



'Winner effect' without winning: Unresolved social conflicts increase the probability of winning a subsequent contest in a cichlid fish

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ABSTRACT

Previous winning experience increases the probability of winning a subsequent contest. However, it is not clear whether winning probability is affected only by the outcome of the contest (winning or losing) or whether fighting experience itself is also sufficient to induce this effect. We investigated this question in the East African cichlid fish *Pundamilia* spec. To create an unresolved conflict we allowed males to fight their own mirror image prior to a real fight against a size-matched non-mirror-stimulated control male. When males fight their own mirror image, the image's response corresponds to the action of the focal animal, creating symmetrical fighting conditions without the experience of losing or winning. We found that mirror-stimulated males were more likely to win an ensuing contest than control males. Interestingly, in this species mirror stimulation also induced an increase in circulating androgens, which is consistent with the hypothesis that stimulation of these sex steroids during aggressive encounters may prepare the animal for subsequent encounters. Our results suggest that fighting experience alone coupled with an androgen response, increases the likelihood of winning, even in the absence of a winning experience.

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1. Introduction

Almost all animal species respond to social challenges, such as territorial intrusion [1]. It has long been known that experiencing aggressive encounters affects the chances of winning future contests, independent of intrinsic fighting ability: winning a fight increases the probability of winning a subsequent contest (winner effect), whereas the experience of defeat has the opposite effect [2,3; reviewed in 4,5]. The winner effect can help to shape emerging social structures and the establishment of dominance hierarchies [2,3]. Despite the ubiquity of the winner effect, little is known about the physiological and cognitive processes that govern its formation [6,7]. Research indicates that the perception of victory is necessary to increase success in a later encounter [8], but it is also possible that fighting experience alone even in the absence of winning is sufficient.

The neuroendocrine system responds to social challenges and thus is well suited to mediate the winner effect [e.g. 1,7,8]. In males of a wide range of vertebrates, aggressive behavior is accompanied by a (transient) increase in androgens [9]. This correlation between androgens and the social environment is generally thought to modulate subsequent agonistic and reproductive motivation, in that it prepares the animal for future competitive situations ('Challenge Hypothesis',

[9]; for recent reviews see [10–13]). Winners often exhibit elevated post-encounter testosterone levels, and several studies indeed suggest that in winners testosterone acts as a reinforcer of aggressive behavior in subsequent encounters, enhancing the chances of future victories [14,15]. However, most research suggests that the perception of winning itself, rather than fighting behavior alone, is instrumental in short-term increases in androgens and driving the winner effect [2,5,8,9]. It is unclear, however, whether the perception of victory is necessary to increase androgen levels and success in a later encounter or whether fighting experience alone even in the absence of winning is sufficient.

To examine this question, we allowed males of the Lake Victoria cichlid fish *Pundamilia* to fight their own mirror image or a non reflecting similar object before fighting a real fight. In mirror-elicited fights the focal male is given fighting experience without experiencing victory or defeat, i.e., the contest remains unresolved, because the mirror image's response is perfectly symmetric to the actions of the focal animal [16]. This paradigm allowed us to test the hypothesis that the experience of an unresolved (mirror-stimulated) fight increases the probability of winning a future encounter with a real opponent (Experiment I). In order to investigate whether success in later encounters after mirror fighting is mediated by androgens, we investigated in Experiment II whether escalated fighting during a mirror-stimulated fight is sufficient to induce an increase in circulating androgens. Previous research on this second question yielded contradictory results. Oliveira et al. [16] and Hirschenhauser et al. [17] failed to detect an androgen response

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during a mirror fight in the Nile tilapia *Oreochromis mossambicus* and the Japanese quail (*Coturnix japonica*), respectively. However, these authors measured androgens in urine and feces, respectively, which are often assumed to correspond well with circulating levels [18–20]. However, several factors can affect the interpretation of such measures, such as the unclear temporal relationship between acute androgen responses in the circulation and their subsequent release into the environment via the digestive or excretory systems; the frequency of urination and defecation, which can depend on social cues; the metabolized nature of the hormones released this way; and possible cross-reactivity to metabolites from other steroid hormones [20–22]. It is therefore interesting to note that males of the cichlid fish *Astatotilapia burtoni* exhibited increased levels of circulating androgens during a mirror fight [23; see also 24]. This later result is consistent with our prediction that during a mirror fight hormones should increase in anticipation of further escalation of the fight against an equally strong opponent, likely increasing winning probability in later encounters.

