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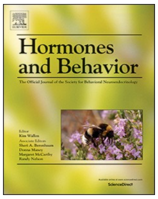

Differential modulation of courtship behavior and subsequent aggression by octopamine, dopamine and serotonin in male crickets

Jan Rillich[a,*], Birk Rillich[b], Paul A. Stevenson[a]

[a] *Institute for Biology, Leipzig University, Leipzig, Germany*
[b] *Institute for Biology, University of Rostock, Rostock, Germany*

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ABSTRACT

Aggression is a behavioral strategy for securing limited resources and its expression is strongly influenced by their presence and value. In particular, males are generally thought to guard females after mating to ward off other males, but the underlying control mechanisms are unknown. Here, we investigated the role of amines on male courtship behavior and its subsequent effect on male-male aggression in crickets (*Gryllus bimaculatus*). Contrary to the guarding hypothesis, female presence alone had no immediate effect on male-male aggression. Furthermore, confirming studies on other species, prior female contact, but not necessarily courtship or copulation, promoted subsequent male-male aggression in subordinate, but not socially naive crickets. This promoting effect of female contact is transient and slowly wanes after her removal. Selective aminergic receptor antagonists revealed that the promoting effect of prior female contact on male-male aggression is mediated by octopamine (OA), as well as by serotonin (5HT) acting most likely via $5HT_1$ and/or $5HT_7$ like receptors. This contrasts the role of $5HT_2$-like receptors in maintaining reduced aggressiveness after social defeat. Furthermore, while dopamine (DA) is necessary for the recovery of aggression in subordinates after defeat, it appears to play no part in female induced aggression. Male courtship, on the other hand, is selectively promoted by DA and 5HT, again most likely via $5HT_1$ and/or $5HT_7$ like receptors, but not by OA. We conclude that OA, DA and 5HT each differentially modulate different aspects of courtship and aggressive behavior in a context specific fashion.

1. Introduction

Conspecific aggression is considered to be an offensive behavioral strategy directed towards a competing member of the same species that is adapted to secure some resource at minimal cost (Stevenson, 2019). Conspecific aggression can thus increase individual fitness and reproductive success (Cunningham and Birkhead, 1998). In many species, including humans therefore, males compete aggressively for females, and later guard them against intruding males to secure their reproductive investment (Andersson, 1994; Alcock, 1994; Buss, 2002). It is, however, poorly understood how the nervous system translates reproductive investment into aggressive behavior (review: Hsu et al., 2006). Here we investigate in crickets, how small (e.g female contact only), or greater investments (e.g. courtship and copulation) influence aggression towards male intruders, and how this is controlled by biogenic amines.

Crickets (*Gryllus bimaculatus*) have advanced to a model system for analyzing the experience dependent control of aggression by neuromodulators (reviews: Stevenson and Rillich, 2016, 2017). In these insects, experiences including flying, winning, male contact and shelter occupancy each increase male aggression via the action of octopamine (OA), the invertebrate analogue of noradrenaline. Aggression in crickets is also influenced in various ways by experience with females. In *Acheta domesticus*, males housed nightly with females tended to be less aggressive than those deprived of females (Brown et al., 2007), and similarly male *G. pennsylvanicus* are less likely to win an encounter after successful copulation (Judge et al., 2010). On the other hand, the mere presence of a female increases aggression in male subordinate *A. domesticus* (Killian and Allen, 2008). Also in *G. bimaculatus*, female presence, but not her scent alone, increases agonistic displays and this correlates positively with mating success (Tachon et al., 1999). Successful mating in crickets involves the transfer of a nutritious spermatophore. This investment is widely thought to cause the male to become aggressive towards the female after copulation to prevent her from eating this nuptial gift (Loher and Rence, 1978; Sakai et al., 2017), and also to guard her by fighting off intruding male competitors (Wynn and Vahed, 2004). There is also evidence that octopamine plays a role in controlling courtship behavior in crickets. The levels of this amine in

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* Corresponding author at: Faculty of Life Sciences, Institute for Biology, Leipzig University, Talstr. 33, Leipzig D-04103, Germany.
E-mail address: rillichj@gmail.com (J. Rillich).

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the haemolymph increase after flying, fighting and courtship (Adamo et al., 1995), and flying increases both aggression (Hofmann and Stevenson, 2000; Stevenson et al., 2005) and courtship (Dyakonova and Krushinsky, 2008). Octopamine also increases male copulatory readiness (Matsumoto and Sakai, 2000), and shortens the pause in calling song production that normally occurs after copulation (Nagao et al., 1991; Ureshi et al., 2002). On the other hand, in *Drosophila*, mutant males deficient in OA show normal courtship towards a female (Zhou et al., 2008) and OA is now thought to bias brain function to favor aggression rather than courtship (Watanabe et al., 2017). Other studies in *Drosophila* point increasingly to the amines serotonin and dopamine as main players in controlling courtship behavior in male insects (Becnel et al., 2011; Pooryasin and Fiala, 2015; Zhang et al., 2016). The importance of biogenic amines for the promoting effect of female-male interactions on male-male aggression, however, is not known for any insect to our knowledge.

In our study we treated male crickets with several different antagonists selective for insect octopamine, dopamine and serotonin receptors, and evaluated their effects on specific elements of courtship behavior (female contact, courtship song, spermatophore transfer), and how these aspects of courtship then influence aggression. Our findings reveal that OA, DA and 5HT each differentially modulate different aspects of courtship and aggression depending on behavioral context.

