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Hormonal anticipation of territorial challenges in cichlid fish

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In many territorial species androgens respond to social interactions. This response has been interpreted as a mechanism for adjusting aggressive motivation to a changing social environment. Therefore, it would be adaptive to anticipate social challenges and reacting to their clues with an anticipatory androgen response to adjust agonistic motivation to an imminent social challenge. Here we test the hypothesis of an anticipatory androgen response to territorial intrusions using classical conditioning to establish an association between a conditioned stimulus (CS = light) and an unconditioned stimulus (US = intruder male) in male cichlid fish (*Oreochromis mossambicus*). During the training phase conditioned males (CS−US paired presentations) showed a higher decrease in latency for agonistic response toward the intruder than unconditioned males (CS–US unpaired presentations). In the test trial, conditioned males showed an increase in androgen levels (i.e., testosterone and 11-ketotestosterone) relative to baseline, in response to the CS alone. This increase was similar to that of control males exposed to real intruders after CS, whereas unconditioned males showed a decrease in androgen levels in response to the CS. Furthermore, conditioned males were significantly more aggressive than unconditioned males during the post-CS period on test trial, even though the intruder male was not present during this period. These results reveal the occurrence of a conditioned androgen response that may give territorial males an advantage in mounting a defense to upcoming territorial intrusions, if the ability to readily elevate androgens does not co-vary with other traits that bear costs. (AQ: B, AQ: C)

aggression | androgens | associative learning | social challenge

Fn1 In many territorial species males respond to social challenges with a rise in androgen levels as proposed by the "challenge hypothesis," which seems to be a way of adjusting the agonistic motivation to changes in the social environment (1, 2). The challenge hypothesis has been tested in a wide range of vertebrate species and has recently been extended to invertebrates (3, 4). (AQ: D) According to this hypothesis, the circulating androgen levels should be higher in periods of social instability, like the establishment of breeding territories and/or mating. This androgen response to social challenges would help to modulate subsequent agonistic and reproductive motivation and therefore shape the expression of social behavior as a function of prior social experience (2, 5). The evidence to support this reciprocal model between androgens and social behavior is robust with several studies showing that social interactions can affect androgen levels and many others illustrating the role of androgens on the expression of social behavior (for a recent review of the available literature see ref. 6). The role of androgens as modulators in adjusting behavior to the social context has been studied in several social phenomena such as the winner-looser effect (7), the dear-enemy effect (8), the audience effect (9), and the bystander effect (10), and in all these social phenomena transitory changes in hormonal levels induced by social challenges appear to be the likely mediators of the behavioral output (for a review see ref. 11). Through this bidirectional relationship between androgens and behavior, the social interaction in which individuals take part influences their androgen levels and androgens will themselves modulate perceptual, motivational, or cognitive mechanisms that influence behavior (2, 11), therefore optimizing behavioral performance in future social interactions. It would thus be adaptive for the individuals to predict social challenges and to respond to their clues with an anticipatory increase in androgen levels. Even though the conditioned release of androgens has already been shown after conditioning of mating behavior (12), the hormonal anticipation of territorial challenges has never been tested. Signaled presentation of food, conspecific rivals, predators, or sexual partners elicits natural anticipatory behaviors directed to the signaling stimuli (13). Classical conditioning has been suggested to mimic naturally occurring situations in which imminent social interactions are signaled through visual, chemical, or mechanical changes of the surrounding environment. In fact, in the territorial fish *Trichogaster trichopterus* it has been shown that males learn to associate environmental cues with territorial disputes, which translates into a competitive edge for conditioned males (14). However, little is known about the mechanisms through which it occurs. To test whether the defensive advantage of conditioned males is androgen related we used a classical conditioning paradigm to test how androgen levels [i.e., testosterone (T) and 11-ketotestosterone (11-KT)] in conditioned cichlid males (*Oreochromis mossambicus*) respond to signaling stimuli. We aimed at promoting an association between the presentation of a light (conditioned stimulus, CS) and a male territorial intrusion (unconditioned stimulus, US), thereby inducing associative learning in a socially challenging situation for *O. mossambicus* territorial males. Males were submitted to 1 of 3 treatments: (*i*) trained with CS and US pairings and tested for the response to CS alone—"conditioned" group; (*ii*) trained with CS and US pairings and tested for the response to the US after CS (i.e., territorial intrusion signaled by light)—"real intruder" group; (*iii*) trained with unpaired presentations of the 2 stimuli and tested for the response to CS alone—"unconditioned" group.

