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Social context rather than behavioral output or winning modulates post-conflict testosterone responses in Japanese quail (*Coturnix japonica*)

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ABSTRACT

Testosterone regulates the expression of sexual and aggressive behavior in male vertebrates and treatments with testosterone may promote territorial aggression and winning in dyadic contests. Conversely, individual testosterone levels respond to sexual or aggressive interactions and the social environment. Post-conflict testosterone in winner males though appears to be more complex than simply reflecting conflict outcome. Expression and degree of post-conflict testosterone responses may adapt to additional modulators such as repeated winning experience, audience presence, opponent's fighting ability, and self-assessment. We present simulated intrusion experiments with male Japanese quail using mirror-elicited aggression and fights with real opponents ('direct challenge'). We recorded agonistic behavior and measured immunoreactive testosterone metabolites (TM) non-invasively from individual droppings. Frequencies of initiated agonistic behavior were similar whether towards the mirror or in direct challenge tests, although some of the males were behaviorally non-responsive to the mirror ('mirror submissives'). However, there was no TM response to the mirror test in both, mirror fighters and mirror submissives, thus independently of behavioral output. After direct challenges TM levels were elevated in all males (focal males winning or conflict unresolved after 30 min), hence independently of conflict outcome. Thus, in male quail a combination of physical stimuli and the individual perception of own and opponent's fighting ability explained the expression of post-conflict TM responses rather than behavioral performance, conflict outcome, or any of these factors alone. In sum, our results emphasize that the degree of androgen responsiveness to agonistic behavior is fine-tuned by components related with social context and environment.

1. Introduction

In male vertebrates testosterone regulates spermatogenesis, the expression of secondary sexual characters, and sexual and agonistic behavior. Administration of testosterone may affect aggressive behavior and territorial song rates in birds, and potentially promote winning in dyadic contests by stirring up existing dominance hierarchies. Conversely, androgens are generally responsive to sexual and agonistic interactions in male vertebrates and literature from fish to man suggests a high degree of mutual interaction between androgens and behavior [1–4]. In line with this, the social environment (for example, the availability of receptive females or group density) may also modulate individual male testosterone levels [5–7]. Furthermore, testosterone may be viewed as the physiological mediator of the trade-off between investing in male–male aggression or in paternal care [8]. The androgen responsiveness patterns to sexual and paternal behavior observed in birds [3,9] were generally also predictive of the hormonal response patterns in teleost fish [4,10,11]. However, in particular for the agonistic context (i.e., androgen responsiveness to male–male agonistic interactions), even within taxa a general consensus seems to be confounded by various factors related to the social environment and context [4,12–15].

Surprisingly, the function of increased testosterone levels in response to specific behaviors remains unresolved despite the taxonomic breadth of this phenomenon. Presumably, the major function of testosterone increases after agonistic interactions may be to sustain or reinforce the individual's motivation to engage in high-intensity fights [16–18] and at the same time boost sexual functions. The degree of the testosterone responses to territorial challenges co-varies with stimulus intensity, i.e., the duration and the number of actors involved [13,19]. In humans as in fish, high androgen levels are generally related with the experience of winning an aggressive encounter, and winning or losing alters the state of specific neurotransmitter systems of the brain [20–23]. Androgen levels may even be elevated in bystanders watching conspecifics fighting [24,25] and in anticipation of competitive sports games [26]. However, the predicted high androgen levels in winners are not always confirmed in the literature and the effect

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sizes of avian studies are generally smaller than with fish or mammals [4]. In male Japanese quail (*Coturnix japonica*), for example, victory or defeat in dyadic encounters was not simply reflected in plasma testosterone levels [27] although the winning experience explained acute testosterone responses [2,28]. Also in male Californian mice (*Peromyscus californicus*) only repeated winning experience rather than a singular winning event, i.e., behavioral history, resulted in the predicted testosterone response [18]. Other variables of behavioral experience, such as the familiarity of opponents [29,30], audience effects [5,31], and the individual's self-assessment relative to the fighting ability of the opponent [32,33] may potentially influence the androgen responsiveness to agonistic interactions. From comparative results in mice post-conflict testosterone was also suggested to covary with territoriality and population density [18,34]. Thus, post-conflict testosterone in winner males appears to be more complex than simply reflecting conflict outcome. Altogether, the social context of a conflict seems to fine-tune the degree of post-conflict testosterone responsiveness [12].

