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Winning and losing in public: Audiences direct future success in Japanese quail

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ABSTRACT

Among vertebrates, winning a fight enhances the probability of future victories and vice versa and the role of post-conflict testosterone in mediating this 'winner effect' is widely accepted. In a series of staged fights of Japanese quail (*Coturnix japonica*) we tested both opponents' pre-fight and post-conflict testosterone, behavior and dominance status after returning to their social groups. We found that the presence of a familiar mixed-sex audience during the encounter modulated both the testosterone response and the long-term success after a fighting experience. 'Public losers' but not 'public winners' lacked a post-conflict testosterone response, whereas without an audience both winners and losers increased testosterone metabolite levels. Long-lasting winner and loser effects exclusively occurred when the performance information was perceived by a mixed-sex audience. In further experiments we manipulated the testosterone responsiveness of either the loser or the winner. An artificial post-conflict testosterone surge after having lost a fight effectively reversed the loser effect in Japanese quail. In contrast, the 'winner effect' was not changed by blocking testosterone after the fight. Overall, male Japanese quails' post-conflict testosterone was connected to the audiences and thus, own or the observers' perception of the challenge rather than to winning or losing a fight.

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Introduction

Testosterone, the major sex steroid of male vertebrates, is profoundly involved in the regulation of sexual and agonistic behavior. In many species so-called 'winner and loser effects' have been observed: winning a fight enhances the probability of future victories and losing a fight increases the likelihood of future defeats (Hsu et al., 2006; Rutte et al., 2006). Winner and loser effects are adaptive when the costs for participating in a subsequent encounter are reduced by behaving according to the most recent social experience (Lehner et al., 2011). Testosterone seems to mediate the winner effect, but its role is not well understood. Conversely, the social environment can have strong effects on testosterone secretion, which may in turn influence subsequent behavior. For example, aggressive encounters can elicit testosterone surges in fighting males, an effect that seems to be conserved throughout the vertebrate taxa including humans (Hirschenhauser and Oliveira, 2006; Mazur and Booth, 1998; Wingfield et al., 1990; but see e.g. Apfelbeck and Goymann, 2011; Goymann, 2009). Increased post-conflict testosterone levels during social instability are believed to serve the persistence of aggressiveness in future territorial encounters (Ramenofsky, 1985; Wingfield, 1994) and to facilitate future winning (Oliveira et al., 2009; Oyegbile and Marler, 2005).

However, the role of post-conflict testosterone in mediating the winner effect is still not clear. Conflict outcome alone does not always explain testosterone changes in winners and losers (e.g., Apfelbeck and Goymann, 2011; Earley and Hsu, 2008; Sachser and Pröve, 1984; Trumble et al., 2012; van der Meij et al., 2010). After fights of male guinea pigs (*Cavia aperea f. porcellus*), the challenged resident males exhibited higher testosterone responses than intruder males independently of winning or losing (Sachser and Pröve, 1984). In California mice (*Peromyscus californicus*), testosterone mediated future winning ability only in combination with a previous winning experience and the 'home advantage' of residents (Fuxjager and Marler, 2010; Fuxjager et al., 2009). Oliveira et al. (2009) suggested that winner and loser effects rely on different mechanisms in cichlid fish (*Oreochromis mossambicus*): the winner effect was androgen-sensitive while the longer lasting loser effect may have involved more permanent neuronal changes. In humans, the opponents' psychological state (i.e. the opponent's self-efficacy) can modulate the testosterone response of the respective winner or loser, i.e. reduce the testosterone increase in winners or increase the testosterone response in losers (van der Meij et al., 2010). In sum, androgen hormone action is currently thought to mediate the winner effect (Fuxjager et al., 2010; Oliveira et al., 2009). However, a meta-analysis throughout all vertebrate taxa revealed that the relationship between testosterone and dominance was generally affected by social and contextual factors; furthermore, studies comparing testosterone of winners and losers had particularly low effect sizes in birds (Hirschenhauser and Oliveira, 2006).

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According to the 'challenge hypothesis' (Wingfield et al., 1990), the magnitude of the testosterone response to an aggressive encounter differs due to the mating system and is a function of the stability of the social environment. In times of social instability males are more frequently involved in social interactions and this is often accompanied by increased testosterone levels. Japanese quail (*Coturnix japonica*) are promiscuous galliform birds with a hierarchical social structure among males and among females of a group (Mills et al., 1997). Previous tests have shown that Japanese quail had increased testosterone metabolite levels after interacting with a simulated territorial intruder (Hirschenhauser et al., 2008). These post-conflict changes in testosterone metabolites were not explained by conflict outcome alone: birds in staged fights that remained unresolved after 30 min had similar high post-conflict testosterone metabolite levels as the winners of resolved conflicts. On the other hand, fighting behavior itself can occur dissociated from testosterone in some species (birds: reviewed in Goymann, 2009; and see Apfelbeck and Goymann, 2011; Apfelbeck et al., 2011; Landys et al., 2010; non-avian: Fuxjager et al., 2011; Oliveira et al., 2005) including Japanese quail in mirror-fighting tests (Hirschenhauser et al., 2008). Results from mirror-elicited aggression suggested that information on the opponent's relative fighting ability and thus, the individual perception of the degree of a challenge, influences testosterone levels. Social environment potentially affects the perceived degree of challenge, i.e. the 'excitement' involved in an agonistic encounter and perhaps also contest dynamics and physical exhaustion experienced especially by the loser (Lehner et al., 2011). Current advances of knowledge about the social and cognitive competences of an increasing number of species show that the presence of conspecifics as an audience may substantially alter an individual's behavior and physiology (e.g. Cornelius et al., 2010; Matos and Schlupp, 2005; Zuberbühler, 2008).