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Results

Effect of Associative Learning on Territorial Defense. The effect of classical conditioning training on behavioral performance of territorial males toward the intruders was assessed by scoring the duration and latency of agonistic behaviors (displays and fights) in all animals during the territorial intrusion. All individuals reacted aggressively toward the intruders in all training trials (see supporting information (*SI*) Movies S1–S3). ZSV1 ZSV2 ZSV3 Comparisons between groups relative to the duration of aggressive behaviors on first and last training trial by repeated measures ANOVA AQ: E showed no significant effects of group ($F_{2,21} = 0.222$, $P > 0.05$),

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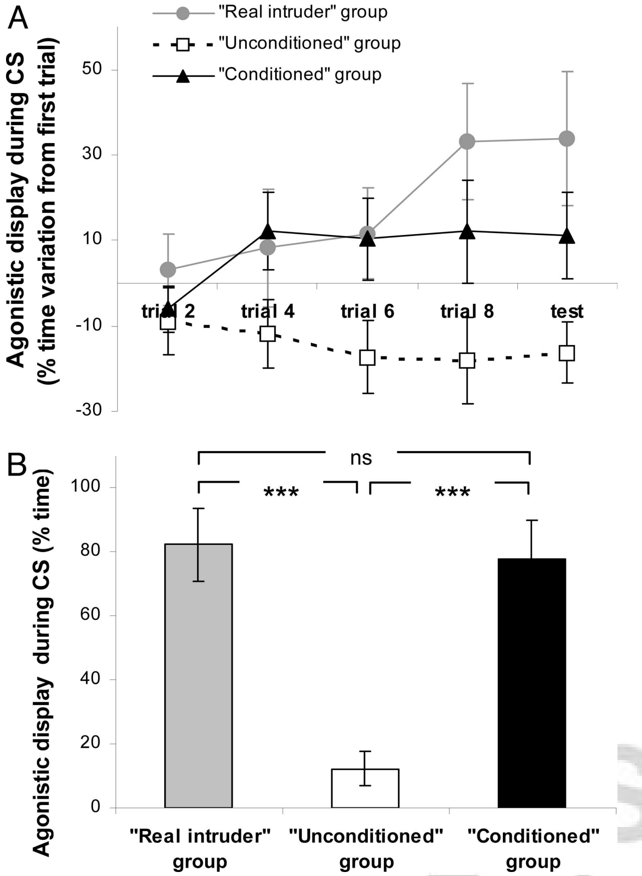

Fig. 1. Aggressive behavior of territorial males toward the CS light: during training conditioned and real intruder groups exhibited significantly more agonistic displays to the CS than unconditioned fish. (*A*) Variation in CS-directed displays from first trial ($trial_n$-$trial_1$) along training, expressed as percentage of total CS time. (*B*) Duration of CS-directed aggressive displays during test trial, expressed as percentage of total CS time. *P* values refer to Tukey HSD tests (*, 0.05; **, 0.01; ***, 0.001; NS, >0.05). Error bars represent ±1 SEM. For all data, *n* = 8 per group.

