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Article

# Octopamine in Male Aggression of *Drosophila*

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## Summary

**Background:** In mammals and humans, noradrenaline is a key modulator of aggression. Octopamine, a closely related biogenic amine, has been proposed to have a similar function in arthropods. However, the effect of octopamine on aggressive behavior is little understood.

**Results:** An automated video analysis of aggression in male *Drosophila* has been developed, rendering aggression accessible to high-throughput studies. The software detects the lunge, a conspicuous behavioral act unique to aggression. In lunging, the aggressor rears up on his hind legs and snaps down on his opponent. By using the software to eliminate confounding effects, we now show that aggression is almost abolished in mutant males lacking octopamine. This suppression is independent of whether tyramine, the precursor of octopamine, is increased or also depleted. Restoring octopamine synthesis in the brain either throughout life or in adulthood leads to a partial rescue of aggression. Finally, neuronal silencing of octopaminergic and tyraminergic neurons almost completely abolishes lunges.

**Conclusions:** Octopamine modulates *Drosophila* aggression. Genetically depleting the animal of octopamine downregulates lunge frequency without a sizable effect on the lunge motor program. This study provides access to the neuronal circuitry mediating this modulation.

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## Introduction

Vertebrates require noradrenaline to display aggression (for a review, see [1]). For example, dopamine β-hydroxylase knockout mice lacking noradrenaline hardly show any aggressive behavior [2]. The effect of noradrenaline is suggested to be biphasic: Slight increases in noradrenaline level lead to enhanced aggressive behavior, whereas strong elevations suppress aggression [1].

Less is known about the role of octopamine (OA) in arthropod aggression, but its effects seem to be equally complex. In crustaceans, OA injection leads to a submissive-looking body posture [3, 4]. In crickets, injection of the OA agonist chlordimeform causes normally submissive losers of fights to re-engage in fighting faster than sham-injected animals [5]. Likewise in honeybees, injection of two OA agonists, XAMI and DCDM, biases the likelihood of aggressive display toward non-nestmates over nestmates [6].

In *Drosophila*, agonistic encounters of males and females are composed of a variety of both offensive and defensive components, some of which are displayed more often in one sex than in the other [7–10]. For example, "lunging," i.e., rearing on the hind legs and snapping down on the opponent, is characteristic of males, whereas "low posture fencing," i.e., pushing each other with the legs, is displayed by both genders. Up to now, two studies investigated the role of OA in *Drosophila* aggression. Both used a mutant for tyramine β-hydroxylase (TβH), an enzyme converting tyramine (TA) to OA. Mutant $T\beta h^{nM18}$ flies lacked OA but showed about 10-fold-increased TA levels in the brain [11]. Taking various aggressive behavioral components into account, Baier et al. [12] observed in fights between white-eyed $T\beta h^{nM18}$ and wild-type males a decrease of aggressive behavioral patterns in the mutant. In contrast, focusing on the males' behavioral choice between aggression and courtship, Certel et al. [13] did not report a general decrease in aggression for $T\beta h^{nM18}$ males when fighting against each other (S. Certel and E. Kravitz, personal communication). However, if males approached other males by vibrating their wing(s), which occurred in about three encounters per 30 min recording period, $T\beta h^{nM18}$ males less often showed a transition to aggressive behavior than did wild-type males.

Here, we report on an automated recording of *Drosophila* male aggression that allows a high throughput under standardized conditions. The software detects one of the key features of aggression: the lunge. With this tool we demonstrate that (1) small differences in body size influence the outcome of a fight in favor of the larger male, (2) walking activity correlates positively with lunge frequency, and (3) flies mutant for the *white* gene, a member of the ABC transporter gene family, are profoundly impaired in aggression not only because of the deteriorated optics of their eyes but also due to the missing gene function in the central nervous system. Excluding the influences of these factors that had confounded a previous study [12], we show that males without OA display hardly any lunge behavior, even though execution of the lunge motor program is largely indistinguishable from that of wild-type males. Presumably, an elaborate pattern of OA, and possibly TA, levels in time and space is required to enable flies to express wild-type aggressive behavior.

## Results

### Automated Recording of Lunges

Quantifying the rich repertoire of *Drosophila* aggressive behavior by manually evaluating and interpreting video recordings is a time-consuming and demanding task. We therefore developed an automated evaluation tool that detects a single, distinct component of *Drosophila* male aggression, the lunge, in video clips of *Drosophila* behavior. The lunge is a striking feature of male aggression that does not occur in other behavioral contexts. Within a lunge, three phases can be distinguished. During the first phase, the attacking fly rises on his hind legs, lifting his long body axis by 49.2 ± 1.2°. He then snaps down on his opponent (phase 2), with his head reaching a velocity of 254 ± 11.8 mm/s (means ± SEMs; n = 25) and his body reaching forces of about twice his body mass. Finally, the attacking male tries to grab his opponent with his forelegs and, if successful, pulls him toward his own body (phase 3; not always present; Figures 1A and 1B; see also Movie S1, available online; for recording and automation, see the Experimental Procedures and Figure S1A).