In a series of staged fights with Japanese quail we tested the effect of the presence of both a familiar and an unfamiliar mixed-sex audience on agonistic behavior and post-conflict testosterone levels of winning and losing males, and the persistence of their dominant status within the group. Second, we manipulated the post-conflict testosterone changes of either winners or losers of staged fights as in Oliveira et al. (2009) to test whether socially induced testosterone is needed for mediating the winner and/or the loser effect in Japanese quail. We temporarily blocked testosterone action (Hau and Beebe, 2011; Soma et al., 1999) in males after winning, or we experimentally elevated post-conflict testosterone in males after losing the staged fight. If testosterone was needed for maintaining the males' dominance status, we expected that winning males with blocked post-conflict testosterone action would not remain dominant in their social group (despite of having won the staged fight) and that losing males with artificially elevated post-conflict testosterone levels would remain dominant in their social groups (despite of having lost the staged fight).

Methods

All experiments were carried out in accordance with the EU Directive 2010/62/EU for the use of laboratory animals and with permission of the Austrian Amt der Steiermärkischen Landesregierung (FA10A-78Hi3/08-5).

Animals and mixed-sex audience experiments

Japanese quail are small, domesticated galliform birds. They are promiscuous and if breeding occurs, parental care is purely maternal (Mills et al., 1997). We have previously observed increased levels of excreted testosterone metabolites in response to simulated territorial intrusions (Hirschenhauser et al., 2008). We conducted staged fights between two male Japanese quail for testing the effect of the presence of a familiar mixed-sex audience on agonistic behavior and post-conflict testosterone levels of winning and losing males. Dyadic fights between

male Japanese quail without an audience served as a control (sample sizes in Table 1). The quail were housed in social groups composed of two males and three females. Two of such social groups were housed in adjacent cages (each 200 × 65 × 70 cm) separated by removable wooden divisions. Males of adjacent groups were of similar age and size (body mass ratio = 1.1 ± 0.01 mean ± s.e.m.) and had no previous experience with each other. On average the age of the tested birds ranged between 7 and 14 months, and their body mass was 278.3 ± 6.2 g. All birds had ad libitum access to standard food, water and shelters at all times. Interactions among group members were observed at least twice daily and the groups were separated if ongoing disputes or serious fighting occurred. After an 'initial dominance phase', the dominant male of each group was separated from its group for the duration of the staged fight. The subordinate male and the females were present behind a wire-mesh as an audience visible to the separated males ('audience group'; Fig. 1) or behind opaque divisions as a control ('no audience group'). Removal of the wooden division started the staged encounter between the two separated (previously dominant) males. Agonistic interactions between opponents were recorded for ≥ 30 min until there was a clear winner and loser. A 'loser' status was assigned when one male continuously escaped from the attacking opponent (the 'winner'). Interactions were terminated by re-inserting the wooden division.

Behavior records

Behavior was sampled from video-tapes during each experimental phase (Fig. 1). During staged fights we recorded latency time to first attack and first escape, which opponent had initiated the fight and frequencies of attacking the opponent. To document the opponents' interactions with the audiences, i.e. peeping through or pecking at the wire-mesh in front of the audience, we scan-sampled with which of the two audiences the two focal males interacted right before the staged fight phase was terminated ($n = 25$ fights with audience; Table 1). Directly after winning or losing the staged fight, each opponent was allowed to return to its group and agonistic interactions among the group members were recorded for 20 min ('same day'). Three days after the staged fights at the same time of day, groups were recorded again for monitoring longer-term effects of winning and losing. From these video tapes we measured male-chasing-male behavior for both the previously dominant and the previously subordinate male of each group by continuous recording. We used frequencies per minute to compare agonistic interactions between winner and loser groups, and between same day and third day. We assessed the dominance relationship between the two males of a group three days after the staged fight based on agonistic displacements in the 20 min video recordings. The male chasing and pecking the other male was termed dominant, and a withdrawing male was subordinate.

Post-conflict testosterone

We measured both the winners' and the losers' post-conflict testosterone changes using excreted androgen metabolites from individual droppings (Hirschenhauser et al., 2008). Using droppings is

Table 1

Overview of sample sizes for each treatment group. Note that numbers are n fights; each fight involved four males and 6 females (two focal dominant males and two social groups each with one subordinate male and three females).

	No treatment	Increased testosterone of losers	Blocked testosterone of winners
Audience	7 ^a	10	8
No audience	9		

^a One male did not provide a dropping within 240 min; therefore, testosterone metabolite data for the audience group are based on $n = 6$ fights (i.e. 6 winners and 6 losers), behavioral data were available from $n = 7$ males.

