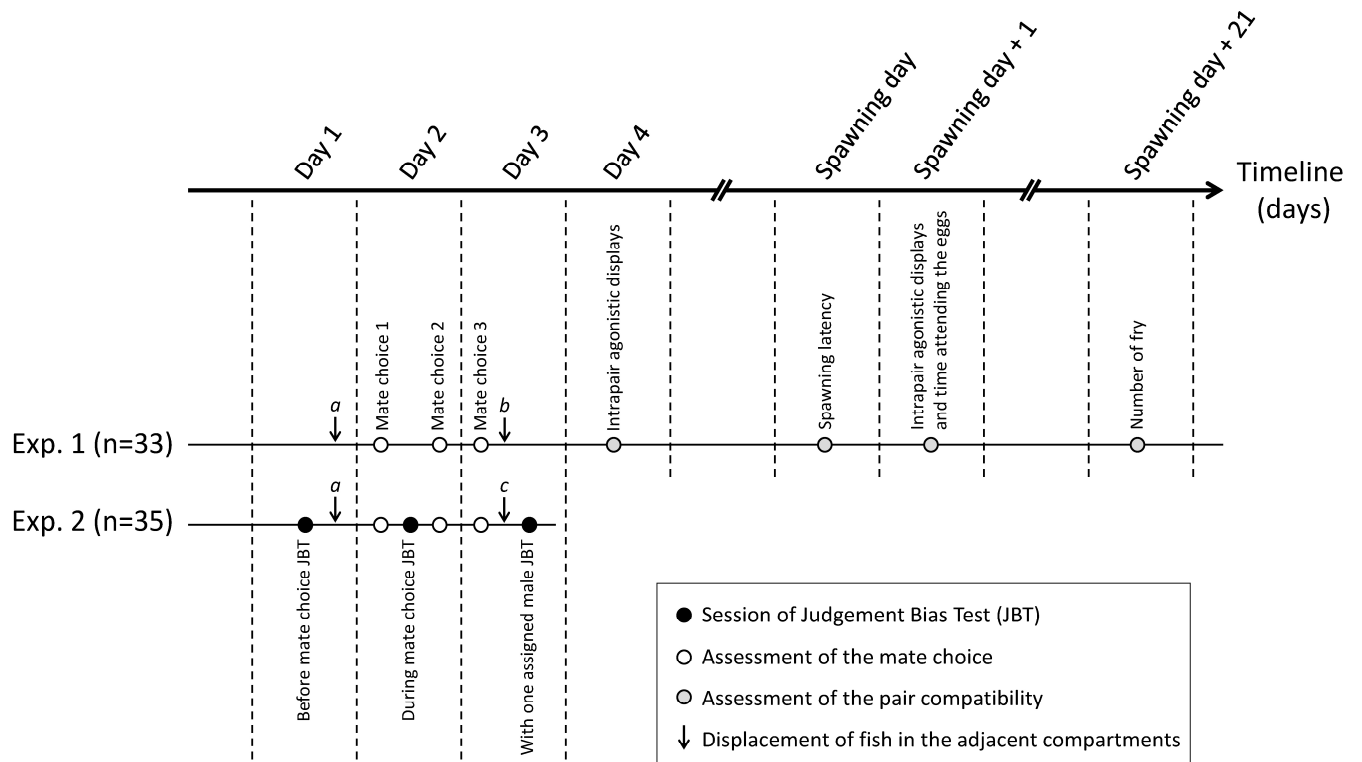


Figure S1. Timeline representing the sequence of events for each experiment. The 68 females were randomly divided into two experimental groups. Experiment 1 (n = 33 females) was designed to assess the ability of the binary choice test to accurately identify the preferred male for each female and predict the long-term reproductive success of the pair. At least one week prior to the experiment, the females were housed with two females, one in each adjacent compartment (Fig. 1). At the end of the first day (event ‘a’), the adjacent females were removed and replaced by two males. The observations of female preference for a male in binary a choice test (open circle) were then performed three times to assess the repeatability of this measure. Immediately after the third observation period of mate choice, the female was randomly assigned either her preferred partner (n = 16) or her non-preferred partner (n = 17). The assigned male was introduced into the female’s compartment, while the other one was removed from his compartment (event ‘b’) for several measures of pair compatibility until 21 days after spawning (grey circle).