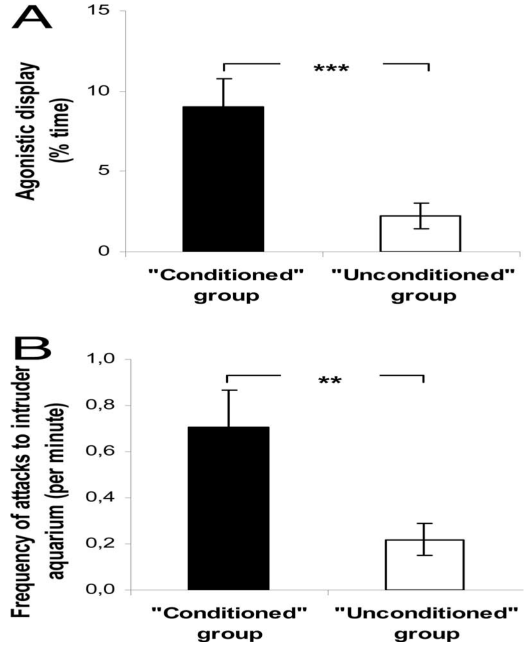

Fig. 2. Classical conditioning results in anticipatory aggressive behavior of conditioned but not of unconditioned territorial males during post-CS period in test trial. (*A*) Duration of agonistic displays expressed as percentage of total post-CS time. (*B*) Number of attacks to intruder aquarium per minute. *P* values refer to 1-way ANOVA effect of group (*, 0.05; **, 0.01; ***, 0.001; NS, >0.05). Error bars represent ±1 SEM. For all data, *n* = 8 per group.

AQ: F no effect of trial ($F_{2,21}$ = 2.54, $P > 0.05$), and no effect of
AQ: G group–trial interaction ($F_{2,21}$ = 0.222, $P > 0.05$) [Percentage of time (±SEM) engaged in agonistic behavior: first trial, conditioned group = 61 ± 14.8%, real intruder group = 68 ± 13.2%, unconditioned group = 54 ± 17.3%; last trial, conditioned group = 70 ± 14.5%, real intruder group = 82 ± 11.5%, unconditioned group = 79 ± 13.5%]. During the conditioning training, latency for the first aggressive behavior toward the intruder decreased from the first to the last training trial both in all groups. However, a smaller decrease in latency was found in the unconditioned group relative to the conditioned groups ($F_{2,21}$ = 6.757, $P < 0.05$; Tukey HSD post hoc comparisons: unconditioned vs. conditioned, $P < 0.05$; unconditioned vs. real intruder, $P < 0.05$; conditioned vs. real intruder, $P > 0.05$) [normalized variation in latency (±SEM) for the first aggressive behavior on last training trial ($[8^{th} - 1^{st}]/1^{st}$): conditioned group = −0.87 ± 0.07, real intruder group = −0.93 ± 0.03, unconditioned group = −0.34 ± 0.2].

Another relevant element of the conditioning process was the CS-elicited conditioned response. During the training phase, males of the conditioned and real intruder groups tended to increase their aggressiveness toward the CS light, whereas males from the unconditioned group showed a decrease relative to the
F1 first CS presentation (Fig. 1*A*). Comparisons between groups for
AQ: H the duration of agonistic displays during CS in the test trial revealed a significant effect of group (1-way ANOVA, $F_{2,21}$ = 15.035, $P < 0.001$; Tukey HSD post hoc comparisons: unconditioned vs. conditioned, $P < 0.001$; unconditioned vs. real intruder, $P < 0.001$; conditioned vs. real intruder, $P > 0.05$; Fig. 1*B*), while no differences were observed between groups in the first trial (1-way ANOVA, $F_{2,21}$ = 2.057, $P > 0.05$).

Additionally, in the test trial, during the post-CS period, conditioned fish spent a higher percentage of time in agonistic display (1-way ANOVA, $F_{1,14}$ = 17.112, $P < 0.001$) (Fig. 2*A*) and F2 showed higher frequency of attacks toward the empty intruder aquarium than males from the unconditioned group (1-way ANOVA, $F_{1,14}$ = 10.425, $P < 0.05$) (Fig. 2*B*), even though neither one of these 2 groups was exposed to the intruder male in the testing trial.

Effect of Associative Learning on Circulating Androgen Levels. Changes in both 11-KT and T levels from pretraining (baseline) to posttest differed significantly among groups (1-way ANOVA, 11-KT: $F_{2,18}$ = 4.64, $P < 0.05$; T: $F_{2,19}$ = 5.33, $P < 0.05$): conditioned males and males exposed to real intruders showed an increase in androgen levels following the test trial whereas unconditioned males showed a decrease (Tukey HSD post hoc comparisons for both 11-KT and T: unconditioned vs. conditioned, $P < 0.05$; unconditioned vs. real intruder, $P < 0.05$; conditioned vs. real intruder, $P > 0.05$; Fig. 3 *A* and *B*). F3