Winner and loser effects, information on the relative fighting ability of the opponent (resource holding potential [35]), behavioral experience [33], as well as self-assessment [36] are probably mediated by neuroendocrinological changes [30] with the effect of winning being usually less detectable than the effect of losing [33,37]. One example for the complex mechanism underlying androgen responsiveness to agonistic behavior has been reported from cichlid fish. Male Mozambique tilapia (*Oreochromis mossambicus*) clearly responded with elevated androgen levels to fighting with a territorial intruder [11]. When confronted with a mirror the same fish fought extensively towards its own mirror image but the corresponding androgen response was absent [32]. In addition to the missing tactile, acoustic and olfactory cues, two types of 'non-physical' information are missing in mirror-elicited aggression: (a) the assessment of the relative fighting ability of the opponent, as the mirror opponent's 'response' is in perfect symmetry and temporal alignment with the actor; and (b) who will be the winner of the contest as there will be no outcome of the conflict. Both, the relative fighting ability, as well as the conflict outcome (winning versus losing) are potential key moderators of the androgen responses we measure after agonistic interactions.

The presented experiments on the effect of male–male aggression on post-conflict androgen levels in Japanese quail were based on two issues: first, to test for the lack of a hormonal response to mirror-elicited aggression (as proposed for fish) in an avian species; second, to test whether the social context (i.e., experimental set-up with females in audience) was sufficient to alter the relatively weak winner–loser androgen responses of male quail that have been observed previously [2,28,38]. We used a non-invasive approach by determining levels of excreted androgen metabolites from individual droppings as integrative measures of systemic androgen levels [39–41]. There have previously been studies using fecal testosterone measures in quail [42,43]. To test whether our assay system measured systemically relevant androgen metabolites in male quail droppings we present the excretion patterns of androgen metabolites after treatment with gonadotropin releasing hormone (GnRH) or saline Ringer solution. Simulated intruder tests ('direct challenge') were designed for male Japanese quail and the observed androgen responses compared with mirror-elicited aggression. If information on the fighting ability of the opponent (relative to own) and / or physical stimuli were required to fully express the predicted androgen response to the agonistic conflict, we expected to observe no androgen response to the mirror test. In contrast, if the outcome of a conflict, i.e., winning or losing were determining the post-conflict testosterone response, we expected elevated androgen levels in winners of the 'direct challenge' tests. Together these data may contribute to unravel the complex components of an aggressive encounter that determine and fine-tune post-conflict testosterone responses.

2. Methods

2.1. Study animals and hormone assays from quail feces

Japanese quail were kept in 100×100×100 cm cages under a long-day photoperiod (16 h of light and 8 h of dark, lights on at 0730 h). Temperature ranged from 18 °C to 22 °C, food and water were provided ad libitum.

We measured immunoreactive 17ß-hydroxyandrogen metabolites (testosterone metabolites; TM) from individual droppings using an enzyme immunoassay (EIA) with a group specific antibody raised in rabbits against 5α-androstane,-3α-ol-17-one-3α-hemisuccinate linked to bovine serum albumin. As standard testosterone and as label a biotinylated testosterone derivative (5α-androstane-3ß-17ß-diol-3HS) were used [44]. The standard curve ranged from 0.8 to 62 pg TM per well. Fecal samples (0.1 g) were extracted with 1 ml water plus 1.5 ml methanol by vortexing (30 min) and after dilution an aliquot was used in the assay. For determination of intra- and inter-assay variations homogenized pooled samples were used. Mean intra-assay coefficient of variation was 9.5% and mean inter-assay coefficient of variation was 7.6%.