Prior to Experiment 2 (n = 35 females), each female was trained to learn the positive and the negative signals during daily sessions (see electronic supplementary material, Appendix S2, for details about the training protocol). During Experiment 2, the female preference for a male (open circle) was assessed following the same protocol as in Experiment 1. In addition, we assessed the effect of the assigned male on her affective state using three successive daily sessions of the judgement bias test (JBT, solid circle). The training sessions and the first JBT session (‘before mate choice’) were performed in the presence of two females, one in each adjacent compartment. Then, as in Experiment 1, the adjacent females were removed and replaced by two males at the end of the first day (event ‘a’). The second JBT session (‘during mate choice’) was performed in the presence of these two males. On the third day, the females were randomly assigned either their preferred (n = 17) or non-preferred (n = 18) male; this male remained in his compartment, while the other male was removed (event ‘c’). Then, the third JBT session (‘with one assigned male’) was performed.

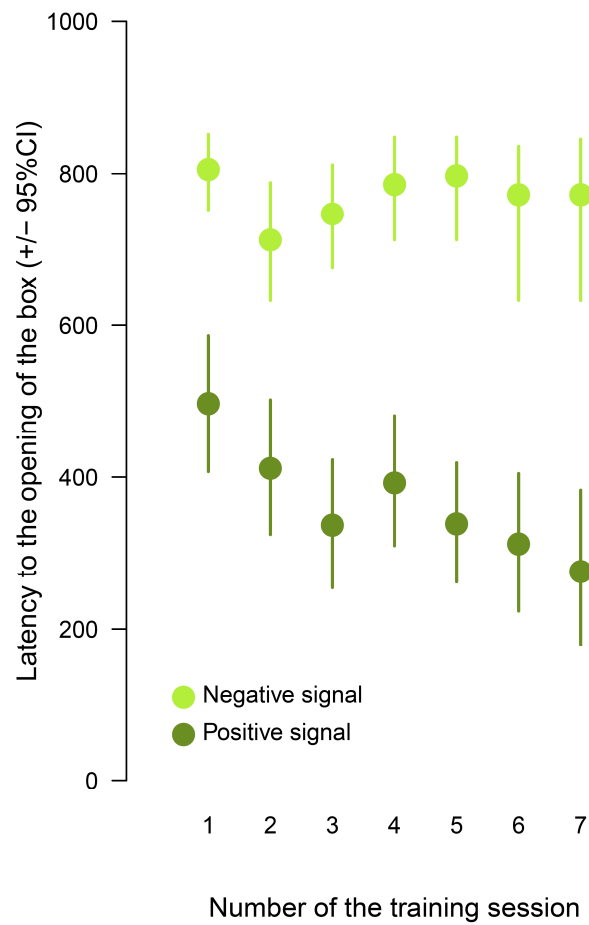


Figure S2. Learning curves for the positive and negative signals used in the judgment bias test. Prior to Experiment 2, the fish were individually trained to discriminate between the positive and negative signals. The latency time (in seconds) to the opening of the box was significantly affected by the interaction between the signal (positive or negative) and the number of sessions (mixed-effects Cox model for repeated measures with the individual as a random factor: $\chi^2_1 = 10.02$, $p = 0.0015$). The latency significantly decreased over time for the positive signal ($\chi^2_1 = 20.00$, $p < 10^{-5}$) and remained constant for the negative signal ($\chi^2_1 = 0.223$, $p = 0.64$).