Discussion

In the present study we have shown that territorial cichlid males learn to anticipate territorial intrusions and that this anticipation also triggers an increase in androgen levels that would then facilitate a male's ability to respond to an upcoming aggressive challenge. This is the first time that the conditioning of an androgen response to territorial intrusions has been demonstrated in vertebrates. Earlier studies with rodents had already showed that males can learn to anticipate opportunities for

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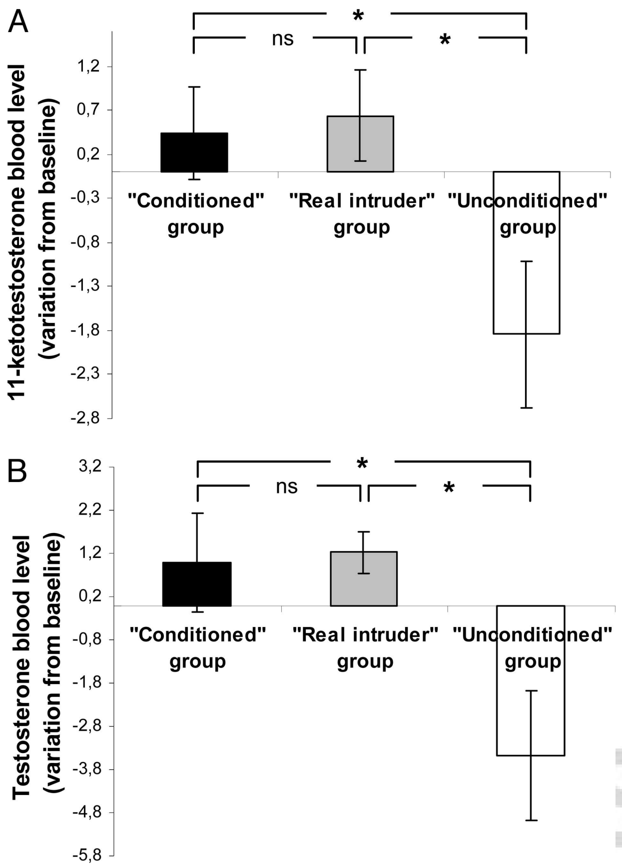

Fig. 3. Classical conditioning results in anticipatory increase in androgen blood levels in conditioned and real intruder groups of territorial males. (*A*) Variation in testosterone plasma level relative to baseline levels, after test trial. (*B*) Variation in 11-ketotestosterone plasma level relative to baseline levels, after test trial. *P* values refer to Tukey HSD tests (*, 0.05; **, 0.01; ***, 0.001; NS, >0.05). Error bars represent ±1 SEM. For all data, $n = 8$ per group.

sexual behavior and prepare themselves physiologically by elevating luteinizing hormone (LH) and testosterone levels (12). Therefore, the conditioning of endocrine responses by socially relevant CS stimuli may be a more general mechanism used by vertebrate males to adjust their internal state to anticipated social contexts. Classical conditioning has been shown to occur in a wide range of species, with multiple conditioning paradigms (see ref. 15 for a review). The generally described behavioral effects of conditioning are CS-elicited conditioned responses and a more efficient response toward the US. Once the CS–US association is learned, the CS itself activates the representation of the US so that behaviors typically directed toward the US are directed to the initially neutral CS (14, 15, 16). This pattern was fully observed in the current study. During the training phase conditioned males (i.e., males from both the conditioned and real intruder groups) decreased the latency of response to the territorial intrusion and acquired aggression directed to the CS itself. On the last training trial, conditioned males rapidly approached the CS in the frontal display posture in anticipation of the forthcoming social interaction, and thus were able to confront their rivals already in a threatening posture. In the test trial, when tested with the CS alone (i.e., in the absence of a real intruder), conditioned males, but not unconditioned ones, exhibited aggressive displays toward the empty aquarium, suggesting that conditioned males were anticipating an intrusion and mounting a defense for an imminent territorial challenge, which they have learned to be signaled by the light (CS). Concomitantly, an increase in T and 11-KT levels was also observed in conditioned males after the test trial, which was similar to that elicited in the real intruder group. This indicates that the CS alone induces an androgen response of similar magnitude to that elicited by a territorial intruder, which means that conditioned males are also anticipating the presentation of the intruder after seeing the CS by raising their androgen circulating levels. Interestingly, males of the unconditioned group, where the presentation of the CS and the US was unpaired, exhibited a significant decrease in androgen levels. Because in this group the CS is always followed by a 2-hour intrusion-free period during training, males apparently learned to anticipate a period of social inertia and therefore drop their androgen levels. This suggests the occurrence of conditioned inhibition according to the Rescorla-Wagner model of conditioning (17). The endocrine responses in both the conditioned and unconditioned groups are probably underlying the differences in their behavioral performance during the post-CS period in the test trial. Hollis (14), using a discrimination protocol, trained male fish with both a CS^{+}, which signaled a territorial intrusion, and a CS^{-}, without association to a social stimuli. The presentation of the CS^{-} led to an inhibition of aggressiveness in conditioned males, as we report here for unconditioned (i.e., unpaired trained) males. In summary, positive signaling stimuli increase the conditioned male's defensive ability, and signals predicting periods free from social challenges allow males to loosen up their defense in times and places where challenges have low probability of taking place (14). Together this positive and negative associative learning may contribute to the optimization of territorial defense.