2. Methods

2.1. Experimental animals

Mediterranean field crickets, *Gryllus bimaculatus* (de Geer) of both sexes were reared together during larval development under constant standard conditions at Leipzig University (22–24 °C, relative humidity 40–60%, 12 h:12 h light:dark regime daily feeding on bran and fresh vegetables). Freshly moulted adult males and females were removed from this larval stock and then kept as separate stocks of approximately 40 individuals of each sex under the same conditions until mature (2–3 weeks post moult). They thus had no sexual experience before the experiment. Two days prior to experimentation, individual males were isolated from other males and kept in individual glass jars with ample food and water and are referred to as “naive”. Their weight at the time of the experiments was 1.32 ± 0.23 g (mean, standard deviation) and ranged from 0.93 to 2.02 g. Females were taken from the stock of virgin females on the day of experimentation. All experiments were performed during daylight hours, avoiding times when aggression tends to be depressed (just after midday and on generally dreary days; cf. Stevenson et al., 2000) at an ambient temperature of 20–25 °C. All animal treatments complied with the principles of laboratory animal care and the German law on the protection of animals (*Deutsches Tierschutzgesetz*).

2.2. Courtship behavior

For each trial, a single male cricket was placed together with one female in one side of a clear Perspex-glass arena (l. w. h.: 16 × 9 × 7 cm), with a sand-covered floor, and divided halfway along its length by an opaque sliding door. During a 20 min observation period we then noted whether or not the male contacted the female, and whether this was followed by the male generating the characteristic courtship song, with or without subsequent transfer of a spermatophore to the female during a copulatory act in which the females actively mount a courting male (see Sakai et al., 2017).

2.3. Post-courtship aggression

Unless otherwise stated, access to another male placed in the opposite half of the arena was enabled by removing the dividing door 20 min after the initial observation period to record courtship. This male was of equal size (< 5% weight difference) but made hyper-aggressive by previously flying it in a wind stream for 3 min shortly beforehand (cf. Hofmann and Stevenson, 2000). Such hyper-aggressive males always defeated the test crickets in dyadic contests, and thus served as a standard for evaluating the full aggressive potential of the latter (see also Stevenson and Rillich, 2013, 2015). To this purpose we noted both the time taken until the test cricket retreated (persistence), and the escalation level scored on a scale of 0–6 (Stevenson et al., 2000): Level 0: mutual avoidance without aggression. Level 1: one cricket attacks, the other retreats. Level 2: antennal fencing. Level 3: mandible spreading by one cricket. Level 4: mandible spreading by both crickets. Level 5: mandible engagement. Level 6: grappling, an all-out fight.

2.4. Pharmacological treatments

To evaluate the roles of biogenic amines in courtship and aggression we applied different aminergic receptor antagonists. Their selectivity and most effective dosages that influence cricket aggressive behavior, without any obvious detrimental effect on general motility, has been determined in numerous prior investigations (Rillich and Stevenson, 2011, 2014, 2015, 2017, 2018). Based on these studies, the following drugs (Sigma Aldrich, Deisenhofen, Germany) were applied: The octopamine-receptor blocker (OAR-bl) epinastine hydrochloride (Roeder et al., 1998), the insect dopamine-receptor blocker (DAR-bl) fluphenazine dihydrochloride (Degen et al., 2000), the two serotonin receptor blockers ketanserin (+)-tartrate salt and methiothepin mesylate salt. Ketanserin is generally considered to be an insect $5HT_2$ receptor blocker ($5HT_2$R-bl), whereas methiothepin is less selective, but binds primarily to $5HT_1$-like receptors, so that we refer to it here as a $5HT_{1+}$ receptors blocker for brevity ($5HT_{1+}$R-bl, Vleugels et al., 2015; see discussion for details). All drugs were first dissolved in dimethyl sulfoxide (DMSO), and diluted to give a final concentration of 10 mM in 5% DMSO with insect saline (content in mM: NaCl 140, KCl 10, CaCl2 7, NaHCO3 8, MgCl2 1, *N*-trismethyl-2-aminoethanesulfonic acid 5, d-trehalose dihydrate, pH 7.4). Each test animal received a single 20 μl injection of one drug into the haemocoel via the pronotal shield using a microsyringe (Hamiliton®, Bonaduz, Switzerland). Control animals received vehicle only. The behavioral effects of drugs and vehicle were evaluated in parallel experiments 30–60 min after application.

2.5. Data analysis

All statistical tests were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) running on a Macintosh computer (Apple Computers, Cupertino, CA, USA). The median and the interquartile range (IQR) were calculated for non-parametric data sets. Non-parametric tests were also performed on duration since the data failed D'Agostino and Pearson omnibus normality tests, even after log transformations. The Kruskal-Wallis (K-W) test with Dunn's multiple comparisons test (Dunn's test) was used to test for significant differences in the distributions between multiple groups. The χ^2 test was performed to compare the relative occurence of various aspects of male courting behavior: for one experiment 5 groups were compared (Fig. 3), so we applied the Bonferroni correction to alpha to control the family wise error rate ($*p < 0.01$, $**p < 0.002$, $***p < 0.0002$). Each test cricket was used for only one experiment, and the total numbers (n) used for each experimental test group are indicated in the figures.