2.2. Effect of GnRH on androgen excretion

The measurement of TM from quail droppings is based on the assumption that the patterns of excreted metabolites reflect the respective changes of testosterone circulating in the blood. [39]. To show the effect of a physiological stimulus on the TM patterns measured in quail droppings we injected nine males with 0.3 µg gonadotropin releasing hormone (GnRH, RECEPTAL, Hoechst-Roussel Vet. Wiesbaden [40,45]) into the pectoral muscle. A minimum of one dropping per individual within 1 h prior to the GnRH application (mornings between 0900 and 1000 h; as individual baseline) and all fecal matter after the stimulus was continuously collected until 8 h after the stimulus (avg±S.E. 16.9±0.7 droppings per individual). As controls nine males were treated with 50 µl saline Ringer solution and went through the same sampling procedures as the GnRH-treated males (11±0.6 droppings per individual). Treatment times were similar in both groups to avoid potential confounding by diurnal variation of behavior (i.e., crowing and mating [46,47]) and gonadal hormones [2,46,48]. We compared mean TM levels in droppings from GnRH and control treatment males during intervals of 2, 4, 6, and 8 h after treatment. However, as GnRH is expected to elicit a short elevation in TM the effect may be averaged out by calculating means. Therefore, we also depicted the peak levels of excreted TM during each time interval. The GnRH experiments were approved by the Austrian national committee for the use of live animals in research (BMBWK 66.006/0014) and adherent to European ethical guidelines [49]. We observed no adverse effects caused by the GnRH treatment.

2.3. Simulated intrusions: experimental set-up

Quail were kept as groups of one male and three to four females in the cages and conditions described before. For the simulated intrusion experiments each cage was separated into a male and a female half by a wire-barrier to avoid physical injury [50]. The tested quail groups were observed in this set-up for 16 months as part of a descriptive long-term study and therefore, focal males were familiar with their female group and habituated to the housing conditions, to the presence of a human observer and the procedure of collecting fecal samples. All males were in acoustic contact with each other as they were kept in one large room, but focal males (residents) and intruders were raised in different groups and never had been in visual or physical contact. Thus, previous fighting experiences and familiarity [29,30,38] were avoided. Fifteen males served as focals, fifteen additional males as intruders. All tests were performed in the presence of the focal

Table 1
Results of the two-way repeated measures ANOVA calculations for the GnRH-effects on mean and peak excretion of androgen metabolites in quail droppings, as well as for the simulated intrusion experiments with mirror performance and conflict outcome as independent categories

	GnRH and mean TM response	GnRH and peak TM response	Mean TM after simulated intrusions due to mirror test performance?	Mean TM after simulated intrusions due to conflict outcome?
Repeated factor (within-subjects effect)	Time from treatment		Glass–Mirror–Intruder	Glass–Mirror–Intruder
	$F(4,89)=0.7$	**$F(4,89)=4.8$**	**$F(2,44)=7.9$**	**$F(2,44)=7.4$**
	$P=0.6$	**$P=0.002$**	**$P=0.002$**	**$P=0.003$**
Independent factor (between-subjects effect)	GnRH or control treatment		Fighting–Submissive	Winner–Unresolved conflict
	$F(1,16)=1.2$	$F(1,16)=2.0$	$F(1,13)=0.01$	$F(1,13)=1.1$
	$P=0.3$	$P=0.2$	$P=0.9$	$P=0.3$
Interaction of the two factors	$F(4,89)=0.1$	$F(4,89)=0.8$	$F(2,44)=0.4$	$F(2,44)=0.02$
	$P=0.9$	$P=0.5$	$P=0.7$	$P=0.9$
Pairwise comparisons (Holm–Sidak)	2 h after GnRH treatment		Glass–Mirror	
		$t(9)=2.7$	$t(15)=0.8$	$t(15)=0.8$
		$P=0.009$	$P=0.5$	$P=0.4$
	2 h after control treatment		Mirror–Intruder	
		$t(9)=1.2$	**$t(15)=3.8$**	**$t(15)=4.3$**
		$P=0.2$	**$P<0.001$**	**$P<0.001$**

Significant effects ($P<0.05$) are depicted as bold letters.

males' female group. Focal and intruder males of comparable age, size and reproductive state (well developed cloacal glands with active foam production [51]) were chosen.