2. Methods

2.1. Animals

We used 105 lab-bred, 18–24 months old adult males belonging to the haplochromine cichlid fish *Pundamilia* spec. from Lake Victoria. Haplochromine cichlids are polygynous, female mouth brooders without a pair bond and with strong male territoriality [25]. All aquaria contained a gravel substrate and were connected to a central biological filter system with continuously circulating water which was maintained at a temperature of 25 ± 2 °C. A natural tropical light: dark cycle of 12:12 h was maintained. The fish were fed flake food six times per week and a mixture of ground shrimps and peas twice per week. All experiments were carried out under animal experiment licenses (DEC 3137 and DEC 4335A) granted by the University of Groningen and in compliance with current laws in The Netherlands.

2.2. Experiment I

2.2.1. Procedure

Males were individually housed in separate compartments with visual but no physical access to other males at least 1 week prior to the experiments. At least 3 days before each test we placed pairs of size-matched males with no history of prior interactions in 100 liter test aquaria, which were subdivided by an opaque partition into two compartments, each of which contained a PVC tube as shelter. Thus, each male had no visual access to other males. Prior to combat, one randomly chosen male in each pair ($n = 40$) was presented with a mirror at one end of the compartment for 15 min; the corresponding control male was exposed to a sheet of black non-reflective glass instead to control for the presence of a novel object in the aquarium. The fighting behavior of the fish was recorded for 15 min using a focal continuous recording method. All aggressive displays (gill or frontal display and lateral display) and attacks (individual butts and bites at the mirror) were scored. We then removed the mirror/glass, and immediately lifted the opaque partition between the two experimental fish and the ensuing fight was observed from behind a blind. We identified winner and loser, defined as the winner chasing the loser at least three consecutive times [26]. We then immediately separated the fish. If there was no resolution within 30 min (fight duration, mean \pm SE = 792 ± 88 s, $n = 40$), we separated the fish and called it a tie.

2.3. Experiment II

2.3.1. Procedure

For 7 days prior to the experiment, each male ($n = 25$) was housed in isolation in a 50 l aquarium with a PVC tube as a shelter. On Day 7,

the fish were randomly divided into two groups; the first (experimental group, $n = 13$) was presented with a mirror at one end of the aquarium for 20 min; the second (control group $n = 12$) was exposed to a sheet of black non-reflective glass instead to control for the presence of a novel object in the aquarium.

We recorded the fighting behavior of the fish for 20 min using a focal continuous recording method. At the end of the 20-min test, we removed either the mirror or the sheet of black non-reflective glass. We then collected blood from the caudal vein using a 1 ml syringe and 0.5×16 mm needles. From approximately half of the fish in each group blood was collected within <5 min after the end of the test. Blood collection from the remaining animals occurred 30 min after the end of the test. We chose for these two time points to determine the temporal dynamics of the androgen surge after a mirror fight. Each sample was centrifuged at 12,000 rpm for 10 min to isolate the plasma portion of the blood, which was then stored at -80 °C until assayed for hormones.

We measured circulating levels of two androgens, T and 11-KT, in blood plasma using enzyme immunoassays (T: Assay Design, Ann Arbor, MI; 11-KT: Cayman Chemical Ann Arbor, MI) following protocols established by and exactly as described in Kidd et al. [27]. We used 7.2 and 3.6 μ l blood plasma per sample to assay T and 11-KT, respectively. The intra-assay coefficient of variation (CV) was 1.58% and 2.57% for the T and 11-KT, respectively. The inter-assay CV was 6.56% and 4.46% for T and 11-KT, respectively. Cross-reactivity for these kits and sensitivities are shown in Kidd et al. [27].

2.4. Analysis

Hormone, aggression and fight duration data were log transformed ($\ln x + 1$) prior to analyses to meet assumptions of parametric testing. In Experiment I, we tested which males (mirror stimulated or control) had higher probabilities of winning using a binomial test. Hormone levels were compared between fish from the experimental and the control group, using ANOVA with time of sampling (0 or 30 min) and treatment as explanatory factors. We report means \pm standard error. All quoted probabilities are for two-tailed tests of significance.

3. Results

3.1. Experiment I

Seven of 40 mirror-stimulated males did not show any response (neither agonistically nor submissively) toward their own mirror image. Of those seven individuals, three won and four lost the subsequent encounter. Eight of the 40 dyadic contests ended in a tie (i.e., remained unresolved after 30 min, without a clear winner). In the remaining 25 fights, 18 mirror-stimulated males defeated the control male in the subsequent fight; this winning success rate (72%) was significantly higher than expected by chance (binomial test, $p = 0.042$, $n = 25$). Neither the duration of the fight (Wald = 0.20, $df = 1$, $p = 0.65$), nor the rate of aggression against the mirror image (logistic regression: attack rate: Wald = 0.31, $df = 1$, $p = 0.58$; display rate: Wald = 0.65, $df = 1$, $p = 0.42$) significantly affected the probability of winning the ensuing fight.