To have the software identify lunges in image sequences, it was essential to confine flies to a horizontal arena surrounded by high glass walls covered with Fluon, rendering the walls too slippery for flies to hold on (Figure 1C). In this way, overall aggression was high because flies could not avoid further encounters. All encounters were recorded.

The software program we developed for this study records the number of lunges for each fly in a certain time interval. In addition, it provides information such as the distance the fly walked, his size, and the time he spent on the food patch and in the periphery. Because the lunge has been reported to be the most frequent behavior by which an opponent is displaced from the food patch [8], the number of lunges of a male may serve, at least to some extent, as an indicator of his overall aggressiveness.

To evaluate the reliability of the software, the same clips were analyzed twice with respect to the number of lunges: once by the software and once "by hand." The software is designed to minimize false-positive assignments (counting frame sequences wrongly as lunges). This leads to a slightly larger number of false negatives (missing lunges; Figure S1B). The software underestimates the occurrence of lunges by about 11%, as indicated by the slope of the red line in Figure 1D. This value is independent of the lunge frequency (Figure S1B). Importantly, it is also largely independent of genotype (Figure S1C). Only if a genotype results in a high percentage of nonfighting males does the overall error rate differ from that of wild-type because for nonfighting males, the number of lunges can only be overestimated (Figure S1C).

Overestimating lunge frequency for nonfighting males can hide subtle differences between genotypes. Therefore, we added a "lunge view" software program that enables the investigator to focus only on those frame sequences that contain lunges according to the "lunge count" software. The investigator can then decide whether the selected frame sequences indeed represent lunges, thereby eliminating false positives.

### Walking Activity and Body Size, but Not Time of Day, Affect Fighting

To determine baseline aggressive behavior of wild-type flies in our paradigm, CantonS (CS) males were tested. Independent of the time of day (p = 0.17; n per hour = 8–32), a pair of five-day-old CS males performed 3.85 ± 2.82 lunges/min (mean ± SD; n = 191), demonstrating the high variability already observed in other paradigms of *Drosophila* male aggression [8, 14, 15]. In the present study, aggression was recorded from the 15$^{th}$ to the 30$^{th}$ min, constituting a period when flies already had settled into the arena and displayed constant aggression at a level indistinguishable from that of the two subsequent 15 min time bins (data not shown).

The total number of lunges performed by a pair of males correlated positively with their overall walked distance, i.e., the more the two flies walked the more lunges they performed. This correlation could be demonstrated for numerous genotypes (Figure S2). The pairs of flies were regarded as one

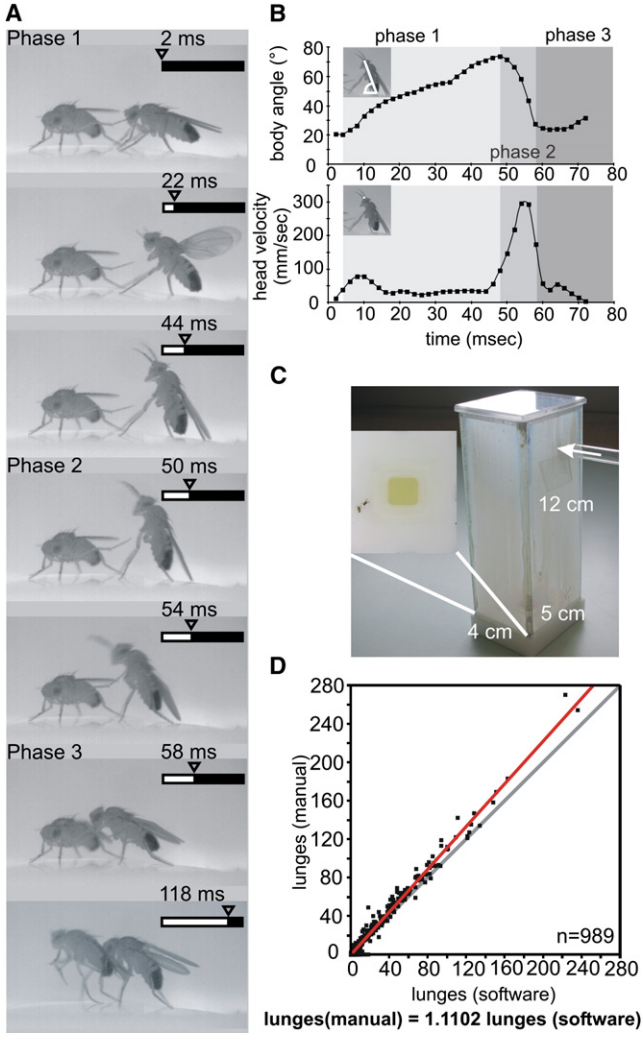


Figure 1. The Lunge and Its Automated Evaluation

(A) The lunge.