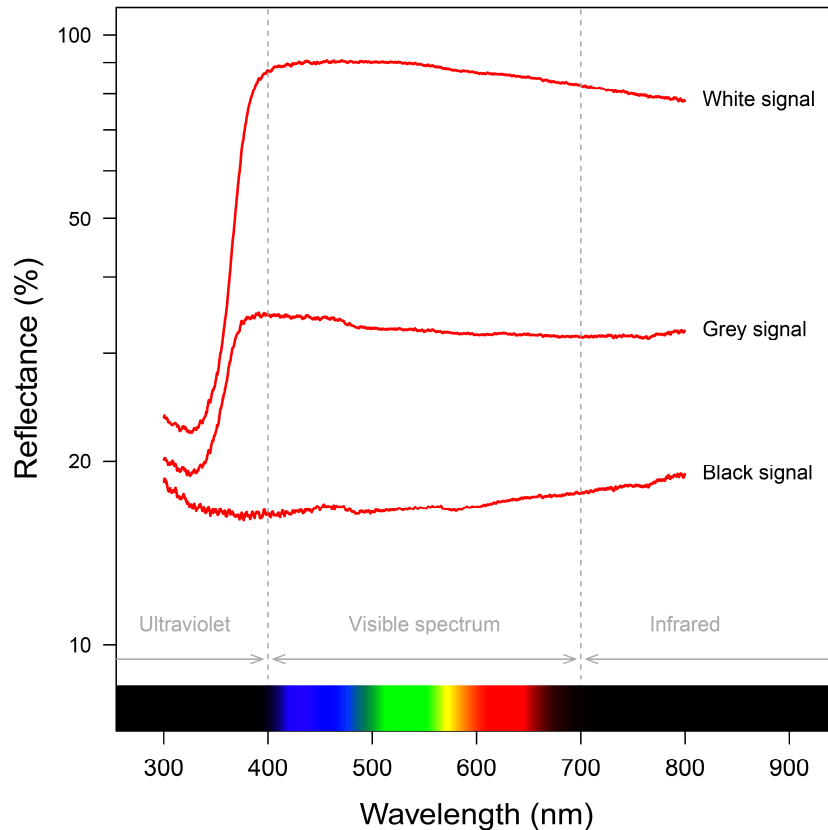


Figure S3. Spectral reflectance curves of each of the three types of lids used in the judgement bias tests.

The ambiguous signal was positioned halfway between the position of the positive and negative signals. The ambiguity of the signal also came from the grey colour of the lid, which was a shade between the black and white lids used for the positive and negative signals. Cichlid fish have the rhodopsine Rh1 gene expressing rod photoreceptor cells, allowing perception of shades of grey (Trezise & Collin 2005). The Weber-Fechner law (Nieder & Miller 2003) states that the perceived brightness P is proportional to the logarithm of the measured intensity I for the stimulus: $P = k \times \log(I)$. The regular brightness gradation (black, grey, and white) was thus calibrated along a logarithmic scaling resulting in visually equidistant steps such that the ratio of perceived brightness between black and grey signals $P_{Black}/P_{Grey} = 0.81 \pm 0.05$ (mean \pm sd) was similar to that between grey and white signals $P_{Grey}/P_{White} = 0.83 \pm 0.04$. Brightness was measured using a spectrophotometer (USB2000+, Ocean Optics, IDIL Fibres Optiques, Lannion, France). Following the methods by Biard et al. (2017), the lid samples were illuminated at an angle of 90° with a deuterium-tungsten lamp (DH2000), and the reflected light was measured at the same angle with a $200 \mu\text{m}$ fibre optic reflection probe (QR200-7-SR-BX). An opaque black tube was fitted at the end of the measuring probe to exclude ambient light and standardize the measuring distance (3 mm). The percentage of reflectance was measured every 0.333 nm in the 300-800 nm spectral range. For each value of the wavelength λ , the reflectance $R(\lambda)$ was calculated relative to pure white (Spectralon Diffuse Reflectance Standard, WS-1-SL) and pure dark (generated by closing the shutter of the spectrometer) references, as $R(\lambda) = 100 \times [(\text{sample-white})/(\text{white-dark})]$. Two randomly chosen lids were analysed for each colour with three independent measures per lid (the probe was lifted and then placed back on the sample again). When these values for the reflectance across the spectrum range were consistent over the repeated measures (Repeatability = 0.98, 95%CI = [0.94; 1], $p < 10^{-5}$), the reflectance curves were averaged for each colour and plotted on a logarithmic scale according to the Weber-Fechner law. The brightness was calculated as the average reflectance over the spectral range.