To understand the magnitude of statistically significant effects we also calculated the effect size as given by the commonly used Cohen's d and also by Probability of Superiority, A which is somewhat more appropriate for non-parametric data (Table 1) following the suggestions of Ruscio and Mullen (2012). Cohen's d, the standardized mean difference between two groups, was calculated as the difference between the group means divided by the within-group standard deviation. The resultant effect size d is interpreted as follows: 0.2 = small effect,

Table 1

Table giving the error probability, *p* for comparisons between test groups that were statistically significantly different (Dunn's test), together with estimates of the effect sizes as given by Cohen's *d* (*d*) and by the Probability of Superiority (*A*) for data depicted in Figs. 1, 2 and 3. Both estimates indicate that all statistically significant effects found in our study are not trivial.

Tested groups			Escalation level			Persistence		
			p	*d*	*A*	*p*	*d*	*A*
Fig. 1	♀Not offered	Contact only	**	0,95	0,75	**	0,77	0,77
	♀Not offered	+Courtship	***	1,45	0,87	***	0,96	0,90
	♀Not offered	+Sperm transfer	***	1,52	0,89	***	1,18	0,92
Fig. 2	♀Not offered	♀Absence (0 min)	***	1,13	0,81	***	0,97	0,83
	♀Not offered	♀Absence (5 min)	***	1,00	0,81	***	0,97	0,78
Fig. 3	♀Not offered + vehicle	♀Contact + vehicle	***	1,09	0,81	***	0,95	0,82
	♀Not offered + DAR-bl	♀Contact + DAR-bl	*	0,79	0,71	*	0,72	0,72
	♀Not offered + $5HT_2$-bl	♀Contact + $5HT_2$-bl	*	0,61	0,69	**	0,72	0,71
	♀Contact + vehicle	♀Contact + OAR-bl	***	0,78	0,72	***	0,83	0,74
	♀Contact + vehicle	♀Contact + $5HT_{1+}$-bl	***	0,73	0,72	***	0,74	0,74
	♀Not offered + vehicle	♀Not offered + $5HT_2$-bl	*	0,74	0,71	*	0,80	0,72

(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

0.5 = medium effect, 0.8 = large effect. The Probability of Superiority, *A* was calculated from the product of the number of control animals and test animals for pairwise comparisons (n control $*$ n test), minus the Mann-Whitney U-value, divided by the product (n control $*$ n test). The result gives the probability that a randomly sampled score from e.g. a test group is larger than a randomly sampled score from the control group. Both methods indicate that all significant differences found are not trivial.

3. Results

3.1. Courtship in socially naive and subordinate crickets

When offered access to a female, all socially naive males ($n = 54$, Fig. 1B), approached and physically contacted the female with their antennae within the 20 min observation period. After this initial physical contact, males engaged in either no further contact (46%), or subsequently generated the characteristic courtship song by stridulating with their forewings (30%), but did not actually copulate with the female. On some occasions though, singing males transferred a spermatophore to the female during a copulatory act (24%). Compared to these performances, those of subordinate males, that lost an aggressive interaction with a hyper-aggressive male 30 min previously, were not statistically significant different (female contact only: 40%; contact with song only: 29%; contact with song and sperm transfer: 31%; $n = 65$, χ^2 tests compared to naive for all performances: $p > 0.39$, Fig. 1B′).

3.2. Post-courtship aggression

We next evaluated the effect of the different courtship experiences on the male's subsequent aggressiveness in the presence of the female towards a hyper-aggressive intruding male, against which they always lost. Firstly, and as a control, we noted that socially naive males with no prior female contact escalated to the physical level of mandibular engagement (median escalation level 5, IQR 3–5, $n = 42$, Fig. 1C, white bars) and persisted for some 7 s against the intruder (median duration, IQR 3.75–11). Compared to these performances, we found no significant effect of female presence on subsequent aggression, regardless of whether they only contacted the female, generated courtship song or transferred sperm (K-W test: p-escalation = 0.313; p-persistence = 0.143; Fig. 1C, D).

Contrary to this, female contact (for 20 min) had a promoting effect on the aggressiveness of crickets that were defeated by a hyper-aggressive male 10 min before access to the female, i.e. 30 min after defeat (K-W test: p-escalation < 0.001; p-persistence < 0.001; Fig. 1C′, D′, Table 1). Firstly, control losers that had no contact to females are typically non-aggressive (cf. Stevenson and Rillich, 2013) and retreated immediately on confronting the male intruder (level 1: 67%, median escalation level 1, IQR 1–2; median persistence: 0 s IQR 0–2, $n = 30$; white bars). Compared to this, and confirming early observations of Killian and Allen (2008), even losers that only contacted the female (grey bars) were more aggressive and escalated to level 5 (median, IQR 1–5, level 1: 31% of $n = 26$) and persisted 8 s (median, IQR 0–11) significantly different to control (Dunn's test compared to control: p-escalation < 0.01, p-persistence < 0.01). Losers that generated the courtship song, and transferred sperm tended to escalate higher and longer but the aggressiveness was not statistically different to losers that did not court (e.g. for loser that transferred a spermatophores, median escalation level: 5, IQR 5–6; median duration: 9 s IQR 8–15.75, level 1: 10% of $n = 20$).