(B) Based on the lunge sequence depicted in (A), the two graphs show the alterations of body angle (upper panels) and head velocity (lower panels) over time. The body angle is the angle enclosed by the long body axis and the horizon (see inset). Head velocity was calculated on the basis of the white point shown in the lower inset.

(C) The experimental setup. In the middle of the floor, a 1 cm$^2$ hole is filled with a mixture of agarose, apple juice, and sugar.

(D) The software underestimates the amount of lunges by ~11% (red line). The x axis represents the number of lunges detected by the software, whereas the y axis indicates the number of lunges counted by hand. Each data point represents one male. For comparison, the gray line shows the ideal detection of every single lunge (slope equals 1).

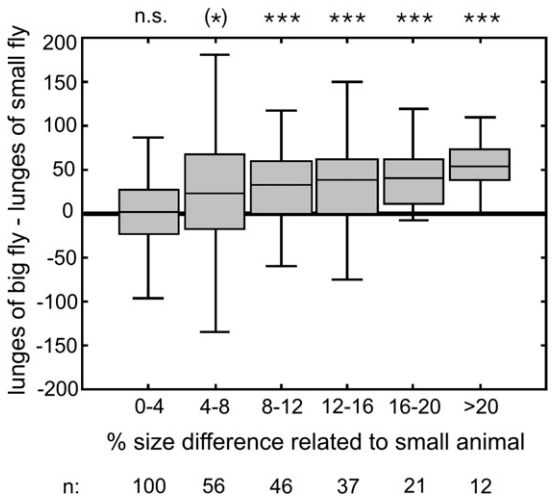


Figure 2. The Effect of Body Size on the Course of a Fight

The black line indicates the median, the boxes extend to the 25th and the 75th percentiles, and the error bars range from the minimum to the maximum without outliers. Size differences between two males from 8% up influence the outcome of a fight, i.e., the bigger fly is likely to lunge more frequently than the smaller fly. Size was measured from the dorsal side as the two-dimensional area of the fly (compare to Figure S1A). The size differences between two males were calculated with respect to the smaller male (x axis). The difference in lunges was determined by subtracting the number of lunges of the smaller fly from the number of lunges of the bigger fly (y axis). Data from three different genotypes were pooled and subdivided into 4% bins. Each bin was tested for a significant deviation from zero with the Wilcoxon-Sign-Rank test.

unit (total lunges and total distance walked) because the interactions were strongly dependent upon both flies. Not just the dominant fly approached the subordinate one; the subordinate fly often returned to the food patch, thereby eliciting new attacks. We decided to normalize lunge frequency to walking activity for two reasons. First, variance was strongly reduced by this step. A pair of five-day-old CS males performed 16.4 ± 6.6 lunges/m (mean ± SD; n = 191). Second, in mutant studies differences in lunge frequency between genotypes might be a side effect of differences in walking activity rather than a result of alterations in aggressiveness. However, because walking activity and aggression might be regulated by separate mechanisms, the lunge count software allows for evaluation of the two separately, if necessary.

The two males did not lunge equally often within the recording period. In 156 of 172 pairs that performed at least 10 lunges, one male performed more than 70% of all lunges. As in many other species, in *Drosophila* the size difference between two males strongly influences which male wins more aggressive encounters [8, 16–19]. The effect is most obvious when the weight difference between the opponents is pronounced (~50%) [18]. Our data show that a size difference of just 8% (measured as the projection area from above) results in the bigger fly being likely to lunge more often than the smaller fly (Figure 2). Because the 8% difference in body size cannot be detected by the human eye, fights were always set up between males of the same genotype in order to avoid a confounding influence of size when investigating the effect of a specific genotype.

## Mutant *white* Males Are Impaired in Aggressive Behavior

Many transgenic fly lines are generated and kept in a *white* mutant background. We therefore examined the role of the *white* (*w*) gene in aggressive behavior. Males mutant for the null allele $w^{1118}$ [20] were strongly impaired in aggression, lunging at a rate of only 3% of wild-type male levels (Figure 3). Providing $w^{1118}$ males with a *mini-white*$^{+}$ transgene had differing effects but never resulted in a full rescue of wild-type aggression (Figure S3A).