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Two unexpected observations regarding the predictions of classical conditioning have occurred in this study:

(*i*) Males from the unconditioned group (i.e., unpaired CS–US presentation) also showed a decrease in latency for the first aggressive behavior toward the intruder in the last training trial. This may result from the experimental conditions given to our territorial males that may have led them to perceive each intrusion as a victory—the fact that intruders were always of smaller size, the interaction always occurred in the tank of the focal fish, and the removal of the intruders from the focal male's territory after the US period could be interpreted as a retreat. This interpretation is supported by evidence from the literature that shows that a victory in an aggressive interaction elicits a decrease in latency to aggressive behavior in future agonistic interactions (18, 19). Nevertheless, even though a decrease in latency in unconditioned males was observed, a significantly larger decrease in latency was found in the conditioned groups. AQ: I

(*ii*) In contrast to other conditioning studies of aggressive behavior (14), no differences were found in aggressiveness toward the intruder between males of the conditioned and unconditioned groups. However, on the test trial, during the post-CS period, conditioned males showed a higher frequency of attacks toward the intruder's aquarium and a higher percentage of time spent in agonistic display than the unconditioned males.

Together, these 2 observations suggest that the classical conditioning-induced improvement in territorial defense in *O. mossambicus* results from an increase in vigilant state and not from an increase in fighting ability of conditioned males.

In *O. mossambicus* learning that some environmental cues predict the appearance of a rival may have adaptive value. In this territorial species male fitness depends to a large extent on the ability of males to establish and defend breeding territories. Therefore, any factor that improves territorial defense increases male's probability of keeping its territory and guarantees access to females. Although flashing lights are not to be found in nature, other naturally occurring stimuli exist that may act as conditioning stimuli in the wild. Many visual, mechanical, and chemical cues from intruder males detected by territory owners,