We next tested whether or not the continued presence of a female is required for the promoting effect on loser aggression (Fig. 2B, C). For this, we first compared two groups of male losers that both had no prior contact, and matched them against hyper-aggressive males either in the absence, or in presence of a female during the encounter. In the latter case, we ensured that the loser actually contacted the female briefly and immediately before the contest with the intruder, to provide the experience of female presence. This revealed that female presence alone had no promoting effect on male aggression (*U* test: p-escalation = 0.428, p-persistence = 0.632).

We next compared the aggressiveness of male losers with no prior contact to a female, to those that had female contact for 20 min previous, but which encountered the intruding male in the absence of the female, either directly after removing the female, or after a delay of 5 or 10 min. This revealed that prior contact to a female promotes male aggression even when the female is no longer present (K-W test: p-escalation < 0.001; p-persistence < 0.001; Fig. 2B′, C′, Table 1). Thus, when tested immediately after removing the female, losers were significantly more aggressive than controls that had no female contact (Dunn's test: p-escalation < 0.001, p-persistence < 0.001). Interestingly though, the aggression enhancing effect of prior female presence was still evident 5 min after removing the female (Dunn's test compared to control: p-escalation < 0.001, p-persistence < 0.001), but not after 10 min, when aggressive escalation and persistence were at the same low level as submissive losers.

3.3. Effects of amine-receptor blockers on courtship

Treatment with vehicle (5% DMSO in insect saline) had no appreciable effect on male courtship behavior when tested 30–60 min after application. During the 20 min observation period, naive adult males

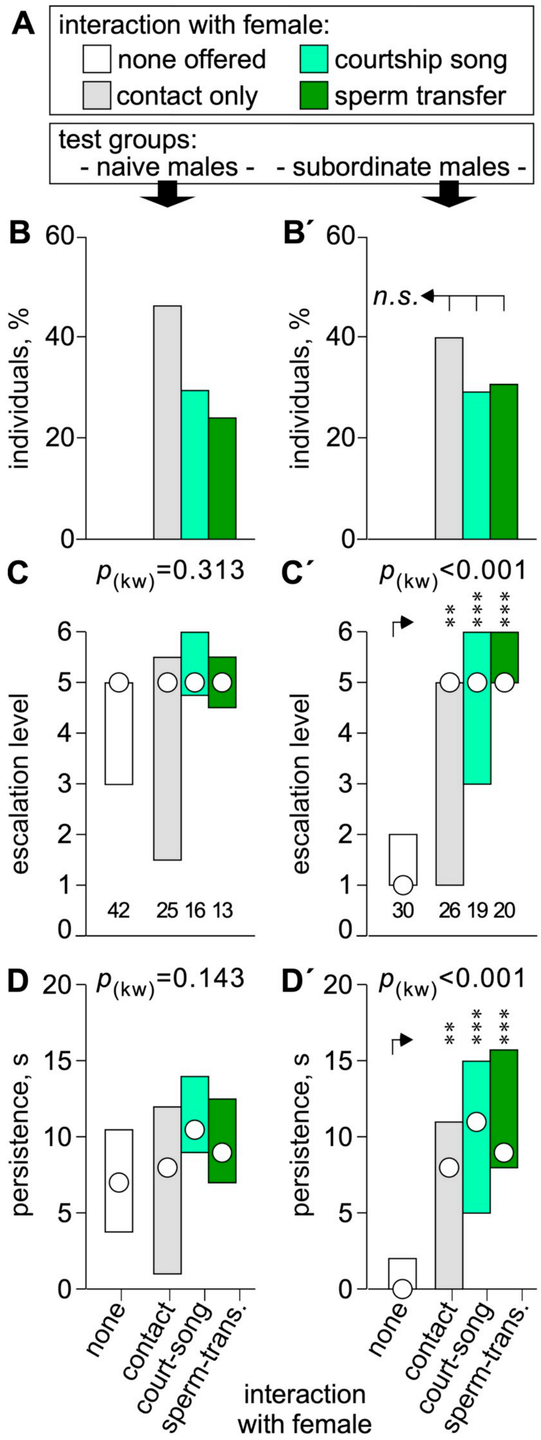

Fig. 1. Elements of male courtship behavior and their influence on subsequent aggression. A Key to test groups. Naïve (left charts) and subordinate ("losers", right charts) male crickets were placed together with a female for 20 min and their highest level of courtship behavior exhibited, and subsequently the male's aggressive response towards a hyper-aggressive intruding male in the presence of the female. Grey bars: female contact only; light green: contact followed by courtship song; dark green: contact with song and sperm transfer. White bars: controls giving the aggression of crickets that experienced no prior contact to a female. B, B′ Bars charts giving the most intense element of courtship behavior displayed by males (% of individuals). C, C′ Bar charts giving the escalation level and D, D′ persistence against hyper-aggressive opponents (circles: median, bars: interquartile range, IQR; *n* is given above the x-axis). Differences in courtship between naive and loser crickets were tested by χ^2 test, and are not significant (n.s, $p > 0.39$). Significant differences in C, C′, D and D′ are indicated above the bars by the *p*-value from Kruskal-Wallis-tests ($p_{(kw)}$) and asterisks for Dunn's multiple comparisons test (**$p < 0.01$, ***$p < 0.001$). Effect sizes are given in Table 1.