Mutant $w^{1118}$ flies lacking the characteristic red pigmentation of the eyes are visually impaired [21, 22]. Indeed, an intact visual system is required for normal aggressive behavior, as blind $norpA^{P24}$ hemizygote [23, 24] and motion-blind homozygous $ninaE^{17}$ males [25–27] performed significantly fewer lunges per meter than wild-type Berlin (WT-B) males (<10%; for both, $p < 0.00001$). Consequently, we asked whether to show aggression males needed the *white* gene function in vision for proper pattern contrast in the eye. For a tissue-specific knockdown, we used the eye-specific GMR-GAL4 line [28] to drive a UAS-RNAi-*white* transgene. These males showed only a light coloring of the adult eye, and aggression was almost completely abolished (Figure 3A). In an inverse experiment, we rescued the eye-color phenotype in males carrying a GMR-*white* construct in a $w^{1118}$ mutant background. Interestingly, with flies fighting at 28%–65% of wild-type level, the aggression was only partially restored independent of the number of constructs and their location (Figure 3B). This suggests that an intact visual system is required for proper aggressive behavior. Because the flies' eye colors were dark red but still clearly distinguishable from wild-type CS males, this experiment did not rule out that the lower-than-WT level of aggression reflected an incomplete restoration of contrast transfer in these eyes.

On the other hand, *white* gene function might be required in tissues of the fly other than the pigment-producing cells in the eye. The latter idea is supported by findings of Campbell and Nash [29], who detected *white* messenger RNA in $so^{1}$ flies by using RT-PCR. Mutant $so^{1}$ flies have neither eyes nor ocelli and should therefore lack pigment-producing cells. Also, in a place-learning paradigm in complete darkness (heat box), $w^{1118}$ null mutant flies are impaired [30]. To test whether the *white* mutation affects neurons outside the eye, we combined various GAL4 drivers (*Ddc*-GAL4; *TH*-GAL4, *Tdc2*-GAL4, MB247-GAL4; NP6510-GAL4, NP6561-GAL4) expressing GAL4 in groups of neurons in the central brain with the UAS-RNAi-*white* transgene. Indeed, diminishing *white* expression in these cells reduced the frequency of lunges to varying degrees ranging from 5%–48% of wild-type level (Figure S3C). These results suggest that *white* exerts its effect not only in pigment-producing cells but also in other parts of the brain, some of which are involved in the control of aggression.

## Males with Reduced OA Level, but Elevated TA Level, Show Reduced Aggression

Scoring various components of *Drosophila* aggressive behavior, Baier et al. [12] report severely reduced aggression in $T\beta h^{nM18}$ males. $T\beta h^{nM18}$ mutant flies lack tyramine β-hydroxylase (TβH), an enzyme converting tyramine (TA) to octopamine (OA). These flies have no detectable levels of OA, whereas TA levels are elevated by about 10-fold [11]. These authors, however, had used $T\beta h^{nM18}$ males carrying the additional $w^{1118}$ null mutant allele and tested them with red-eyed control males. As shown above, the $w^{1118}$ null mutation by itself leads to profoundly reduced aggression. Furthermore, even after backcrossing the $T\beta h^{nM18}$ flies to $w^{+}$, mutant males were still about 8% smaller than wild-type males ($p = 0.0039$) (Figure S4C). Hence, the body-size difference might have contributed to