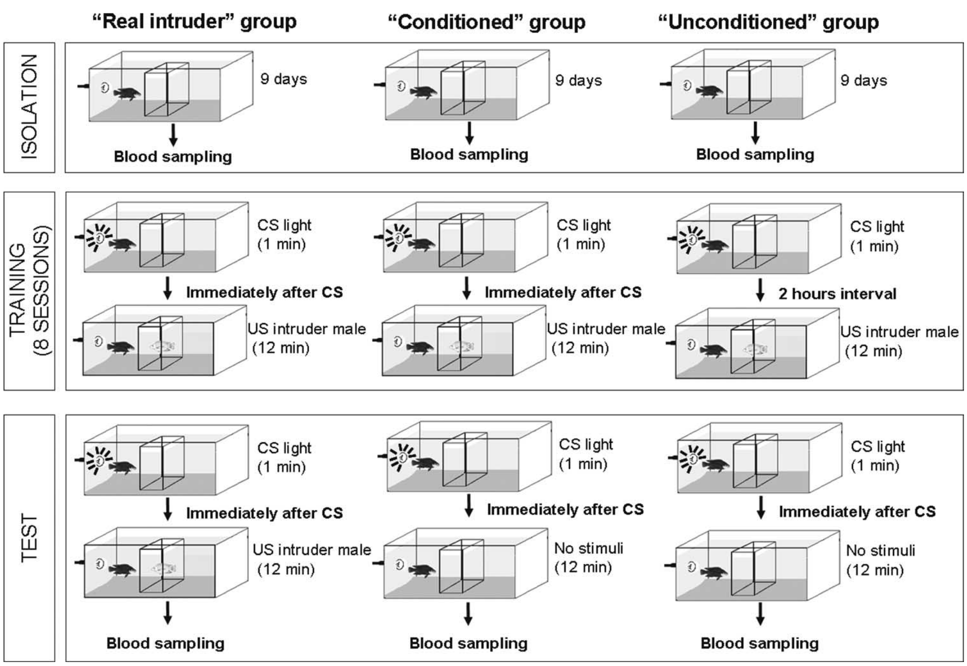

Fig. 4. Schematic representation of the training protocol for each of the 3 experimental groups: real intruder, conditioned, and unconditioned.

like moving shadows, approaching color patterns, and disturbances of underwater vegetation can play a CS role (14). It is thus reasonable to point out the biologically relevant role that classical conditioning may have on natural biological systems. In this study we show that, in this cichlid species, a CS light that signals a territorial intrusion optimizes the vigilance state of conditioned males. By enhancing agonistic motivation and modulating neuronal mechanisms of learning and memory in challenging situations and by relaxing defense in socially stable periods, endocrine anticipation of rival intrusions might optimize territorial defense and avoid the costs of high androgen levels (20) at times when these are unnecessary.

Methods

Animals and Housing Conditions. Adult *O. mosssambicus* (Pisces: Cichlidae) males were kept in mixed sex groups in 240-L aquaria at the animal housing facilities of the Instituto Superior de Psicologia Aplicada, Lisbon, Portugal. Water was continuously aerated and kept at a temperature of 26 °C ± 1 °C, and the photoperiod regime was 12 L:12 D. Animal care and all experimental protocols were approved by the national authorities (Direcção Geral de Veterinária, Portugal).

Conditioning Procedure. For this experiment, 24 reproductively active adult males were selected. Fish of comparable size and age were kept in social isolation for 9 days before the experiment to minimize previous social experience effects on behavior and androgen levels. On the last 2 days of the isolation period fish were transferred to the training aquaria for territory establishment. Conditioning training proceeded on the next 4 days and on the
F4 14th day the test trial was performed (Fig. 4). Conditioning aquaria consisted of 200-L aquaria visually isolated from the external environment, with a 16-L aquarium centrally positioned inside it and a 20-W light externally located next to each aquarium's left wall (Fig. 4).

For the conditioning procedures the light was used as the conditioned stimuli (CS) and a male territorial intrusion was used as the unconditioned stimuli (US). The presentation of the CS lasted for 1 min and was immediately followed by the US. The territorial intrusion (US) lasted for 12 min after which intruders were removed from the aquaria by the experimenter. Intruder males, about 10–20% smaller than territorial males, were assigned to experimental fish and kept inside 16-L aquaria in the experimental male's territory during intrusions to prevent injuries. Intruders were smaller than resident males to make sure the latter would not experience a decrease in their territorial status during the course of the training trials. Intruders remained isolated during the whole experiment except during intrusions and a rotating scheme was promoted during training so that none of the experimental males would receive 2 sequential presentations of the same intruder, avoiding possible habituation effects (21). Male fish were divided into 3 groups with different training (n = 8 per group). Males of the conditioned and real intruder groups received 2 daily paired presentations of the CS (light, 1 min) and US (male territorial intrusion, 12 min) during 4 days; males of the unconditioned group received 2 daily unpaired presentations of the CS and the US (2 h apart) (Fig. 4). On the test trial, conditioned and unconditioned males were only presented with the CS followed by a 12-min post-CS period (corresponding to the intrusion period during training, but without any intruder) to assess the behavioral and hormonal conditioned response elicited by the CS alone (Fig. 4). In turn, on test trial males of the real intruder group were again exposed to a paired presentation of both stimuli as a control for androgen response to a real territorial challenge (Fig. 4).