Each focal male ($N=15$) was tested in three different 30-min experiments and a minimum interval of one day (maximum two days) was allowed between consecutive tests with different stimuli (i.e., glass, mirror, or intruder male). This enabled the homogenization of day time and minimum bias of the hormonal data due to diurnal variations [2,46,48]. Frequencies of initiated agonistic behaviors, such as threats followed by pecks and grabs (directed at the head, neck or body [2,38]) or chases were continuously recorded for 30 min in blocks of 5-min intervals. 1) In the mirror test a 17×21 cm mirror was placed in the male's compartment and the focal male was then allowed to interact with its mirror image for 30 min. 2) To control for the novel object the focal males were then tested in the presence of an equally sized piece of glass. 3) The third test introduced a size-matched intruder inside the focal male's compartment ('direct challenge'). The focal male was identified as a winner in dyads when the other male had ceased from attacking and was searching for escape at the end of the 30-min test. Throughout the entire test period the females were present at the other side of a wire mesh partition.

Individual droppings were used to measure baseline levels and response patterns of fecal immunoreactive testosterone metabolites before and after the presentation of the stimuli. Observations began in the morning (0900 h). As soon as a fresh dropping was collected to determine baseline levels of excreted androgen metabolites, the presentation of a stimulus was started. During the tests no samples were collected, to avoid interference with the ongoing interactions. After removal of the stimulus, droppings were continuously sampled until 4 h after the challenge (avg±S.E. 5±0.5 droppings per individual).

2.4. Data processing

The time delay of steroid excretion varies widely between species and is faster in birds than in mammals [52]. In birds, as in mammals, renal excretion via uric acid is faster than excretion via feces [53–55]. Based on fecal excretion, we expected to measure specific response patterns after a species specific delay time [41,56]. To assess gut transit time in Japanese quail, we marked standard food with edible food dye and allowed the birds ($N=11$) to feed on it for 15 min. Subsequently, all droppings were collected continuously and tested for color content by diluting the sample in methanol. Excretion of the color markers started at 88 (±9) min after food intake and lasted for a period of 165 (±12) min (which was well within the range of transit times reported from radiolabel metabolism tests in other bird species [2–5 h in *Anser* sp. [56]; 1.3–2.7 h in *Saxicola torquata* [40]; 1–4 h in *Tetrao tetrix* [57]; 6 h in *Strix occidentalis caudalis* [58]). This specified the effective dropping sampling interval we used to measure steroid responses to any stimulus. Therefore, in the experiments TM response patterns were calculated from all droppings collected after 90-min post-stimulus, whereas individual baseline TM levels were estimated from samples collected between −60 and 90 min. TM response patterns based on individual peak TM levels among all droppings excreted post-stimulus were finally expressed as percentage of the individual baseline TM levels. This data processing should minimize the potential bias caused by the fact that the 'fight or flight' response of the autonomic nervous system slows down gut transit time (as opposed by the 'rest and digest stage' [59]), which in turn may alter levels of excreted hormone metabolites [60]. Although male quail indeed defecated more frequently during the GnRH and food dye tests (1.9±0.1 in both tests) than during the social stimuli tests, a comparison of the observed dropping production indicated no major difference between

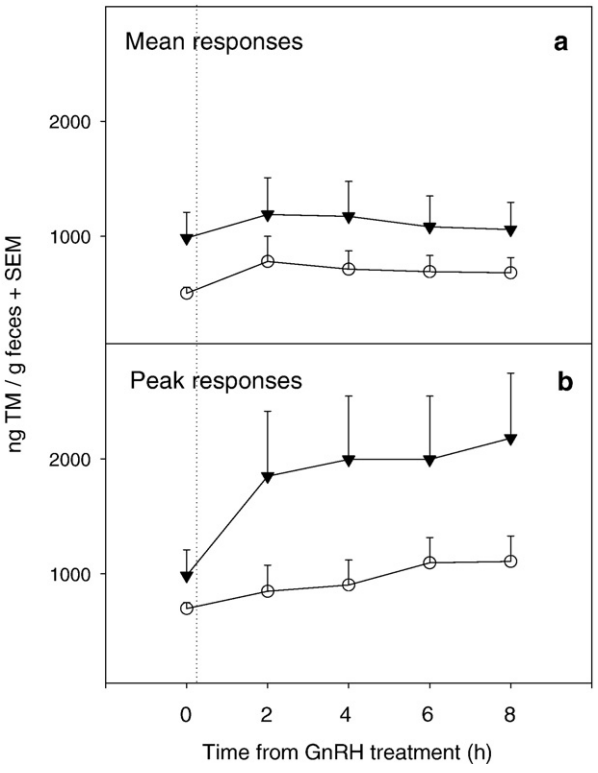

Fig. 1. Patterns of excreted TM in droppings of male quail after treatment with GnRH ($N=9$; filled triangles) and in control males treated with Ringer solution ($N=9$; open dots). (a) Mean and (b) peak levels of excreted TM per two-hour intervals are presented.