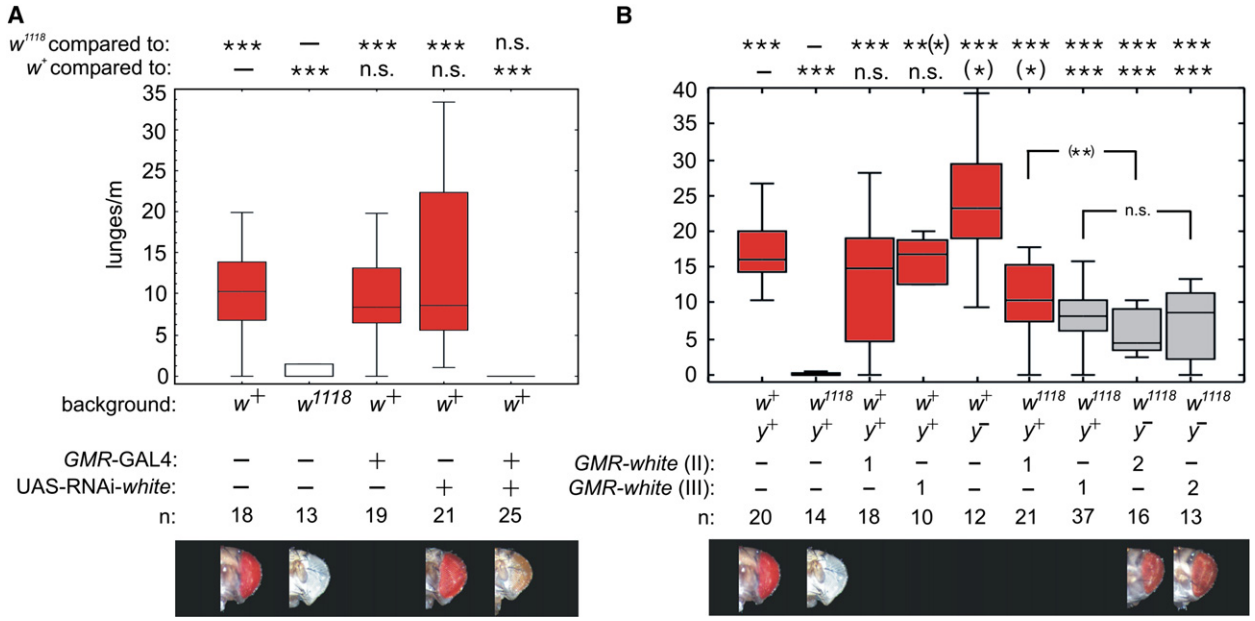


Figure 3. The Impairment of $w^{1118}$ Null Mutants in Aggressive Behavior

Plot parameters are as described for Figure 2.

(A) In $w^{1118}$ null mutant males aggression is almost abolished ($p = 0$), a phenotype that can be mimicked by knocking down *white* expression only in the eye ($p = 0.06$; last column).

(B) Rescue experiments with two different insertions of the same *GMR-white* construct (one being located on the second chromosome and the other on the third) only partially restored aggressive behavior independent of whether one or two copies were present (for all $p < 0.00005$). Genotypes represented in red boxes do not differ from $w^{+}$ with respect to their aggressive behavior, whereas genotypes represented in white boxes are not distinguishable from $w^{1118}$. Grey boxes show genotypes different from both $w^{+}$ and $w^{1118}$ regarding their aggression. Where informative, the eye color of a male of a specific genotype is illustrated below its corresponding box plot.

the decreased aggression as well. To test whether reduced aggression was indeed due to the $T\beta h^{nM18}$ mutation and independent of body size, we measured it in pairs of mutant males and in our automated recording setup counting only lunges. Aggression was still almost completely abolished (Figure 4A).

In contrast to our results, Certel et al. [13] did not report a general decrease in aggression compared to wild-type males when $T\beta h^{nM18}$ males fought against each other (S. Certel and E.A. Kravitz, personal communication). To exclude genetic background as the cause for this discrepancy (their $T\beta h^{nM18}$ mutant stock had been independently crossed into $w^{+}$ background [13]) we tested their stock in our paradigm. These males displayed profoundly fewer lunges per meter compared to wild-type males (Figure S4D). However, with a remaining level of 17% of wild-type, males of their $T\beta h^{nM18}$ mutant stock were more aggressive than males of our $T\beta h^{nM18}$ mutant stock, which displayed hardly any aggressive behaviour.

On the basis of published effects of OA, we tested two hypotheses that might explain the strong decrease in aggression observed for $T\beta h^{nM18}$ males. (1) During jumping, distance and force production of $T\beta h^{nM18}$ flies is only ~50%–60% of wild-type level [31]. Consequently, $T\beta h^{nM18}$ males might be incapable of executing lunges. However, a quantitative high-speed analysis measuring 12 parameters of lunges did reveal only a single small difference between lunges of CS and $T\beta h^{nM18}$ males: While rising up on their hind legs, $T\beta h^{nM18}$ males did not elevate their body as much as wild-type males (−26%; $p = 0.005$). In other words, only the frequency, but not the execution, of lunges seemed to be affected. (2) As mentioned in the introduction, injection of the OA agonist chlordimeform into crickets causes normally submissive losers to re-engage in fights faster [5]. Therefore, appropriate levels of OA might be required to motivate former losers to fight again. If $T\beta h^{nM18}$ males establish a hierarchy within the first 15 min and the loser thereafter avoids to re-engage in further fights, lunges might become a rare event. To test this hypothesis, the first 15 min immediately after pairing the flies were analyzed. Right from the beginning, $T\beta h^{nM18}$ males performed hardly any lunges ($p < 0.0001$), indicating a general loss of aggressiveness independent of former experiences.

We investigated whether restoring OA in $T\beta h^{nM18}$ males would increase the frequency of lunges. This would strengthen the assumption that it is indeed the lack of OA that elicits the low-aggression phenotype. $T\beta h^{nM18}$ females are sterile, and fecundity can be restored by feeding octopamine [11, 32]. Moreover, feeding OA successfully rescues a memory deficit of $T\beta h^{nM18}$ flies [33]. In that study, OA should have crossed the insect blood-brain barrier because it was supposed to have its effect in the mushroom body, a structure of the central brain. We provided 5 mg/ml OA in normal fly food either throughout the whole life span or only during adult life. Neither treatment restored aggression in $T\beta h^{nM18}$ males compared to wild-type males (for both, $p < 0.0001$). The same feeding protocol, however, reverted female sterility independent of the onset of OA supplement ($p = 0.42$ and $p = 0.64$), indicating that OA was ingested and still active in the fly.