Behavioral Sampling. A focal continuous recording method was used for behavioral sampling of both events and states during CS presentation and territorial intrusion period using Palm Pilot (Palm, Inc.) with FIT-System software (J. Held). The following agonistic behaviors were scored: full display (the fish stands in front or laterally to the intruder or the light with extended fins and opercula); partial display (the fish stands in front or laterally to the intruder or the light with extended fins); hitting (a rapid swim followed by a hit toward the intruder's aquarium); bite (a bite movement directed toward the intruder aquarium); tail beating (a slap of the tail); circling fight (rapid swim around the intruder aquarium); mouth fight (fish swim vigorously and hit against intruder aquarium with fully opened mouth) (for a detailed description of agonistic behavior of this species see ref. 22). Hitting, biting, and tail beating were scored as events and their frequency per minute was calculated. All of the other behaviors were scored as states, relative to their duration, and the percentage of total sampled time spent in those behaviors was calculated for each CS or US period as total duration of aggressive behavior. Changes in latency or duration of agonistic responses were calculated relative to the first trial. All trials were video recorded for posterior measurement of response latencies for the above-described behaviors and the latency for the first aggressive behavior was scored.

Blood Sampling and Hormone Assays. Before blood sampling, animals were anesthetized with MS-222 (Sigma) until deep anesthesia was obtained, which took less than 1 min. After blood sampling, fish were kept in highly oxygenated aquaria until complete recovery from anesthesia. The whole procedure took less than 5 min. Blood was drawn from the caudal vein (≈0.1 mL), centrifuged for 10 min at 3,000 rpm, and the plasma stored at −20 °C until further processing. Circulating levels of the 2 major fish androgens, 11-KT and

T, were determined by RIA. Sample processing and RIA methods for assessing blood androgen levels were described elsewhere (23–25). The 11-KT antibody used was kindly donated by David Kime (University Sheffield, UK) and its cross-reactivity was given elsewhere (26). The T antibody used was developed by John Merrit. The intra- and inter-assay coefficients of variation for 11-KT and T were 1.38 and 3.39 and 3.43 and 1.80, respectively.

The first blood sampling was performed on the seventh day of isolation period to establish a baseline for the androgen levels (associated with absence of social interactions); a second sample was taken immediately after the test trial, on the last day of the experiment (Fig. 4). To control for diurnal variations of hormone levels (10) each fish was sampled at the same time for both blood collections. The treatment effect on androgen levels was expressed as the change between the test trial and baseline ($T_{test} - T_{isolation}$ or $11\text{-}KT_{test} - 11\text{-}KT_{isolation}$).

Statistical Analysis. Between-group comparisons of total androgen variation, variation in latency for aggressive behavior, variation in duration of aggressive behavior, and frequency of aggressive events were made by 1-way ANOVA using each group as 1 level. ANOVA results were followed by post hoc Tukey HSD test to search for specific differences between groups. The difference between groups along training trials was tested by repeated measures ANOVA, using each trial as 1 level in the repeated factor, and group as an independent factor. Two-sided *P* values were used and the significance level was set to 0.05. Data are shown as mean ± standard error of the mean.

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Supporting Information

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Movie S1. 8th training session of a ''conditioned'' fish, here represented by a sample period of the initial 15 sec after light (CS) onset followed by the first 60 sec of the interaction between the focal male and the intruder (US). ''Conditioned'' male react to the CS light by approaching it and with dorsal fin display. At the beginning of the US social challenge, ''conditioned'' male immediately approach the intruder in full display and quickly adopt the dark territorial color pattern, circling the intruder and aggressively advancing towards him with biting attempts and tailbeats.

SV1 Movie S1 (MOV)

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Movie S2. 8th training session of an ''unconditioned'' fish, here represented by a sample period of initial 15 sec after light (CS) onset followed, 2 hours later, by the first 60 sec of the interaction between the focal male and the intruder (US). The unconditioned male doesn't react to the light onset. When the intruder male is presented, even though the aggressive repertoire is displayed (full display, circling and tailbeating), the focal male shows long latency in displaying it and in a less intensive way. During the overall US period ''unconditioned'' males performed equally aggressive as the ''conditioned'' ones, even though differences for the initial latencies were observed. Territorial interactions usually escalate over time and ''unconditioned'' males took more time than ''conditioned'' males to reach a full exhibition of territorial defense behavior.

SV2 Movie S2 (MOV)