the test situations (glass and mirror test: 1.6±0.1; direct challenge: 1.7±0.1 droppings per hour).

Hormonal data are presented as means per individual±standard errors in the text and the figures. Data on GnRH-treatment effects and post-conflict patterns of TM met normal distribution assumptions (Kolmogorov Smirnov tests, mean TM after GnRH: $Z=0.7$; $N=90$; $P=0.7$, peak TM after GnRH: $Z=0.9$; $N=90$; $P=0.4$; TM after simulated intrusions, mean TM responses: $Z=1.0$; $N=45$; $P=0.3$, peak TM responses: $Z=1.1$; $N=45$; $P=0.2$) and were, therefore, tested parametrically using Two-Way Repeated Measures ANOVA with post-hoc Holm–Sidak adjustments for multiple pairwise comparisons. The behavioral data differed from normal distribution (Kolmogorov Smirnov test: $Z=1.7$; $N=45$; $P=0.005$), were calculated as geometric means and interquartiles per category and analyzed non-parametrically using Friedman rank ANOVA and Wilcoxon signed-rank tests. Probabilities were adjusted to the number of multiple comparisons with identical data using the post-hoc Bonferroni procedure. All probability tests are given two-tailed. Statistical analyses were conducted using the SPSS for Windows 15.0.1 and SigmaStat 3.5.