$T\beta h^{nM18}$ males carrying a wild-type $T\beta h$ cDNA downstream of the *hsp70* promoter (*hsp-Tβh*) were used to show that the $T\beta h$ locus is responsible for the behavioral changes measured here. The heat-shock protocol applied had already been used successfully to rescue the above-mentioned memory deficit of

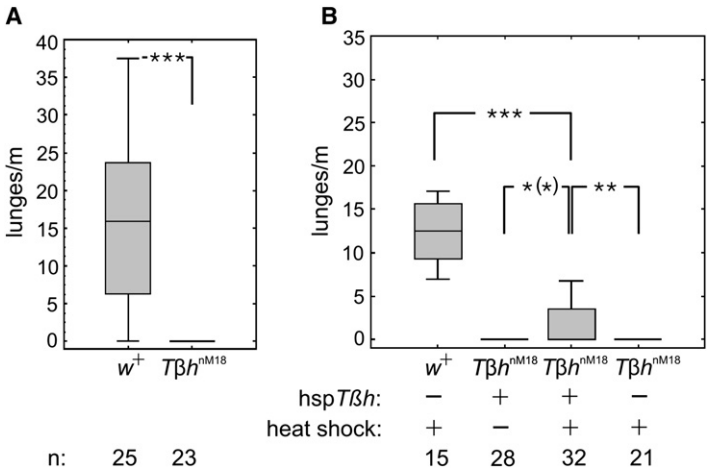


Figure 4. $T\beta h^{nM18}$ Males Lacking OA Display Less Aggression Than Wild-Type Males

Plot parameters are as described for Figure 2.

(A) In $T\beta h^{nM18}$ males aggression was almost abolished compared to wild-type males (p = 0).

(B) Heat-shock-induced expression of *Tβh* in adult $T\beta h^{nM18}$ mutant males partially restored aggression compared to both males of the same genotype without heat shock (p = 0.004) and to heat-shocked $T\beta h^{nM18}$ males lacking the hs-*Tβh* construct (p = 0.003).

$T\beta h^{nM18}$ flies [33]. Heat-shock-induced expression of *Tβh* in adult $T\beta h^{nM18}$ males restored aggression to a small but significant extent compared to both males of the same genotype without heat shock and to heat-shocked $T\beta h^{nM18}$ males lacking the *hsp-Tβh* construct (Figure 4B). 47% of all mutant $T\beta h^{nM18}$ pairs that temporarily expressed TβH in all cells showed at least one lunge, whereas only 14% and 9% of all pairs of the same genotype without heat shock and of $T\beta h^{nM18}$ males lacking the *hsp-Tβh* construct, respectively, showed at least one lunge. This result substantiates the role of octopamine in modulating *Drosophila* male aggression. Because this partial rescue was hidden in the noise of the software, clips were evaluated manually (see above).

To rescue fecundity in females, a slightly stronger heat-shock protocol was applied. It resulted in a percentage of *Tβh;; hsp-Tβh* egg-laying females that were indistinguishable from wild-type (n = 19–24; due to technical reasons, Fisher's exact test could not be applied; Figure S4G).

The rather poor performance of $T\beta h^{nM18}$ males that temporarily expressed TβH in all cells might be due to the short time window in which TβH was expressed. In the light of immunohistochemical data indicating that there are neurons expressing TA, but not OA [34], misexpression of TβH, alternatively, might change tyraminergic into octopaminergic neurons, which might have deleterious effects on aggression.

### Males Lacking OA and TA Show Reduced Aggression

Because OA-supplemented food did not rescue aggression in $T\beta h^{nM18}$ males, we next examined whether the increased TA, rather than the lack of OA in $T\beta h^{nM18}$ males, might have caused the aggression phenotype. To address this issue, we used mutants of the *tyrosine decarboxylase 2* (*Tdc2*) gene ($Tdc2^{RO54}$). Tyrosine decarboxylase 2 (TDC2) converts tyrosine to TA in neurons. HPLC measurements reveal no detectable levels of TA and OA in $Tdc2^{RO54}$ mutant brains [35]. We used males homozygous for a mutation in the nearby *cinnabar* gene ($cn^1$) as a control because the $Tdc2^{RO54}$ mutant also carried it. $Tdc2^{RO54}cn^1$ males were strongly reduced in aggression compared to $Tdc2^{RO54}cn^1$ heterozygote males and to $cn^1$ males. With the lunge count software, we determined that their lunge frequency was at about 5% of control levels (Figure 5A). This result strongly suggests that in $Tdc2^{RO54}$ and $T\beta h^{nM18}$ males it is indeed the missing OA that causes the aggression phenotype. TA could only still be held responsible if too little TA was as deleterious for aggression as too much.