3.2. Experiment II

All males exposed to a mirror reacted very aggressively to their own image, whereas males exposed to a non-reflective surface did not show any aggressive behavior at all (Fig. 1). Over the course of the testing period, the attack rate, but not the display rate of males in the mirror-stimulated group showed a non-significant upward trend over time (RM-ANOVA with the sum of attacks per five minute interval as repeat, attack: $F(1,12) = 4.095$, $p = 0.07$; display: $F(1,12) =$

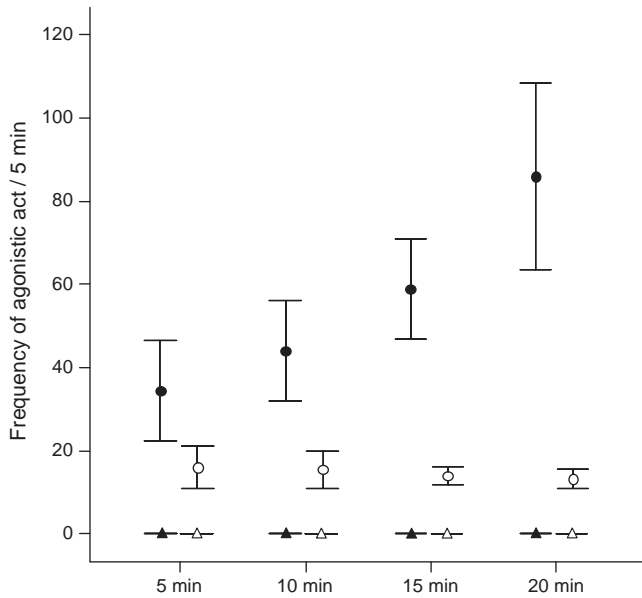


Fig. 1. Escalation in aggressive behavior by *Pundamilia* males in response to their own mirror image, as measured by rates of attacks (filled symbols) and agonistic displays (open symbols) for each five minute intervals (mean \pm SE). The behavior of mirror-treated animals is indicated in circles, the behavior of control animals in triangles ($n = 12$ each for experimental and control groups). Note that one fish from the experimental group was excluded in this graph since his behavior was not quantified in five minute intervals.

0.762, $p = 0.4$). Circulating levels of both T and 11-KT were significantly higher in mirror-stimulated males than in the control males (T: ANOVA: $F(1,24) = 8.440$, $p = 0.008$; 11-KT: ANOVA: $F(1,21) = 7.046$, $p = 0.015$; Fig. 2). T and 11-KT measures were tightly correlated (Pearson correlation, $r = 0.897$, $p < 0.0001$, $n = 24$), with 11-KT concentrations about 40 times lower than those of T. Circulating levels of both androgens seemed highest immediately after the fight compared with 30 min later, but this difference was not significant (effect of time of sampling T: $F(1,23) = 1.092$, $p = 0.307$; 11-KT: $F(1,21) = 3.436$, $p = 0.078$). None of the interaction terms between time of sampling and treatment was significant (T: $F(1,22) = 0.063$, $p = 0.805$; 11-KT: $F(1,20) = 0.546$, $p = 0.468$).

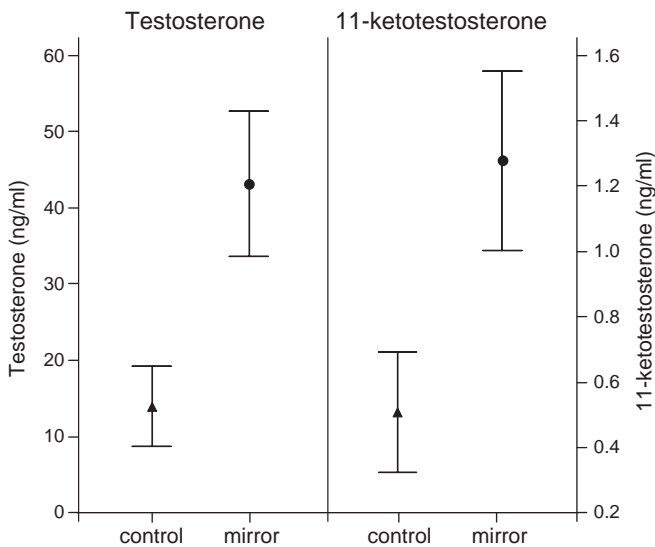


Fig. 2. Variation in circulating levels of testosterone and 11-ketotestosterone (means \pm SE) after exposure to a mirror (circles) or black non-reflective surface (triangles) for 20 min. As androgen levels did not significantly differ between the two time points the combined values from two different time points are shown.