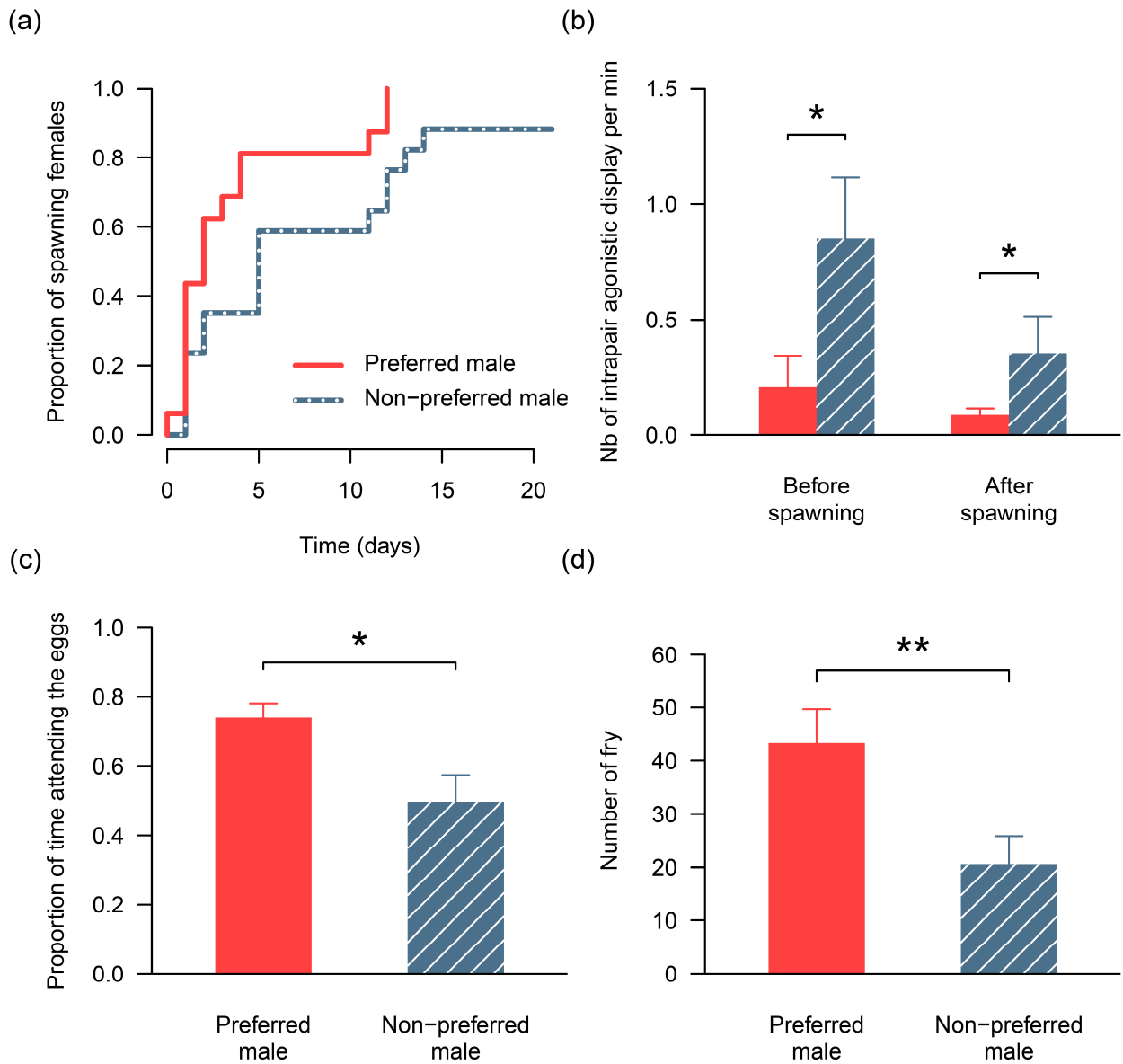


Figure S4: Pair compatibility and reproductive success (Experiment 1) as a function of the assigned male (preferred male in red, non-preferred male in striped blue): (a) spawning latency, (b) proportion of time spent attending the eggs by the female, (c) frequency (number of displays per minute) of aggressive interactions between partners before and after spawning, (d) number of fry three weeks after spawning. The error bars denote the standard error.

Table S1. Experiment 2: effect of the box position and lid colour on the latency to the opening of the box.