Providing mutant $Tdc2^{RO54}$ males with TA/OA-supplemented food during adulthood again did not restore aggression (Figure 5A). The same feeding protocol, however, rescued $Tdc2^{RO54}$ female sterility (Figure S5C). The applied protocol has been demonstrated to restore brain TA and OA levels of $Tdc2^{RO54}$ mutant flies to wild-type levels [36]. Interestingly, Hardie et al. report that feeding only TA could not restore OA levels, "as if ectopically supplied amines were not transported into the appropriate neurons where the metabolic conversion could take place."

To ensure restoration of OA and TA levels within neurons, UAS-*Tdc* was expressed in all tyraminergic and octopaminergic neurons by using *Tdc2*-GAL4. There are two genes encoding for a TDC in flies: *Tdc1* is expressed nonneuronally and *Tdc2* in neurons only [35]. Surprisingly, not *Tdc2* expression, but *Tdc1* expression in *Tdc2*-neurons yielded a small but significant rescue of aggression compared with $Tdc2^{RO54}$ males carrying either only the *Tdc2*-GAL4 transgene or the UAS-*Tdc1* construct (Figure 5B). $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc1* males lunged at a rate of 3% compared to the heterozygote controls, whereas $Tdc2^{RO54}$ males very rarely displayed a lunge.

In general, the aggressive behavior displayed was highly variable. Two separately collected datasets were pooled for Figure 5B; in one of the two experiments $Tdc2^{RO54}$, *Tdc2*-GAL4;UAS-*Tdc1* males were only significantly different to one control. In accordance with previous reports [35, 36] and with our findings on aggression, *Tdc1* expression seemed to be more potent in rescuing female sterility than *Tdc2* expression, with the latter restoring female fecundity only partially (Figure S5F). Strikingly, expressing UAS-*Tdc2* yielded higher OA and TA levels than did expressing UAS-*Tdc1*; in fact, TA levels were even higher than in wild-type flies [35]. Possibly, *Drosophila* male aggression is sensitive to deviations from wild-type OA/TA concentrations, resulting in suppressed aggression.

Feeding wild-type flies OA (5mg/ml) or TA (0.3 mg/ml) did not affect aggression (Figure S6A). Also, overexpression of *Tβh* with the hs-*TβH* transgene had no effect on lunge frequency (Figure S6B). This finding argues that in the $T\beta h^{nM18}$ mutant it is not the excess of TA that is deleterious. Also in the rescue experiments above, the small or missing effects could not be attributed to too high levels of OA or TA.

### Neuronal Silencing of Octopaminergic and Tyraminergic Neurons Reduces Aggression

The finding that rescuing neuronal OA and TA only partially restored aggression points to OA/TA being required either outside neurons or neuronal OA/TA being required at a specific (1) concentration, (2) time point, and (3) place to enable flies to express aggression. To test the importance of tyraminergic and octopaminergic neurons for the control of aggression, these neurons were selectively blocked. Inhibiting action potential generation via UAS-*Kir2.1* expression [37] in *Tdc2*-neurons

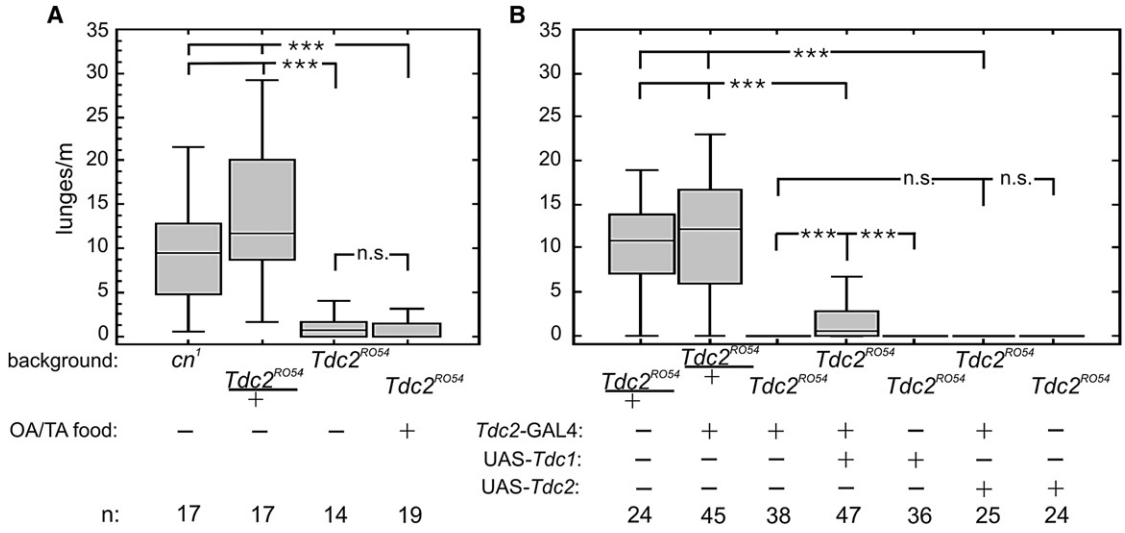


Figure 5. $Tdc2^{RO54}$ Mutant Males Lacking TA and OA Perform Hardly Any Lunges

Plot parameters are as described for Figure 2.