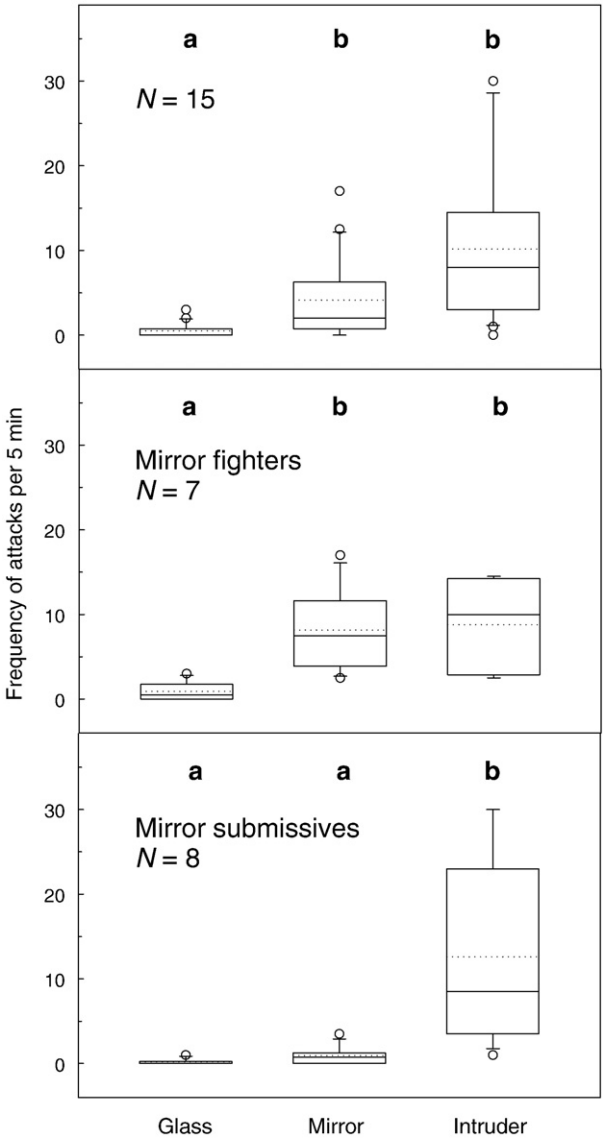

Fig. 2. Frequencies of initiated agonistic behavior ($N=15$ males; upper panel) were almost absent in control tests with glass, while mean attack frequencies towards their mirror image and during the 'direct challenge' were similar. Males that were behaviorally responsive to the mirror test ('mirror fighters'; medium panel) had similar attack frequencies during the direct challenge ($W^{+}=10$; $Z=-0.1$; $N=7$; $P=0.9$). Mirror non-responsive males ('mirror submissives'; bottom panel) vigorously attacked the intruder but not their mirror image ($W^{+}=35$; $Z=-2.4$; $N=8$; $P=0.034$). Boxplots show geometric means (full lines), arithmetic means (dotted lines), 25th and 75th interquartiles, and dots indicate outliers. Different letters specify statistically significant differences.

Table 2
Mean baseline (±S.E.) and post-conflict testosterone (mean and peak response TM levels) and median frequencies of attacks (25th; 75th interquartile) during mirror-elicited aggression and the 'direct challenge'

Category	*N* males	Baseline TM	Mean TM response	Peak TM response	Attacks (frequency per 5 min)	Target of attack (frequency per 5 min)
Mirror test						
Fighting	7	981.3 (±94.6)	565.3 (±98.6)	675.7 (±141.1)	7.5 (4.3; 10.8)	
Submissive	8	754.2 (±192.3)	593.7 (±139.1)	728.7 (±209.4)	0.8 (0.0; 1.1)	
Direct challenge						
Winner	9	515.9 (±62.9)	588.6 (±64.1)	782.6 ((±81.6)	4.0 (2.5; 14.5)	3.0 (0.0; 3.5)
Unresolved conflict	6	701.8 (±126.5)	719.4 (±214.1)	887.3 (±228.3)	11.0 (6.0; 13.5)	6.0 (2.0; 9.0)

TM: mean ng testosterone metabolite per g feces (±S.E.).

3. Results

3.1. Effect of GnRH on androgen excretion

Mean TM levels in droppings of GnRH-treated males after 2, 4, 6, or 8 h were not different from baseline TM levels (within-subjects effect) or from TM in control males (between-subjects effect, Table 1; Fig. 1a). In contrast, the peak TM responses indicated an effect of time after treatment, although the difference between GnRH or control treatment remained non-significant (Table 1; Fig. 1b). Also the interaction effect between treatment and time after treatment was not sufficiently powerful to exclude a random effect on the observed TM patterns (Table 1). However, post-hoc pairwise comparisons indicated that in particular in droppings of GnRH-treated males the peak TM levels were elevated already after 2 h while in the control males treated with saline solution also the peak levels of excreted TM did not differ from baseline TM during any time interval (Table 1; Fig. 1b). Therefore, although not optimal, our data still indicate that GnRH treatment resulted in high peak TM levels from quail droppings. Thus, the presented assay system reflects biologically relevant metabolites and systemic androgen levels in quail droppings with particular sensitivity to peak excretion of androgen metabolites.

3.2. Simulated intrusion experiments

The agonistic performance of focal male quail varied significantly in response to the different test situations ($X^2=11.19$; $N=15$; $df=2$;

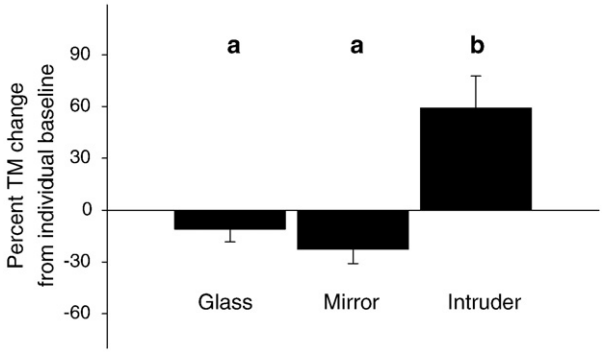

Fig. 3. Percent TM change of individual baseline TM levels in response to the different stimuli in male Japanese quail. Different letters indicate statistically significant differences between categories.