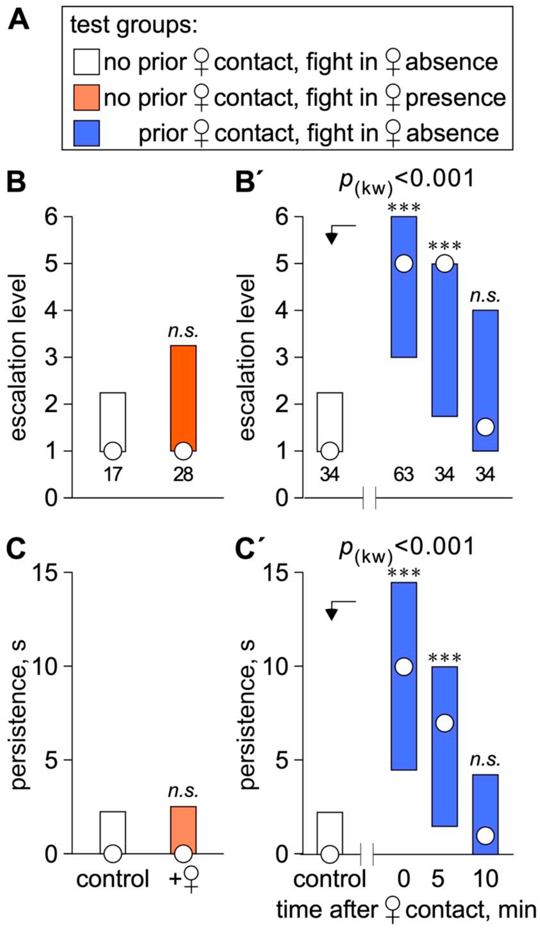

Fig. 2. Influence of prior female contact and continued presence on loser aggression. A Key to test groups. White bars (control): no prior contact and fight in absence of a female, red bars (left side): no prior contact but fight in presence of a female; blue bars (right side): with 20 min prior contact to female, but fight in absence of a female, either immediately after her removing (0 min) or 5 and 10 mins after contact. B, B′ Bar charts giving the escalation level, and C, C′ persistence of losers, 30 min after defeat against hyper-aggressive opponents that had different experiences with a female. Circles: median, bars: interquartile range, IQR; *n* is given above the x-axis in B and B′. Significant differences are indicated above the bars by the *p*-value from Kruskal-Wallis-tests ($p_{(kw)}$) and by asterisks for Dunn's multiple comparisons test (***$p < 0.001$, *n.s.* not significant). Effect sizes are given in Table 1.