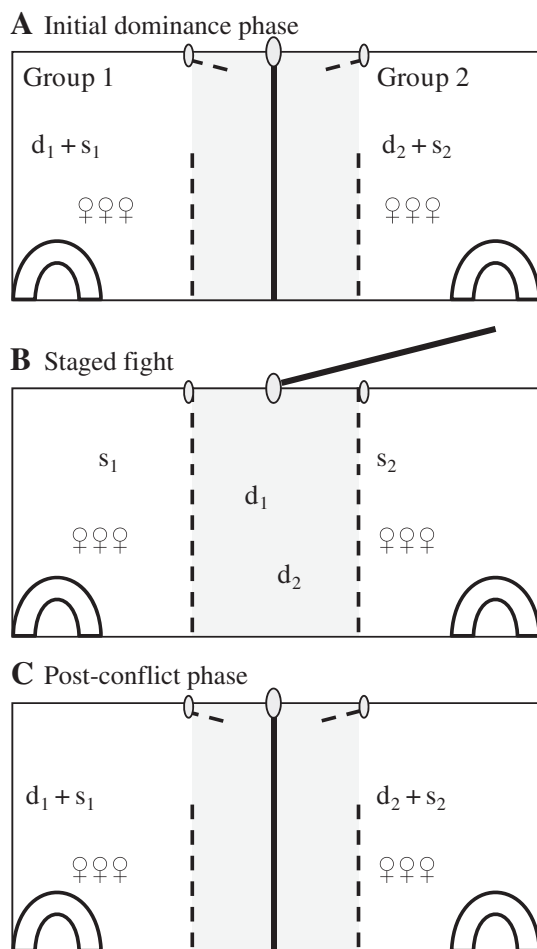


Fig. 1. Schematic view of the experimental set-up. Two social groups were housed adjacently divided by an opaque (removable) division (bold line). Each social group was composed of a dominant male (d_1 , d_2), a subordinate male (s_1 , s_2) and three females (♀♀♀). Shelters as indicated by the reversed U-shape structure, were provided at all times. (A) The 'Initial dominance phase' lasted until the males had clearly sorted who is dominant and subordinate in both groups (29 ± 6 days). (B) 'Staged fight': the dominant males (d_1 and d_2) were separated from their groups by a transparent wire-mesh frame ('audience' condition; $n = 7$ dyads; dashed lines) into the central compartments. Then the opaque division was removed and a direct contest between d_1 and d_2 was allowed until the conflict was resolved. In the 'No audience' condition (control; $n = 9$ dyads), the wire-mesh was replaced by an opaque frame (not shown) and the subordinate males and females of both opponents were not visually informed about the ongoing interactions. (C) 'Post-conflict phase': the opaque division was reinstalled, d_1 and d_2 return to their resident groups. Three days after the fight, we recorded behavior and dominance status in both groups as a (random) sample of longer-term changes. The shaded area marks where the fight took place and where we collected the droppings after the fight.

advantageous for estimating testosterone levels as it avoids handling interference and stress (Heiblum et al., 2000). Samples were collected one day before the experiment (pre-fight level), after the staged fight for measuring the post-conflict response and three days after the staged fight to monitor the short-term nature of testosterone metabolite changes. Sawdust was removed during dropping collection to avoid dust-contamination of fecal material. The birds were continuously monitored until the focal male had released a dropping. The samples were stored -20°C until they were further processed in the endocrinology lab. Based on previous experiences (Hirschenhauser et al., 2008) and an additional evaluation test (see below), we collected droppings from winners and losers between 120 and 240 min after the staged fight to monitor post-conflict testosterone responses. Dropping production was similar in all birds sampled (number of droppings in winners and losers with audience 6 ± 0.3 and 5 ± 0.3 , without audience 6 ± 0.5 and 6 ± 0.4). Hence, variation in dropping production

could not lead to distortions in hormone metabolite concentration measurements (Goymann and Trappschuh, 2011). All tests were conducted in the afternoon to avoid bias due to diurnal variation of hormones.

At the endocrinology lab 0.3 g of homogenized droppings was extracted in 60% methanol by vortexing for 30 min. We used a group-specific enzyme-immunoassay to measure testosterone metabolites (17 β -hydroxyandrogens) as described in Hirschenhauser et al. (2008). Group-specific antibodies specifically bind to steroid derivatives that share a specific functional group (Möstl et al., 2005). The antibody used here was raised in rabbits against 5 α -androstane-3 α -ol-17-one-3 α -hemisuccinate linked to bovine serum albumin and a biotinylated testosterone derivative served as label (Palme and Möstl, 1993). Based on homogenized 'pool samples' and a total of four microtitre plates, the inter-assay coefficient of variation was 1.9, the mean intra-assay coefficient of variation was 8.7%.

Evaluation of effective sampling time and transdermal testosterone treatment

In an additional dataset we tested the time-lag between testosterone secretion and excretion in male Japanese quail by comparing the time-course of transdermal testosterone treatment on testosterone concentration in blood plasma and excreted metabolites in fecal droppings. We monitored (i) after which time interval transdermal testosterone treatment resulted in increased circulating testosterone levels, and (ii) the effective time for collecting fecal droppings, which would reflect circulating testosterone levels in the blood just after a given stimulus, i.e. the staged fight. Transdermal hormone application is routine in human medical care and rarely has been used in animal research (e.g. corticosterone in reptiles: Meylan et al., 2003; melatonin in birds: Goymann et al., 2008). We treated male Japanese quail with 100 μl cream containing 30 μg testosterone propionate (Richter) by gentle application to the skin underneath the feathers at the bird's head ($n = 8$). As control, we sampled eight non-treated additional male Japanese quail in a similar manner (hatched lines). All birds had ad libitum access to food and water at all times and were kept in a long-day light regime (7:00 a.m. to 9:30 p.m.). We performed all experimental treatments at 12:00 a.m. (time zero) to avoid bias of activity and hormonal secretion due to diurnal variation.

To monitor the time course of excreting testosterone metabolites in feces we collected all individual droppings until 5 h after treatment (7.3 ± 0.4 samples in treated and 6.6 ± 0.5 samples in control birds) and one control dropping sample on the third day after treatment. 0.3 g of homogenized droppings was extracted in methanol. We used enzyme-immunoassay to measure excreted testosterone metabolites as described in Hirschenhauser et al. (2008). In an additional dataset we sampled 100 μl blood from the wing veins using heparinized capillaries at zero, 30, 60, 120, 240 and 360 min after testosterone treatment ($n = 8$ creamed and 8 control males). Testosterone was assayed from plasma by radioimmunoassay following the protocol of Goymann et al. (2006).

Increasing testosterone of losers

Directly after staged fights with mixed-sex audience, we treated the losing male ($n = 10$) with 100 μl cream containing 30 μg testosterone propionate before the bird was released to its social group. Transdermal testosterone treatment rapidly increases circulating testosterone levels in blood with a peak after 60 min (see evaluation experiment). Behavior and droppings were sampled before, during and after the staged fights as described above. We tested the effect of high post-conflict testosterone in losers by comparing the dominance status of treated loser males with that of non-treated losers from the initial mixed-sex audience experiments.

Blocking testosterone actions in winners

Quail have been one of the first avian species, in which it was shown that testosterone action and thus, aggressive behavior is regulated by aromatase activity and estrogen receptors in the brain (Schlinger and Callard, 1990). Long-term treatments, i.e. implants with anti-androgen and aromatase inhibitor are often used to experimentally decrease male aggressive behavior (Hau and Beebe, 2011; Marasco et al., 2011; Soma et al., 1999). Typically, circulating testosterone levels of treated males are high, but do not exert effects at the tissue level because androgen receptors are blocked; thus, high levels of circulating testosterone are ineffective at eliciting physiological or behavioral change (Moore et al., 2004; Mougeot et al., 2005).