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Colour × Position	0.82	1	0.37
	Position	1.04	1	0.31
	Colour	0.38	1	0.54
Ambiguous signal:				
Latency	Colour × Position	1.20	1	0.27
	Position	0.17	1	0.68
	Colour	2.19	1	0.14
Negative signal:				
Latency	Colour × Position	0.18	1	0.67
	Position	1.05	1	0.30
	Colour	0.58	1	0.44

Each female was trained to discriminate between a positive and negative signal characterized by a randomly attributed combination of position (on the left or right side of the tank) and colour (white or black lid). The effect of the learned signals on the latency to the opening of the box was assessed in the control contexts using a mixed effects Cox regression model with female identity as a random variable. We followed a stepwise selection model procedure to identify the best model. As the interaction terms (Colour × Position) were non-significant, we then assessed the effect of each variable separately.

Table S2. Experiment 2: latency to the opening of the box as a function of the context ('before mate choice', 'during mate choice', and 'with one male') and the treatment (preferred vs. non-preferred male).

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Context × Treatment	1.62	2	0.44
	Context	2.06	2	0.36
	Treatment	0.50	1	0.48
Ambiguous signal:				
Latency	Context × Treatment	9.29	2	0.0096 **
Negative signal:				
Latency	Context × Treatment	2.12	2	0.35
	Context	0.91	2	0.63
	Treatment	1.13	1	0.29

The females' affective state was repeatedly estimated in three different contexts: 'before mate choice', 'during mate choice', and 'with one randomly assigned male' (either the preferred male or non-preferred male). The first two contexts were used as control contexts to estimate the repeatability of the female's affective state and to verify that the two treatment groups (preferred vs. non-preferred male) did not differ before the third context ('with one assigned male'). The latency to the opening of the box as a function of the signal (positive, ambiguous or negative) was analysed using Cox mixed-effect models with female identity as a random variable to take the repeated measures design into account. For each signal, we followed a stepwise selection model procedure to identify the best model. When the interaction term was non-significant, the effect of each variable was assessed separately. For the two trained signals, the response latencies did not change as a function of the context or the treatment, which was a central assumption in the judgement bias paradigm. Conversely, females changed their response to the ambiguous signal differently across contexts depending on the treatment. As the interaction term was significant, we then analysed the female response separately by context (Table S3) or by treatment (Table S4). Here and elsewhere, the significant terms are highlighted in bold.

Table S3. Experiment 2: latency to the opening of the box as a function of the treatment (preferred male vs. non-preferred male) calculated separately for each context.

a) Context: before mate choice

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Treatment	2.71	1	0.10
Ambiguous signal:				
Latency	Treatment	0.09	1	0.76
Negative signal:				
Latency	Treatment	0.25	1	0.62

b) Context: during mate choice

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Treatment	0.092	1	0.76
Ambiguous signal:				
Latency	Treatment	0.76	1	0.38
Negative signal:				
Latency	Treatment	0.035	1	0.85

c) Context: one assigned male

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Treatment	0.99	1	0.32
Ambiguous signal:				
Latency	Treatment	4.25	1	0.039 *
Negative signal:				
Latency	Treatment	2.06	1	0.15

As there was a significant interaction between the context and treatment (Table S2), the latencies to the opening of the box were analysed separately for each context: before mate-choice (a), during mate-choice (b) and with one assigned male (c). The latency was analysed using Cox models. We verified that that the two treatment groups (preferred male and non-preferred male) did not differ within the first two contexts. Conversely, there was a significant effect of the treatment in the third context and solely for the ambiguous signal: the response latency was significantly longer for the females assigned their non-preferred male than for those assigned their preferred male (HR = 2.52, 95%CI = [1.02; 6.17]).

Table S4. Experiment 2: latency to the opening of the box as a function of the context ('before mate choice', 'during mate choice', and 'with the assigned male') and separately for each treatment (preferred or non-preferred male).

a) Treatment: preferred male

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Context	1.98	2	0.37
Ambiguous signal:				
Latency	Context	1.62	2	0.45
Negative signal:				
Latency	Context	0.10	2	0.95

b) Treatment: non-preferred male

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Context	2.12	2	0.35
Ambiguous signal:				
Latency	Context	9.66	2	0.0080 **
Negative signal:				
Latency	Context	3.60	2	0.17

As there was a significant interaction between context and treatment (Table S2), the latencies to the opening of the box were analysed separately for each treatment. The latency was analysed using mixed effects Cox models with female identity as a random variable in order to take the repeated measures design into account. When the female was assigned her non-preferred male, the context had a significant effect on the latency to the opening of the ambiguous box: her response latency was higher in the third context than in the first two contexts (Tukey *post-hoc* pairwise comparisons “before mate choice” vs. “during mate choice”: $p = 1.00$, “before mate choice” vs. “with one assigned male”: $p = 0.027$, “during mate choice” vs. “with one assigned male”: $p = 0.019$).