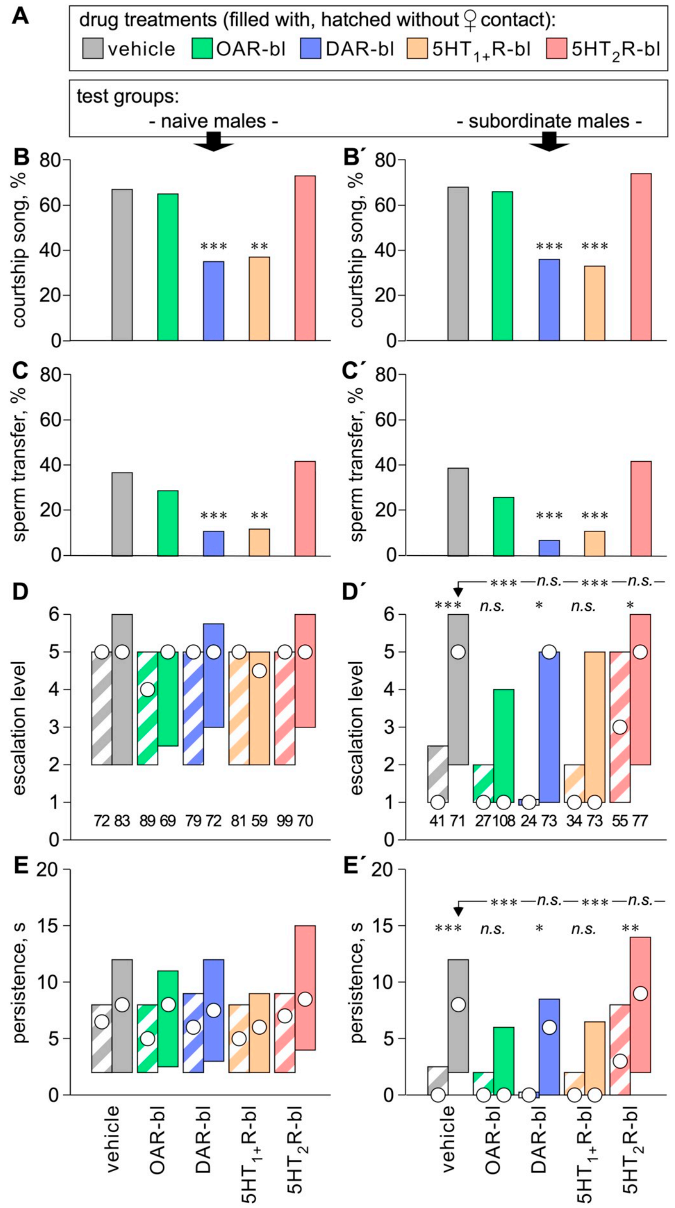

Fig. 3. Influence of amine receptor blockers on courtship and subsequent aggression. A Key to drug treatments and test groups. Grey bars: vehicle (5% DMSO); green bars: OA-receptor blocker epinastine (OAR-bl); blue bars: DA-receptor blocker fluphenazine (DAR-bl); orange bars: 5HT$_{1+}$-like receptor blocker methiothepin (5HT$_{1+}$R-bl); red bars: 5HT$_2$-like receptor blocker ketanserin (5HT$_2$R-bl). Filled bars with, hatched bars without prior female contact. Left side charts for naive crickets, right side for subordinates ("losers"). B, B′ and C, C′ Percentages of males that exhibited courtship song, respectively transferred a spermatophore during 20 min contact with a female. D, D′ and E, E′ Escalation level, and respectively persistence against hyper-aggressive opponents directly after female contact (filled bars in presence, hatched in absence of a female; circles: median, bars: interquartile range, IQR; *n* is given above the x-axis). Significant differences between groups are indicated by asterisks: B, B′ and C, C′ for χ^2-test with Bonferroni correction to alpha for 5 comparisons ($*p < 0.01$, $**p < 0.002$, $***p < 0.0002$); D, D′ and E, E′ from Dunn's multiple comparisons test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, *n.s.* not significant). Effect sizes are given in Table 1.

often generated the courtship song in the female's presence (67% of males, $n = 83$, Fig. 3B), and in 37% of all cases they sang and transferred a spermatophore to the female (Fig. 3C). These performances were also not significantly influenced by prior treatment with receptor blockers directed either against octopamine (OAR-bl, epinastine), or 5HT$_2$-like receptors (5HT$_2$R-bl, ketanserin). Contrasting this, pretreatment with the dopamine receptor blocker (DAR-bl) fluphenazine led to a significant decrease of both, courtship song production (35%, $n = 72$, compared to vehicle, χ^2: 15.8, $p < 0.0001$) and spermatophore transfer (11%, χ^2: 13.9, $p = 0.0002$). A similar suppressing effect on courtship behavior occurred after treatment with the 5HT$_{1+}$-like receptor blocker methiothepin (5HT$_{1+}$R-bl, courtship song: 37%, $n = 59$, compared to vehicle, χ^2: 12.4, $p = 0.0004$; spermatophore transfer: 12%, χ^2: 10.5 $p = 0.0011$).

Similar drug effects on courtship behavior were also observed for losers given access to a female 10 min after defeat (Fig. 3B′, C′). Again, the OA receptor blocker epinastine and 5HT$_2$ receptor blocker ketanserin had no significant effect on the portion of individual males to sing or transfer a spermatophore, compared to vehicle (χ^2: $p > 0.05$, for all comparisons), whereas the DA receptor blocker fluphenazine and 5HT$_{1+}$receptor blocker methiothepin significant reduced the incidence of both aspects of courtship behavior (χ^2: $p < 0.0002$ for all comparisons).

3.4. *Aminergic influences on female induced aggression*

Socially naive crickets treated with vehicle (Fig. 3D, E) were as aggressive towards hyper-aggressive opponents as untreated crickets (see Fig. 1), and their performances were again not influenced significantly by prior contact to a female. Moreover, and confirming our earlier studies (Rillich and Stevenson, 2014, 2015, 2017, 2018), none of the tested amine receptor blockers had a significant effect on the aggressive behavior of naive crickets, regardless of whether they had prior contact to a female or not (Fig. 3, left side).

The situation for losers, however, was markedly different (K-W test: *p*-escalation < 0.001; *p*-persistence < 0.001, Fig. 3D′, E′, Table 1). Firstly, as found for untreated losers (Fig. 1), prior female contact led to a significant increase in both escalation level and persistence in vehicle treated losers towards hyper-aggressive opponents (escalation: Dunn's test: $p < 0.001$; persistence: Dunn's test: $p < 0.001$). Furthermore, prior female contact still had essentially the same aggression promoting effect in losers treated with a DA receptor blocker fluphenazine (escalation: Dunn's test: $p < 0.05$; persistence: Dunn's test: $p < 0.05$) or a 5HT$_2$ receptor blocker ketanserin (escalation: Dunn's test: $p < 0.05$; persistence, Dunn's test: $p < 0.01$). Interestingly though, female presence had no promoting effect in losers treated with a OA receptor blocker epinastine or a 5HT$_{1+}$ receptor blocker methiothepin (Dunn's test: $p > 0.05$) and their aggression was significantly less than vehicle treated losers (escalation level and persistence for both drugs, Dunn's test: $p < 0.001$).

4. Discussion

Mating partners are undoubtedly one of the key resources in the animal kingdom (Hsu et al., 2006), and males, including humans, often secure their mates to ward off other male competitors (Buss, 2002). Insects, including crickets, are no exception, and males are generally considered to guard females after copulation (Alcock, 1994) to prevent them from discarding the nutritious spermatophore (Loher and Rence, 1978) and copulating with other males (Simmons, 1986; Bateman and Toms, 1998; Wynn and Vahed, 2004) and thus protect their energy investment in courtship (cf. Hack, 1998). While this widely accepted idea makes evolutionary sense, it does not conform fully with our experimental findings.