Here we aimed at a temporary, i.e. short-term blockage of testosterone actions in male Japanese quail after winning. Directly after the staged fight with mixed-sex audiences, we intraperitoneally injected the winner male ($n = 8$) with a mixture of 1 ml saline solution containing 0.5 mg flutamide (Sigma F 9397; an androgen receptor antagonist) and 0.5 mg fadrozole (Sigma F3806; an aromatase inhibitor). After treatment the bird was released to its social group, behavior and droppings were sampled as described above. We tested the effect of blocking the post-conflict testosterone actions of winners by comparing the dominance status of treated winner males with that of non-treated winners from the initial mixed-sex audience experiments.

We are confident that our treatment with anti-androgen and an aromatase blocker was physiologically effective in male Japanese quail. In an additional test we found that treatment with the doses described above affected crowing behavior, which is regulated by both testosterone and estrogen (Beani et al., 2000; Chiba and Hosokawa, 2006). Male quail ($n = 14$) treated with flutamide and fadrozole took longer to produce 50 crows (continuously recorded in anechoic recording chambers as in Deregnacourt et al., 2009) than after control treatment with 1 ml saline solution (43.8 ± 12.6 min versus 17.4 ± 2.9 min, respectively; Wilcoxon signed rank test $Z_{14} = 2.0$; $p = 0.048$).

Statistical analysis

We used non-parametric statistics as testosterone metabolite and behavior data were not normally distributed. For comparing

testosterone levels, latency times until first attack and fight durations between fights with audience and fights without audience we used Mann Whitney U tests. Comparisons of testosterone and aggression between winners and losers of the same fight were tested using Wilcoxon tests for matched pairs (Elwood and Briffa, 2010). To calculate effect sizes we used Cohen's d (Cumming and Finch, 2001). Fisher's exact test was employed to test for non-random effects of winning and losing experiences on future dominance status. This test determines whether the proportions of winners and losers remaining dominant significantly differ from a random result (Preacher and Briggs, 2001). The effect of transdermal testosterone treatment was tested with two-way repeated measures ANOVA on log transformed data (Shapiro–Wilk test for normality: $p = 0.2$). All probabilities are given two-tailed and were considered significant when $p < 0.05$.

Results

Staged fights with a mixed-sex audience

The males began to attack each other within 6 s (median, range 1 s to 11 min) after removing the partition and the male that initiated the fight was likely to win the encounter. In fact, fight initiation predicted winning but body mass did not (68 dyads tested at first encounters between the dominant male and the subordinate male during group formation; initiating the fight: $X^2_{68} = 25.9$, $df = 1$, $p < 0.0001$; body mass: $X^2_{68} = 0.0$, $df = 1$, $p = 1.0$). Males fought for 6.9 min (median time between first attack and first escape of loser, range 1.5–36 min), and fight duration was similar with or without the audience (Mann Whitney U test $U_{6,9} = 23.5$; $p = 0.7$), and independent of body mass of losers ($r_{15} = -0.2$; $p = 0.4$), body mass of winners ($r_{15} = 0.01$; $p = 0.9$), as well as body mass differences between winners and losers (asymmetries; $r_{15} = 0.2$; $p = 0.5$). Throughout the staged fight phase, winners attacked losers up to 114 times (median 29 times) compared with losers attacking winners up to 62 times (median 9 times). With the audience, winners attacked losers twice as often than during fights without the audience (median [lower; upper quartile] number of attacks per minute with the audience 1.8 [0.7; 2.8] and without the audience 1.0 [0.4; 2.0]), however the difference was not significant ($U_{6,9} = 16.5$; $p = 0.2$).

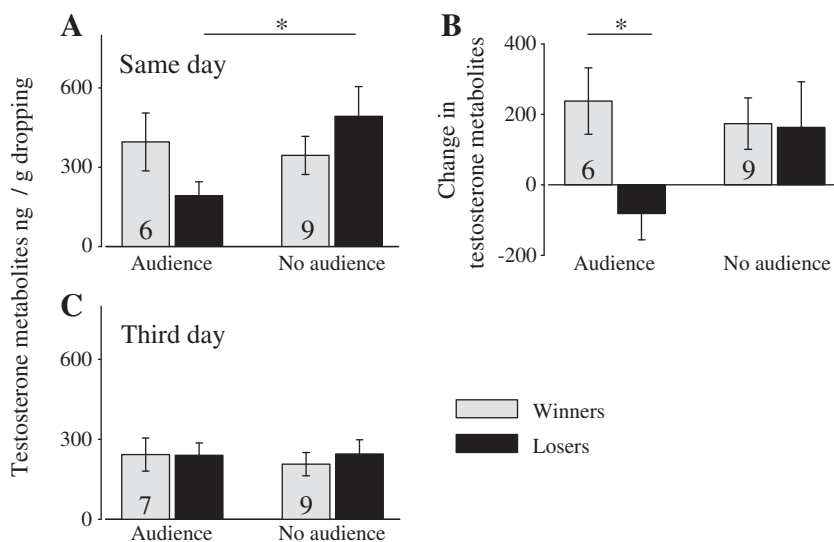


Fig. 2. Male Japanese quail losing in public lack a testosterone response to fighting. (A) Post-conflict testosterone in winners (light bars) and losers (dark bars) of staged fights with or without audience. Excreted testosterone metabolites were measured from faecal droppings collected between 120 and 240 min after the encounter (Electronic Supplementary Content). One male did not provide a dropping within the 240 min interval, therefore, $n = 6$ winners and 6 losers for the 'Audience group'. Bars show mean testosterone metabolites \pm s.e.m., asterisks indicate significant differences between groups. (B) Mean change of testosterone metabolites in response to having won or lost the fight (expressed as the difference between individual pre-fight and post-conflict testosterone metabolite levels) differed when the audience was present ($T_6 = 0.0$, $p = 0.028$, $d = -1.5$) but not without audience ($T_9 = 21$, $p = 0.9$, $d = -0.4$). (C) Three days after the staged fight testosterone metabolite levels did not differ between winners and losers or due to the audience during the encounter (Audience: $T_7 = 0.3$, $p = 0.7$; No audience: $T_9 = 15$, $p = 0.4$). Thus, the post-conflict testosterone response was short-term.