Table 3
Spearman's rank correlation coefficients (*P* values) between agonistic behavior (median attack frequency per 5 min) and baseline TM in droppings before tests, as well as mean and peak TM response levels in droppings within the 4 h after tests ($N=15$ males)

	Baseline TM	Mean TM response	Peak TM response
Mirror test	−0.05 (0.9)	−0.21 (0.5)	−0.16 (0.6)
Direct challenge	−0.02 (0.9)	−0.18 (0.5)	−0.26 (0.4)

TM: ng testosterone metabolite per g feces.

$P=0.004$, Fig. 2). In the control test for the insertion of a novel object (glass) we hardly observed any behavioral response, while the male quail were more actively responding to the 'mirror test' (Wilcoxon test: $W^+=66.0$; $Z=-2.9$; $P=0.006$; Fig. 2). When confronted with their mirror image the test males either responded with instant extensive threats and pecks ($N=7$; compared with glass object: $W^+=28.0$; $Z=-2.4$; $P=0.036$; Fig. 2), or submissively (squatting, trying to escape from the mirror; $N=8$; $W^+=10.0$; $Z=-1.8$; $P=0.1$; Table 2; Fig. 2). The different test situations resulted in similar frequencies of initiated agonistic behavior whether the focal male quail were in the 'mirror test' or in the 'direct challenge' ($W^+=19.5$; $Z=-2.1$; $N=15$; $P=0.1$). Behavioral responses to the 'direct challenge' included threats, pecks and grabs. As result of the 'direct challenge' test we identified nine focal males winning the fight against the intruder (when the intruder male behaved submissively and tried to escape), while none of the focal males lost a trial. The remaining six dyads were rated as 'unresolved conflicts' after 30 min of agonistic interaction (Table 2).

We observed a significant variation in TM levels in response to the different social stimuli tests (Table 1; Fig. 3). After presentation of a novel object (glass) the fecal TM levels remained close to baseline levels and also fighting their own mirror image did not significantly change baseline TM levels (Table 1). Moreover, distinguishing between mirror-fighters and mirror-submissive males did not explain any of the observed variation in both, mean and peak TM responses to the mirror presentation (Tables 1 and 2; peak TM responses: $F(1,13)=0.5$; $P=0.5$). In contrast, male quail showed clear TM responses to agonistic interactions with a real intruder (Table 1; Fig. 3). However, conflict outcome (focal male winning or conflict unresolved) had no clear effect on TM responses (Tables 1 and 2; peak TM responses: $F(1,13)=0.4$; $P=0.5$). In none of the test situations baseline or response TM levels were correlated with rates of agonistic behavior (Table 3).

4. Discussion

Based on measures of excreted androgens our data show three major results: (1) male Japanese quail expressed no androgen response to mirror-elicited aggression. (2) Male quail clearly responded with elevated TM levels to fighting with a real intruder. (3) Conflict outcome alone did not explain the observed variation of post-conflict androgen responses. Thus, fighting behavior itself and conflict outcome alone were not sufficient to engage a post-conflict androgen response. Some information on the relative fighting ability of the opponent and / or physical stimuli seem to be necessary to fully express a post-conflict androgen response. Furthermore, the social context of an aggressive encounter probably modulates the androgen responsiveness and degree of positive reinforcement.

In direct contests animals have the continuous opportunity to rank and assess the opponent's fitness and perseverance and relate this information to own performance and investment. Information on the relative fighting ability (regardless of whether gained as actor or observer [61]) may then be used in future aggressive interactions with those individuals [62]. Mirror-elicited fights, in contrast, do not allow an estimation of the opponent's persistence, as the mirror response is always in spatio-temporal identity with own performance. In line with this, mirror-fighting additionally never results in any outcome of the conflict, that is the fighting individual will neither be the winner nor the loser. Furthermore, mirror-image opponents are deprived of a whole suite of display stimuli, i.e., tactile, acoustic and chemical-olfactory stimuli. Particularly in the fish example [32], the role of those factors was yet unresolved but they were essential covariates of the proposed lack of an androgen response to mirror-fighting. In quail, the olfactory component may be less central than in fish [63], but the effects of acoustic and tactile stimuli in concert with visual cues probably contribute to the motivation and performance. In fact, the results of our mirror test indicate that physical stimuli are needed for the expression of a post-conflict androgen response.