In our first experiment, we found that prior contact and the continued presence of a receptive female had no effect on the aggression of socially naive male crickets towards male intruder, regardless of whether they only contacted the female, generated courtship song, or transferred a spermatophore (Fig. 1C, D). However, as found earlier for *Acheta domesticus* (Killian and Allen, 2008), subordinate *G. bimaculatus* males that had just lost an aggressive contest, and are normally submissive, became highly aggressive after contacting a female (Fig. 1C′, D′). Although there was a trend for subordinates that copulated the female to be even more aggressive than those that merely contacted the female, this was not statistically significant (see also Killian and Allen, 2008). Furthermore, we found that 20 min in the mere presence of a female before a competition, but not during it, was decisive for increasing aggression in male subordinates towards an intruding male (Fig. 2). Prior male-female contact must, however, involve more than just the exchange of antennal signals, since brief antennal stimulation (20 s) with a freshly cut female antenna does not increase aggression in subordinate males, whereas stimulation with a male's antenna does (Rillich and Stevenson, 2015). Contact duration, or visual signals could also play a role, since the mere sight of opponent retreating is sufficient to increase aggression (Rillich and Stevenson, 2011). We conclude, that elevated aggression in male crickets due to female contact cannot be regarded as post-copulatory guarding behavior (cf. Alcock, 1994; Wynn and Vahed, 2004). Nonetheless, although the transfer of sperm is not causal, male crickets have seemingly evolved a strategy where female contact alone leads them in effect to guard their investment in mating.

Similar to this study, we found earlier that occupation of a burrow, as a potential valuable resource, just like female contact, only promotes aggression in subordinate but not socially naive crickets (Rillich et al., 2011; see also Rillich and Stevenson, 2015 on antennal stimulation). This may be because naive males are normally highly aggressive even in the absence of resources (Stevenson and Rillich, 2013), so that any change is statistically difficult to resolve. However, experiences such as flying and winning can both lead to a significant increase in aggression of naive males (Stevenson et al., 2005; Rillich and Stevenson, 2011). Resource effects may, therefore, be more subtile and possibly increase with increasing resource value and possession time. Alternatively, changes in social status might reconfigure neural circuits (cf. Issa et al., 2012), so that subordinates become more sensitive for resources. This might even be adaptive, since losers could stand to benefit more than naives from an increased chance of securing females.

Increased aggression induced by experiences such as flying, winning, residency and antennal stimulation each promote aggression selectively via the action of the biogenic amine octopamine (OA, review: Stevenson and Rillich, 2016). Supporting the finding that OA haemolymph levels increase in males after sexual contact (Adamo et al., 1995), the increase in aggression observed after female contact was blocked by the selective OA receptor blocker epinastine, but not by the DA receptor blocker fluphenazine (Figs. 3 and 4). Interestingly, experience dependent changes in OA-levels (cf. Adamo et al., 1995) and the aggression promoting effect of female contact are both transient events that decay within 5–10 min (Fig. 2B′, C′; see also Rillich et al. (2011) on residency). Thus, female presence only temporarily overrides the effect of losing. We conclude, that female contact leads to the release of OA, which then acts as a neuromodulator to transiently promote subsequent aggression.

It is important to note that OA is acting here in a specific context-dependent fashion, rather than as a general elevator of arousal. Firstly, the OA receptor blocker epinastine did not affect the escalation level or aggressive persistence of naive crickets (Fig. 3D, E). Secondly, corresponding to the finding that OA-deficient mutant fruit flies express normal courtship (Zhou et al., 2008), blocking OA receptors did not affect the incidence of courtship song production or spermatophore transfer in naive or subordinate crickets (Fig. 3B, C).

Our data also reveal that DA has differential modulatory effects on both courtship and aggression that differ to those of OA (Fig. 4). Firstly, and contrary to the OA receptor blocker epinastine, the DA receptor blocker fluphenazine significantly reduced the incidence of both courtship song production and spermatophore transfer (Fig. 3B, C). Similarly, in fruit flies, DA promotes the drive to mate, and lowered DA activity after copulation reduces courtship attempts (Zhang et al., 2016). Secondly, although blocking DA affects neither the expression of aggression in naive crickets (Fig. 3D, E), nor the promoting effect of female contact on loser aggression (Fig. 3D′, E′), DA, but not OA, is necessary for the normal recovery of aggression after social defeat (Rillich and Stevenson, 2014). Our finding that the reduced drive to court and mate induced by DA blockade does not alter the promoting effect of females on male aggression is surprising and implicates that courtship and aggression are controlled independently. Crickets possess genes for 4 different dopamine receptors that are differentially expressed in various tissues, primarily in the central nervous system but also in the Malpighian tubules and testes of males (Watanabe et al., 2013). While their pharmacology still remains to be established, work on other orthopteran insects shows that they are remarkably different to their vertebrate counterparts (Marg et al., 2004). In the absence of more selective drugs, we are unable at present to comment on which subtype(s) the used dopamine antagonist affects. In view of the relatively short latency to its behavioral effect (30–60 min), it seems more likely, however, that this results from activation of dopamine receptors in the brain, rather than in other tissues such as the testes.