All staged fights ended with the opponents interacting with one of the audiences, i.e. peeping through or pecking at the partition/window behind which was the social group of one of the opponents was located. These interactions were either with 'own group' (each of the opponents interacting with its own social group: 32%), or 'opponent's group' (each of the opponents in front of the other's social group window 28%), or 'the loser's group' (both opponents in front of the loser's social group window 32%). In two cases (8%) both opponents interacted with the winner's social group. In sum, a higher proportion of winners interacted with the 'opponent's group' (60% = 'opponent's group' + 'loser's group') than the losers did (36% = 'opponent's group' + 'winner's group').

Changes in testosterone metabolite levels

Before the encounter, levels of testosterone metabolites were similar in prospective winners and losers ($U_{15,15} = 92.0$; $p = 0.4$). After the fight testosterone metabolite levels increased regardless of whether the bird was the winner or the loser ($T_{15} = 52$; $p = 0.7$). However, quail losing in the presence of an audience had significantly lower post-conflict testosterone metabolite levels than quail losing without an audience ($U_{6,9} = 6.0$; $p = 0.012$, $d = 1.2$). In contrast, the winners' post-conflict testosterone metabolite levels did not vary with or without audience ($U_{6,9} = 24.0$; $p = 0.8$, $d = -0.1$; Fig. 2A). To obtain a finer measure of hormone changes due to fighting, we also analyzed the changes in testosterone metabolite levels within individuals before and after the fight: in the audience group, testosterone metabolite concentrations of losers decreased significantly relative to pre-fight levels. In winners with an audience, testosterone metabolites increased during that time period; without an audience, both winners and losers had increased testosterone metabolite levels after the fight. Winners and losers differ significantly in their testosterone responsiveness following staged contests in the presence of an audience, but not in the absence of an audience (Fig. 2B).

Winner and loser effects

To test whether the availability of social information during staged fights with or without an audience affected future social status, we observed both opponents after the fight. Three days after both opponents had returned to their social groups, we recorded the dominance relationships between the two males within each social group, the previous dominant male (returning either as winner or loser) and the previous subordinate male. Returning winners were 100% successful when their group had observed the victory, whereas the majority (71%) of all returning losers lost their previous dominance status (5 of 7) to the former subordinate male in the group (Fisher's $p = 0.021$; Fig. 3A). In contrast, after staged fights without an audience, the winner and loser were equally likely to remain dominant or to lose the dominant status after they returned to their social group (6 and 6 of 9 winners and losers remained dominant; Fisher's $p = 1.4$; Fig. 3A).

Agonistic interactions in the social groups

Before the encounter, dominant males chased subordinate males more frequently than vice versa (audience: $T_{14} = 3$; $p = 0.003$; $d = 1.1$; no audience: $T_{18} = 2$; $p = 0.001$; $d = 0.8$; Fig. 4). Three days after the fight, 'public winners' kept on chasing the subordinate male more often than vice versa ($T_7 = 1$; $p = 0.028$, $d = 0.8$). Public losers however (lacking a post-conflict testosterone response), were chased by the subordinate male of their social group at similar rates than they chased the subordinate male ($T_7 = 2$; $p = 0.2$, $d = 0.8$). In contrast, losers without an audience (with a post-conflict testosterone response) continued to chase the subordinate male of their social group at a higher rate than vice versa ($T_9 = 1$; $p = 0.046$, $d = 0.95$; Fig. 4). Surprisingly, winners without an audience were challenged by their subordinate males at a similar rate than vice versa ($T_9 = 5$;

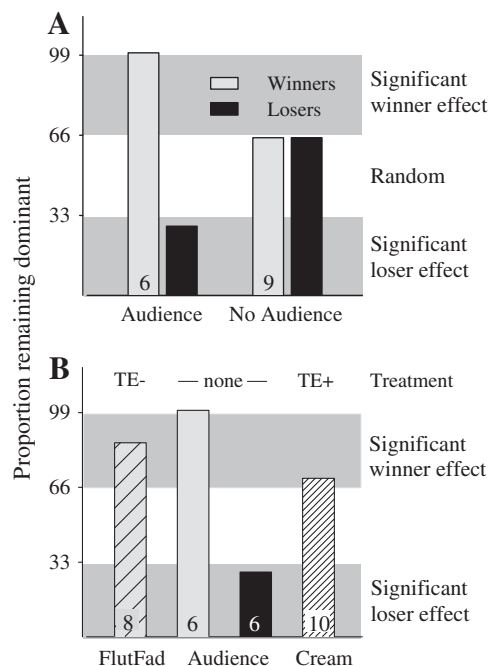


Fig. 3. Social information rather than outcome mediates the winner and loser effects in opponents. Bars show the proportion of previously dominant males, which were dominant over the previously subordinate males after they had returned to their social groups as winner (light bars) or loser (dark bars) of the staged fights. Numbers inside bars indicate sample sizes. Shaded areas mark the statistical thresholds for a significant effect of the experience of prior dominance (winner effect > 66.6%) or prior subordination (loser effect < 33.3%) according to [Begin et al. \(1996\)](#). (A) Staged fights with mixed-sex audience ('Audience') compared with staged fights without audience ('No audience'). (B) Proportion of males remaining dominant when either testosterone responsiveness of the winner was blocked ('FlutFad') or the loser received an artificial increase of testosterone immediately after the staged fight ('Cream'). For comparison with the effects of testosterone treatments the results of non-treated winners and losers of the mixed-sex audience experiment ('Audience') are shown.