Another major feature of mirror-fights is the lack of a size gradient in addition to the lacking behavioral gradient [64]. In the absence of a clear resource holding potential gradient, fights are likely to escalate, in which case the costs involved are unpredictable [65]. During ritualized fights the actors generally assess own and opponent's resource holding potential based on body size, energy efficiency and other definitions of quality to avoid injuries and enhance future reproductive chances [66]. Available information of an opponent's quality must increase with contest duration [65], which is probably determined by the weaker (losing) rival's resource holding potential rather than the gradient between rivals [67]. In the presented direct challenges we chose to use size-matched opponents to provoke a comparable (size symmetrical) challenge situation to the mirror test. The similar size of opponents may also explain why we observed no dyad that ended with the focal male as a loser, rather the resident male was winning or the conflict was extended and remained unresolved after 30 min. Although our study cannot provide a comparison with the androgen responses in losers, unresolved conflicts or having won engaged similar post-conflict androgen responses in male quail, which suggests a more complex mechanism than the anticipated simple pattern of high testosterone in winners.

Particularly in Japanese quail, earlier research reported no consistent (or particularly complex) testosterone differences between males winning or losing dyadic encounters [27,28]. Ramenofsky's [2] experiments indicated some rapid post-conflict testosterone responses during first encounters (social inertia), while serial experience and the formation of social relationships (resolved conflicts) reduced the degree of interaction between testosterone and aggression. Although testosterone implants increased the proportion of fights won in previous subordinates, testosterone was not sufficient to become dominant, i.e. to supersede the actual experience of being a winner or loser [12]. Similarly, Tsutsui and Ishii [27] observed no rank order changes after manipulating testosterone levels of the bottom-ranked individuals in groups of male quail. Also in our study the TM levels in response to mirror-fighting were equally low whether the quail males were actively attacking the mirror image or behaving submissively, while TM responses to the direct challenge occurred in all tested males and were not simply explained by being the winner. Also individual behavioral performance did not co-vary with pre-or post-conflict TM levels. As an alternative, there may exist some intrinsic component responsible for the expression of post-conflict androgen responses. The comparison between an 'unresolved outcome / no winner' situation in case of the mirror test and an 'unresolved outcome / persistent chance to win' in the direct challenge situation suggests an additional role for the persistent chance to win—on top of the opponent's relative fighting ability and physical stimuli.

A number of potential co-factors possibly will have contributed to the observed androgen responsiveness of the male quail to the 'direct challenge'. For example, two additional social context components have probably contributed to the observed expression of post-conflict androgen responses: the audience and the behavioral experience. The tested focal male quail were habituated to the housing conditions as part of a long-term descriptive study with the female group presented behind the barrier (Hirschenhauser, Möstl, unpublished). The fact that the females were present as audience during all test situations may

have added a stimulus-enhancing effect of social context. Furthermore, \the observed TM responses to the 'direct challenge' may relate to the fact that the focal males remained in their 'home' cages ('resident effect' [68]), which supposedly increased aggressivity and maximized the probability of being a winner [38]. This possibly also explains why losing on the side of the focal males did not occur in any of the staged encounters. Even though there are no data for losing in this study, the presented experiments exemplify a combined function for physical contact and the individual and context-dependent assessment of the relative fighting ability for the fine-tuning of post-conflict testosterone. The lack of TM responses to the mirror test probably confirms a role for a behavioral asymmetry between the opponents and for tactile stimuli including physical pain (which both were absent during mirror tests) as critical co-factors for the fine-tuning of post-conflict testosterone. Noticeably, on top of this the social context of a conflict may change the individual perception of own and opponent's resource holding potential, which might explain why conflict outcome alone did not exclusively determine the expression of post-conflict TM responses. In line with this, the social context of a challenge may also profoundly modulate the expression and the degree of post-conflict testosterone responses. The quail results may add an example to the evidence that testosterone responsiveness to agonistic interactions generally involves more complex mechanisms, including behavioral history and cognitive processing of information on social context and environment, rather than merely being a response to the behavioral output, i.e., physical stimuli and fighting behavior. Future investigations are warranted to further disentangle the specific aspects of variability in post-conflict testosterone, such as behavioral history, experience or audience presence.

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