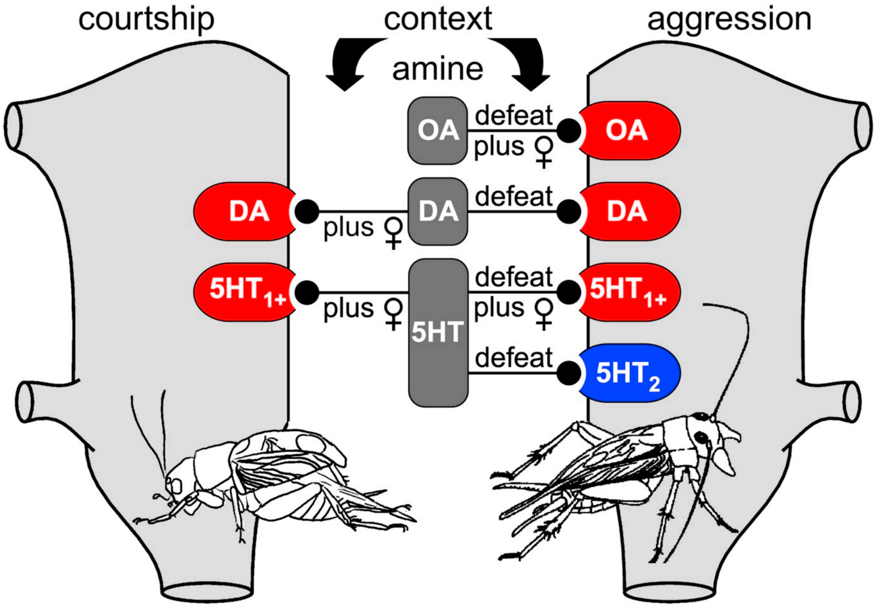

Fig. 4. Pictogramm summarizing the context dependent effects of biogenic amines on courtship and aggression. Aggression (escalation level and persistence) is promoted in subordinate male crickets after defeat by prior contact with a female (plus ♀), and a variety of other experiences (not indicated: physical exertion, winning, residency, male antennal contact; Stevenson and Rillich, 2019). This effect is mediated by octopamine (OA), but not dopamine (DA), acting via specific OA receptors (OA, red). DA also promotes aggression, but only in losers, and is necessary for the recovery of aggression after defeat (Rillich and Stevenson, 2014). Female presence also promotes aggression after defeat via 5HT$_1$ and/or 5HT$_7$ receptors (5HT$_{1+}$, red). Activation of 5HT$_2$ receptors after defeat, in contrast, is involved in maintaining reduced aggression in losers after social defeat (Rillich and Stevenson, 2018). Courtship behavior (courting song production and successful copulation) is promoted in the presence of a female by DA and 5HT acting via DA, and 5HT$_1$ and/or 5HT$_7$ receptors (DA, 5HT$_{1+}$, in red), but not by OA.

Finally, serotonin (5HT) also has differential effects on cricket aggression and courtship, whereby its action seems to depend on the receptor subtype activated. To date, crickets are known to express five 5HT receptor subtypes (two $5HT_1$, two $5HT_{2,}$ one $5HT_7$; Watanabe et al., 2011; Watanabe and Aonuma, 2012), but they cannot be discriminated pharmacologically with currently available drugs. We used two 5HT receptor antagonists that had clearly different effects: ketanserin, which is generally regarded as selective for insect $5HT_2$ receptors (Johnson et al., 2009; Vleugels et al., 2015), and possibly only $5HT_{2B}$ receptors (Thamm et al., 2013; Tedjakumala et al., 2014); methiothepin, which is less selective and blocks all subtypes in combination with ketanserin including $5HT_1$ plus $5HT_{2A}$ and $5HT_7$ (Vleugels et al., 2015), but possibly not $5HT_{2B}$ receptors (Thamm et al., 2013; Tedjakumala et al., 2014). Neither of these serotonergic drugs influenced aggression in socially naive crickets, regardless of whether they had prior access to a female or not. However, ketanserin ($5HT_2$R-bl) increased aggression in subordinates that had no prior female contact after defeat (see also Rillich and Stevenson, 2018), but did not affect the influence of females on subsequent loser-aggression (Fig. 3D′, E′). Methiothepin ($5HT_{1+}$R-bl), in contrast, prohibited the aggression promoting effect of female contact on loser aggression, but did not increase aggression in subordinates without access to a female. With respect to courtship behavior, methiothepin also reduced song and spermatophore transfer, whereas ketanserin did not.

Generally, 5HT is considered to suppress aggression in mammals (Nelson and Trainor, 2007; Clinard et al., 2015), but promote it in invertebrates (Kravitz and Huber, 2003), although it is now becoming increasingly clear that 5HT can have different effects, depending on the behavioral context and the receptor subtype activated. Thus, depending on behavioral context, $5HT_1$ receptor activation tends to promote aggression in humans (Carhart-Harris and Nutt, 2017) and insects (Johnson et al., 2009; Alekseyenko et al., 2014), whereas $5TH_2$ receptor subtypes promote it (rodents: Clinard et al., 2015; fruit flies: Johnson et al., 2009; subordinate crickets: Rillich and Stevenson, 2018; stalk eyed flies: Bubak et al., 2019). With respect to sexual behavior, 5HT is known to enhance the neuronal responses to female sex pheromone in moths (Kloppenburg et al., 1999; Gatellier, 2004), suppress fruit fly mating (Pooryasin and Fiala, 2015) and shortens the sexual inactive stage after copulation in male crickets (Ureshi et al., 2002). However, apart from data pointing to the importance of $5HT_7$ receptors for regulating sexual interest in fruit flies (Becnel et al., 2011), the roles of 5HT receptor subtypes in insect courtship are presently unknown. We propose that 5HT acts via a $5HT_2$ like receptor to inhibit aggressive recovery in losers, without affecting courtship or its effect on aggression, whereas courtship and its subsequent effect on aggression is influenced by 5HT acting via a different subtype that needs to be identified.

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Author contributions

Conceived and designed the experiments: JR BR. Performed the experiments: JR BR. Analyzed the data: JR BR. Contributed reagents/materials/analysis tools: PAS JR. Wrote the paper: JR PAS.

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