Table S5. Experiment 2: latency to the opening of the box as a function of the preference score and the treatment (preferred vs. non-preferred male) in the third context (with one assigned male) and separately for each signal (positive, ambiguous, or negative).

Dependent variable	Fixed effects	χ^2	df	p
Positive signal:				
Latency	Preference score × Treatment	0.24	1	0.62
	Preference score	0.62	1	0.43
	Treatment	0.99	1	0.32
Ambiguous signal:				
Latency	Preference score × Treatment	4.66	1	0.031 *
Negative signal:				
Latency	Preference score × Treatment	0.0017	1	0.97
	Preference score	0.11	1	0.74
	Treatment	2.06	1	0.15

The latency to the opening of the box as a function of the signal was analysed using Cox models. For each signal, we assessed how the female response latency was affected by the interaction between her preference score (measuring the strength of her preference for one male during mate choice test) and the treatment (preferred male vs. non-preferred male). We followed a stepwise selection model procedure to identify the best model. When the interaction term was non-significant, we then assessed the effect of each variable separately. For the positive and negative signal, the response latencies did not change as a function of the preference score and the treatment. Conversely, the interaction term was significant for the response to the ambiguous signal. The response latencies to this signal were thus analysed separately by treatment (Table S6).

Table S6. Experiment 2: latency to the opening of the ambiguous box in the third context ('with one assigned male') as a function of the preference score and separately for each treatment.

Dependent variable	Fixed effects	χ^2	df	p
Treatment: preferred male				
Latency	Preference score	0.54	1	0.46
Treatment: non-preferred male				
Latency	Preference score	4.33	1	0.037 *

There was a significant interaction between the preference score and the treatment on the response latency to the ambiguous signal in the full model (Table S5). We thus assessed the effect of the preference score on the response latency to the ambiguous signal separately for each treatment (either preferred or non-preferred male). The latency was analysed using Cox models.

Table S7. Experiment 1: frequency of intra-pair agonistic displays (Fig. S3c) as a function of the treatment (preferred *vs.* non-preferred male) and spawning status (before *vs.* after spawning).

Dependent variable	Fixed effects	χ^2	df	p
Frequency of displays	Treatment \times Spawning status	0.058	1	0.810
	Treatment	7.73	1	0.0054 **
	Spawning status	4.12	1	0.0425 *

The frequency of intra-pair agonistic displays (number of displays between partners per minute, regardless the identity of the initiator of the interaction) was recorded either before spawning (24h after pairing) or 24h after spawning. These repeated-measure data were analysed using generalized linear mixed models with female identity as a random variable. We followed a stepwise selection model procedure to identify the minimal model. As the interaction term (Treatment \times Spawning status) was non-significant, it was dropped from the model in order to assess the effect of the two variables separately. The frequencies of the displays were significantly smaller when the female was paired with her preferred male than with her non-preferred male (treatment: estimate $\beta = -1.39$, 95%CI = [-2.28; -0.49]), and in presence of the spawn (Spawning status: $\beta = -0.87$, 95%CI = [-1.69; -0.051]).



Movie S1: Illustration of the judgment bias test (Experiment 2). Each female was trained to open boxes covered by a movable lid, and to discriminate between a positive (rewarded with one chironomid larva) and a negative (unrewarded) signal characterized by a randomly attributed combination of box position (on the left or right side of the tank) and lid colour (white or black). For instance in this video, the female has learned that the positive signal was the box on the left side and covered with a black lid, and the negative signal was on the right side and covered with a white lid. Her affective state was assessed as the response to an ambiguous signal placed in an intermediate position between the two learned signals and covered with grey lid. Given the response latency of the female, the speed of the video was increased (see the information on the video).

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