$p = 0.5$, $d = 0.4$; Fig. 4) despite exhibiting a post-conflict testosterone response. As all of these behavioral effects were observed three days after a public winning or losing experience, they represent a longer-term effect. It is noteworthy that on a short-term basis, the patterns were different: on the same day, immediately after the fight, previously dominant males remained dominant after fights with an audience, no matter whether they had won or lost the fight. Without an audience, both returning winners and losers had no clear dominance status in their group (Electronic Supplementary Content).

Transdermal testosterone treatment for losers

Transdermal treatment of male Japanese quail with a testosterone cream increased circulating testosterone levels within 30 min (Fig. 5). Plasma testosterone levels of the treated males were higher than in non-treated control males and reached a peak after 60 min (treatment: $F_{1,14} = 110.4$, $p < 0.001$, time: $F_5 = 1.6$, $p = 0.2$, interaction treatment \times time: $F_{5,68} = 7.0$, $p < 0.001$). The amount of excreted testosterone metabolites was within the naturally observed range in droppings from non-treated winners and losers (Fig. 2). Excreted testosterone metabolites were increased after 120 min and remained high until 240 min after treatment (treatment: $F_{1,14} = 26.1$, $p < 0.001$, time: $F_6 = 14.6$, $p < 0.001$, interaction treatment \times time: $F_{6,60} = 14.2$, $p < 0.001$). Defecation rates were similar in treated and non-treated birds (6 ± 0.4 and 6 ± 0.5 samples in 5 h). Fecal excretion of increased testosterone metabolite levels was delayed by 2–4 h, which represents the interval used in the present

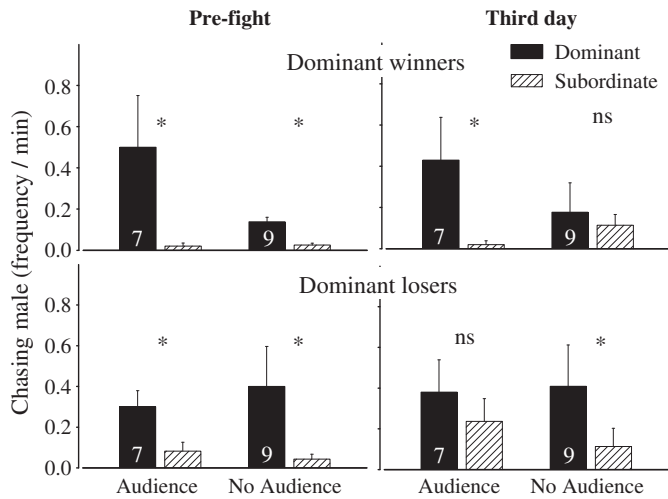


Fig. 4. Losing in public: male quail remember performance information. Bars show pre-fight (open bars) and post-conflict rates of male chasing male (mean \pm s.e.m. from 20 min video recordings) after the previously dominant male had returned as winner (upper row) or as loser (bottom row) of a staged fight with audience (left) or without audience (right). Hatched bars show the frequencies of subordinate males chasing the previously dominant male. Asterisks indicate significant differences (Wilcoxon tests for matched pairs; [32] between chasing behavior of dominant and subordinate males, i.e. one clearly dominant male; 'ns' indicates non-significant dominance relationships with ongoing disputes between the two males in the group. Before the staged fight, all groups had a clear dominant-subordinate male-male relationship. During the post-conflict phase ('Third day') dominant males returning after losing in public were not clearly dominant over the subordinate male in their group, i.e. the previous subordinate males chased returning losers more frequently than returning winners. Without audience dominant losers clearly remained dominant over the subordinate male, but not when the dominant males returned after winning without audience.

study as 'effective response time' for collecting droppings from the opponents after a staged fight.

Testosterone-cream treatment of male Japanese quail after losing the staged fight affected their aggressive behavior substantially. Three days after the losing experience, testosterone-treated males were still very active and exhibited high frequencies of aggressively chasing the subordinate male of the group (Fig. 6); as a consequence the testosterone-treated males remained dominant in their social groups (Fig. 3B). Thus, adding high post-conflict testosterone levels to the losing male experimentally reversed the long-term 'loser effect' with a mixed-sex audience.

Blocking testosterone actions in winners

The treatment of male Japanese quail with anti-androgen and an aromatase blocker resulted in the expected high levels of excreted testosterone metabolites on the day of treatment, as well as three days later (Table 2). In contrast to crowing performance (see methods), agonistic behavior of males treated after winning a fight was not affected and resulted in the same dominance pattern as with non-treated winners (Fig. 6). The proportion of males remaining dominant in their group did not change when the winners' testosterone responsiveness was blocked (Fig. 3B). Thus, in Japanese quail testosterone was not causally linked with the 'winner effect'.

Discussion

Testosterone and the perception of a challenge

In Japanese quail, fighting increased testosterone production in a context-dependent manner. Fighting in general induced high post-conflict testosterone metabolite levels, except in males after losing in public. Artificially elevated testosterone levels significantly increased the proportion of losers remaining dominant in their (informed) groups. The winner's likelihood of remaining dominant in the group was not related to elevated testosterone levels. The hormonal effects of winning and losing in Japanese quail are in contrast to (i) the common notion that testosterone levels are typically higher in winners than in losers (Mazur and Booth, 1998); and (ii) the hypothesis that testosterone mediates the winner effect while the loser effect may not be testosterone-dependent (Oliveira et al., 2009). The effect of an audience on the hormonal responsiveness to winning and losing in Japanese quail underlines the role of the social environment and context as mediators of testosterone responsiveness and future dominance status.