4. Discussion

Previous winning experience increases the probability of winning a subsequent contest [4]. In the present study we have shown that fighting experience alone, even without winning (or losing), is sufficient to increase the likelihood of winning subsequent encounters in *Pundamilia*. In addition, we found that circulating androgen levels increase in response to mirror-induced aggressive behavior, which points to a potential mechanism underlying the mirror-fight effect.

Our findings are consistent with studies that attribute the winner effect largely to the reinforcing effects of androgens [14,15]. In male California mice (*Peromyscus californicus*), exogenous administration of testosterone in castrated individuals following an aggressive encounter led to an increase in aggressive behavior in a subsequent fight [15]. In the cichlid fish *O. mossambicus* treatment with anti-androgens blocks the winner effect [14]. Some studies have suggested that the perception of winning is the driving factor for short-term testosterone release and the winner effect [8,16]. However, our study supports the idea that the experience of winning a contest is not necessary for the winner effect to occur, since fighting under perfectly symmetrical conditions alone is sufficient to increase success in subsequent encounters.

Our results suggest that short-term changes in androgens can affect future aggressive encounters. Oliveira [7] suggested that testosterone enhances selective perception and cognitive processes relevant to fighting. Testosterone can have multiple effects on the brain, for instance due to its conversion into estrogen, which directly promotes aggression, and also modulate brain dopaminergic systems [28].

In our study the staged contest took place immediately after the mirror fight. Though this is a common procedure [28–30, reviewed in 4], other studies tested the winner effect one or more days after the experience [31,32, reviewed in 4]. It is possible that transient increases in testosterone after the (mirror) fight may enhance or sustain appropriate aggressive behavior not only during an encounter [33] but also during subsequent encounters immediately following the previous one, and that the formation of a longer lasting winner effect requires a real winning experience. Future experiments, controlling this time interval as well as winning/fighting experiences should shed more light on this question.

In Experiment I, we found that mirror-stimulated males were more likely to win a subsequent encounter, even in the absence of a real winning experience, than control males. Because we only included those fights in which the mirror-exposed males fought the mirror image, it is conceivable that males who fought their mirror image were intrinsically better fighters [34]. This is, however, unlikely since males that did not fight their mirror image were no more likely to lose the subsequent fight than control animals. The magnitude of the ‘mirror-fight effect’ (22% above chance) is similar to the previously reported winner effect in the cichlid fish *O. mossambicus* (21%, after correcting for intrinsic fighting ability, see [14]). Although we are very cautious with this comparison (due to differences in species and experimental procedures; see [4]), it may well be the case that fighting alone is sufficient for a full winner effect in cichlid fish.

That mirror-stimulated males mounted an androgen response in our study is consistent with findings in another cichlid, *Astatotilapia burtoni* [23]. Mirror-stimulated androgen levels in *Pundamilia* were similar to levels observed in tests with real intruders where focal animals fought a rival enclosed in a transparent tube [35]. During a mirror fight the opponent is viewed as equally strong and circulating androgens should therefore increase in anticipation of further escalation of the fight. This would be consistent with the ‘challenge hypothesis’, which postulates that circulating androgens should increase in periods of social instability, allowing individuals to adjust their agonistic motivation to the ongoing changes in the social environment [9]. However, our findings do not need to be universal across vertebrates. Indeed, they are in

sharp contrast to the findings of Oliveira et al. [16] and Hirschenhauser et al. [17], who did not detect such an androgen response to mirror fights in the Nile tilapia and the Japanese quail, respectively. There are several factors that could explain this discrepancy. Firstly, these two studies were based on urine and fecal measurements of androgens whereas in the present study we measured androgens directly in the circulation [18–22]. Secondly, it is possible that species vary in how their brains process social information and regulate neuroendocrine responses [36]. However, it should be noted that according to Oliveira and Canario [24] this is an unlikely explanation for the discrepancy. Thirdly, there could be methodological differences in the experimental conditions, variation in housing conditions prior to the experiment, etc. Future studies, in particular comparing circulating androgen levels with fecal/urinary levels, should shed more light on this issue.

In conclusion, we have shown that fighting experience alone, even without the experience of victory, increased the likelihood of winning a subsequent encounter in *Pundamilia*. We also found that circulating androgens increased during a mirror fight, possibly in anticipation of further escalation of the fight. A rise in androgens in response to ongoing social interactions – even if they remain unresolved – likely allows an individual to maintain a heightened aggressive motivation in a dynamic social environment filled with potential challenges [9]. At a more proximate level, how steroids act in the brain and increase the probability of success in subsequent encounters remains an exciting question for future research.

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