Results from tests in California mice and cichlid fish suggested a synergistic effect of experience and androgen sensitivity to increase winning chances in future encounters (Dijkstra et al., 2012; Fuxjager et al., 2010; Oliveira et al., 2009). However, experience and memory of social information can modulate social behavior also independently of status signals even if the signals are testosterone-driven (Danchin et al., 2004; Fuxjager et al., 2011). For example, *Anolis carolinensis* lizards responded according to the social rank established during a previous fight and ignored manipulations of the eyespot color that normally signals status in this species (Korzan et al., 2007). In cichlid fish, fighting was coupled

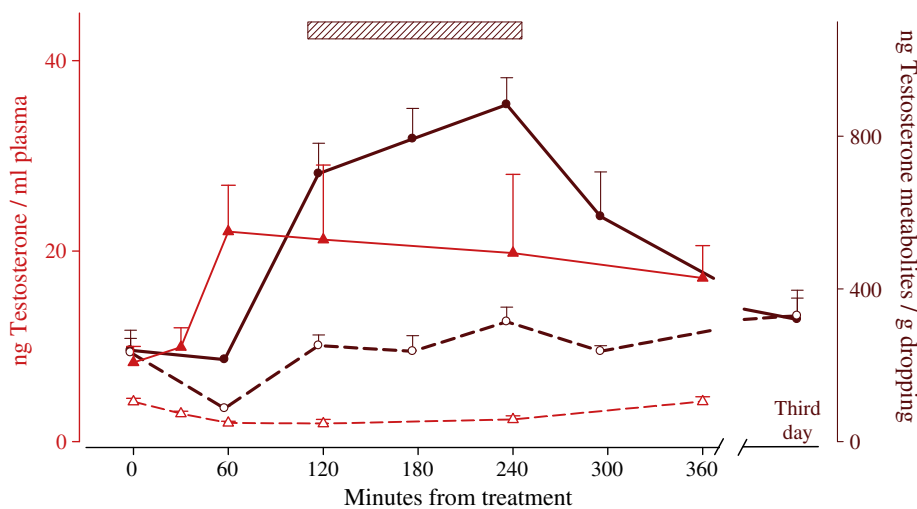


Fig. 5. The effect of transdermal application of testosterone (at time 0) on circulating testosterone levels in blood plasma (filled triangles and thin line) of male Japanese quail. Red lines: circulating testosterone levels in plasma ($n = 8$ males), filled dark dots and dark lines: excreted testosterone metabolites from fecal droppings of the same birds ($n = 7$ males). 'Third day': testosterone metabolites in droppings collected three days after treatment were back to baseline levels. Open triangles (plasma), dots (droppings) and hatched lines show androgen patterns from sham-treated control males. The hatched bar indicates the interval used as 'effective response time' for collecting droppings from the opponents after a staged fight. Plots show means \pm s.e.m.

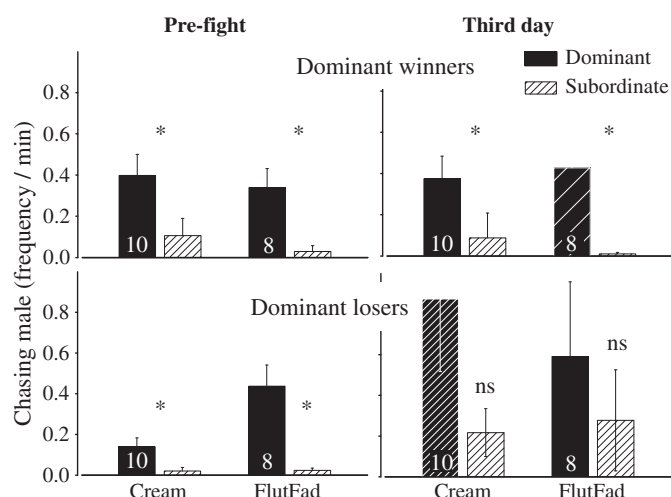


Fig. 6. An artificial post-conflict testosterone surge of losers reversed the 'loser effect' but blocking testosterone responsiveness of winners did not affect the 'winner effect'. All staged fights took place with a mixed-sex audience (see also Fig. 1). 'Cream': the losing males were treated with testosterone cream immediately after the fight (indicated by a narrow white hatch pattern) and the winning male was non-treated. 'FlutFad': the testosterone responsiveness of the winning male was blocked directly after fighting (indicated by wide white hatch pattern), the losing male was non-treated. Asterisks indicate significant differences between chasing behavior of the two males, i.e. a clear dominant-subordinate relationship between the two males in the social group. 'ns' indicates that the relationship is not clearly resolved, none of the males is clearly dominant over the other. During the pre-fight phase the dominant males of all dyads were more often aggressive than the subordinate male ($p < 0.05$). Three days after the fight, winning males were dominant over their subordinate despite of blocking their post-conflict testosterone response ($T_{8,8} = 0, p = 0.031; d = 1.1$); losers with an artificial post-conflict testosterone surge were frequently aggressive, however the difference to the chasing rates of their subordinates remained non-significant ($T_{10,10} = 11, p = 0.2; d = 0.9$). Non-treated losers of the 'FlutFad'-group ($T_{8,8} = 7, p = 0.3; d = 0.8$) were also not anymore dominant on the third day.

with increased androgen levels and increased the likelihood of winning again even without having won before (Dijkstra et al., 2012). Also our results imply that post-conflict testosterone changes play a role in preparing for future territorial or sexual encounters rather than in current interactions or conflict outcome. It is remarkable that a single experience had relatively sustained effects. However, the mutual transfer of social information between the actors and their audience(–s) seems to explain the opponents' future behavior and social status, while testosterone may have been only secondarily involved in the maintenance of dominance status for dominant winners and thus, in mediating the 'winner effect'.

Long-lasting winner and loser effects

Overall, the winner's public experience and/or the observers' memory mediated the 'winner effect' in Japanese quail, and this was independent

Table 2

Mean levels of testosterone metabolites (ng/g dropping ± s.e.m.) excreted in droppings before and after staged fights with audience (240 min and three days after the fight). Effective blocking of testosterone responsiveness in winners (Flut/Fad) is indicated by high post-conflict testosterone metabolite levels; losers treated with testosterone cream had also high post-conflict testosterone metabolite levels (see also electronic supplementary material).

Treatment	Pre-fight	Post-conflict	Three days after staged fight
Winners			
Non-treated	104.5 ± 54.0	314.0 ± 40.8	329.5 ± 67.4
FlutFad (T–)	272.1 ± 32.4	626.5 ± 176.8	502.0 ± 91.1
Losers			
Creamed (T+)	278.8 ± 58.7	887.5 ± 87.5	319.4 ± 59.1
Non-treated	387.1 ± 42.9	396.5 ± 53.8	327.2 ± 60.4

of the winner's testosterone. In tests of fighting California mice without observers, a winning experience increased the expression of androgen receptors in brain areas that control social behavior (Fuxjager et al., 2010); enhanced by transient testosterone release, a winning experience thus may have altered behavior in California mice, e.g. by suppressing submissive behavior during future fights (Gleason et al., 2009). In quail, in particular the loser was affected by the audience, because losing with a mixed-sex audience diminished the male's probability of retaining its dominant status in the long-term. This was due to increased rates of chasing behavior by the previously subordinate male rather than changes in the dominant loser's behavior (Fig. 4). Thus, on the other hand, the 'experience' of the observing subordinate male (from the loser's group) may have suppressed its future submissive behavior in a way similar to the winning California mice. However, this does not explain the role of testosterone: the 'public loser effect' was causally linked to testosterone: 'public losers' had low testosterone levels after the staged encounters (Fig. 2) and experimentally adding testosterone after losing a fight reversed this pattern. The increased testosterone clearly enhanced the treated male's aggressiveness even three days after having lost in public. However, three days after watching the other male's defeat the (previously) subordinate observer male increased his aggressive behavior (Fig. 6) at similar rates as non-treated losers with audience (Fig. 4). These results suggest that performance information – that is by definition detected by an audience (Wagner and Danchin, 2010) – acted as a potent catalyst of future behavior and social status. Further tests are needed to show whether the observed 'audience effects' were a combination of 'observer effects' and 'loser effects', i.e. due to the mutual transfer of information.

In summary, males that exhibited a post-conflict testosterone response were either the winners or took part in fights without an audience, after which they had random chances of remaining dominant or not. In contrast, males with no post-conflict testosterone response were caught in a significant 'public loser effect' (Figs. 2, 3). Although (the lack of) testosterone played a clear role in losing quail, the 'winner effect' was due to an informed audience rather than simply due to high testosterone. In contrast to Oliveira et al.'s (2009) study with cichlid fish, we could not confirm a role of testosterone in mediating the 'winner effect' in Japanese quail, because the dominance status clearly persisted also in males treated with an anti-androgen and an aromatase inhibitor. We are confident that blocking androgen actions with flutamide and fadrozole was effective because the same treatment 'decelerated' crowing behavior of male quail. Therefore, we argue that in Japanese quail the winner effect was not affected by blocking the actions of testosterone because in our experimental set-up the transfer of public information (on winning or losing) had effects superior to testosterone. Possibly, winning and dominance in Japanese quail may not be comparable to winning in cichlid fish. However, if testosterone is part of the mechanisms underlying the observed 'winner and loser effects', we would expect that these mechanisms are evolutionary conserved throughout the vertebrates (Hirschenhauser and Oliveira, 2006; Rutte et al., 2006). Alternatively, testosterone may be the epiphenomenon of another mechanism, such as social information transfer.

Results from studies with humans match our results from Japanese quail to some extent. In humans, testosterone responses to competition are (i) triggered by psychological processes, such as the opponent's self-efficacy rather than own psychological state (van der Meij et al., 2010) and due to positive reaffirmation prior to sports competition (Crewther and Cook, 2012). (ii) Testosterone responses predict the loser's decision to participate in future competitions (Mehta and Josephs, 2006). Also, in humans (iii) unfamiliar audiences enhance the perception of social threats and the stress response (Shalev et al., 2011). In quail this remains to be tested; so far our set-up involved both familiar and unfamiliar audiences. Testosterone plays a subtle role for acquiring and defending one's social status, which evidently includes fear reduction (Eisenegger et al., 2011). We show that in Japanese quail the long-lasting 'public loser effect' was coupled with low testosterone production. The lack of a testosterone response in

quail that lost in a public audience may have led to more 'fearful', 'reactive' or 'risk avoiding' behavior (Mills et al., 1997) and future subordination. For example, if low testosterone production reduced the mobility or the fighting response of the returning losers, this may have had a signaling function. This signal may have provoked status challenging behavior by male competitors (i.e. the previously subordinate spectator).

During the staged fights, performance information was obviously exchanged between the two fighting opponents and their bystanders. There is evidence from many species that third parties not involved in an interaction (bystanders) may extract information from the interaction (Oliveira et al., 1998; Zuberbühler, 2008) and even bystanders of a conspecifics' fight may respond with elevated heart rates and testosterone levels (Oliveira et al., 2001; Wascher et al., 2008). Eavesdropping swordtails (*Xiphophorus helleri*) avoid initiating fights with losers that escalated in the previously watched contest (Earley and Dugatkin, 2002). In turn, the presence and sex of an audience influences male–male aggressive behavior: male fighting fish (*Betta splendens*) fight more aggressively when a familiar male is present than with a female audience (Dziewieczynski and Perazio, 2012; Dziewieczynski et al., 2005; Matos and McGregor, 2002). Our staged fight tests show that in Japanese quail audiences direct future success independently of testosterone, whereas without an audience neither the winning experience nor testosterone alone are sufficient to explain the 'winner effect'. Towards the end of the 'staged fight phase' the opponents clearly interacted with the audiences (through the wire mesh). Particularly the winner males seemed to interact with the 'opponent's' (i.e. the loser's) social group. However, thus far, we cannot disentangle the loser's from the observers' perception. Future work will have to continue (for example, by using one-way mirrors) to control for eavesdropping or, vice versa, exclude the loser's interaction with the audience. Another question for future studies of this phenomenon is, whether the sex of the audience (–s) explains the fighters' testosterone response and the observed long-term consequences. Finally, testosterone manipulations following 'no audience' treatments, as well as investigations of the audience effect in other species (and taxa) would have the potential to reveal the power of the phenomenon observed in Japanese quail. We show that hormonal changes appear to be intimately linked with the social environment in which the conflict takes place.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yhbeh.2013.02.010>.

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