(A) $Tdc2^{RO54}$ $cn^1$ males lacking TA and OA were strongly reduced in aggression compared to $Tdc2^{RO54}$ $cn^1$ heterozygote males and to $cn^1$ males (for both, $p < 0.00003$). Feeding $Tdc2^{RO54}$ $cn^1$ flies TA/OA during the 5 days prior testing did not restore male aggression ($p = 0.46$).

(B) Expressing the nonneuronal *Tdc1* in octopaminergic and tyraminergic neurons of $Tdc2^{RO54}$ $cn^1$ males partially restored aggression compared to control flies without GAL4 ($Tdc2^{RO54}$ $cn^1$; UAS-*Tdc1*) ($p = 0.0002$) or UAS-*Tdc1* ($Tdc2^{RO54}$ $cn^1$ *Tdc2*-GAL4) ($p = 0.0008$). Expressing the neuronal *Tdc2* in the same set of cells did not restore male aggression compared to the two controls ($p = 0.96$ and 0.84, respectively).

mimicked the $T\beta h^{nM18}$ mutant phenotype. That is, *Tdc2*-GAL4/UAS-*Kir2.1* males showed a significant decrease in lunges per meter compared to males both of the driver and of the effector line, with lunges occurring at a rate of about 22% of the controls (Figure 6A). To restrict blockage of tyraminergic and octopaminergic neurons to a small time window, we used the temperature-sensitive UAS-*shibire* transgene [38, 39] driven by *Tdc2*-GAL4. Blocking synaptic transmission only during the experimental period by raising the temperature to more than 30°C almost abolished aggression in *Tdc2*-GAL4/UAS-$shi^{ts1}$ males compared to males of the same genotype fighting at the permissive temperature of 25°C (Figure 6B). However, using the UAS-$shi^{ts1}$ transgene for studying *Drosophila* aggression proved to be difficult due to a general trend of high temperature to reduce aggression. The general reduction in aggression due to high temperature made it difficult to detect differences between genotypes, especially when comparing UAS-$shi^{ts1}$ males with *Tdc2*-GAL4/UAS-$shi^{ts1}$ males at the high temperature ($p = 0.005$), which required a manual evaluation. The marginal decrease in aggression found for UAS-$shi^{ts1}$ males at 25°C compared to *Tdc2*-GAL4 males ($p = 0.047$) is presumably due to the slightly higher walking activity in UAS-$shi^{ts1}$ males because the pure number of lunges was not affected ($p = 0.66$). Despite the problems with using the UAS-$shi^{ts1}$ transgene, the results obtained with both UAS-*Kir2.1* and UAS-$shi^{ts1}$ strengthen our hypothesis that octopaminergic neurons and potentially tyraminergic neurons are necessary for aggressive behavior.

## Discussion

In *Drosophila* as well as other arthropod species, OA is involved in modulating aggressive interactions. We have taken various independent approaches all pointing at an important role of OA in this behavior. First, we have genetically blocked OA biosynthesis at two steps in the metabolic pathway, resulting in strongly reduced male aggression. We have then partially restored aggressive behavior in one mutant by providing the missing metabolic enzyme in all cells via a transgene and in the other mutant by expressing the wild-type gene in octopaminergic and tyraminergic neurons. Finally, we have shown that aggression is suppressed when either action potential formation or synaptic transmission are blocked specifically in these neurons.

The first indication that OA might play a role in modulating *Drosophila* male aggression came from a study by Baier et al. [12], who observed in mutant $T\beta h^{nM18}$ males a deficit in various aggressive behaviors when put together with control males. $T\beta h^{nM18}$ males are, on average, 8% smaller than wild-type CS flies. According to our data, this size difference alone would account for a substantial reduction in lunge frequency. Of even greater importance, their $T\beta h^{nM18}$ flies also carried the $white^{1118}$ mutation and therefore had white eyes, whereas their opponents were red eyed. The $white^{1118}$ mutation by itself leads to a phenotype indistinguishable from the $T\beta h^{nM18}$ mutation because both almost completely abolish aggression. In conclusion, Baier and coworkers arrived at the right conclusion but, in retrospect, had no evidence.

In contrast to our finding, Certel et al. [13] did not report a general decrease in aggression for $T\beta h^{nM18}$ males when fighting against each other (S. Certel and E. Kravitz, personal communication), presumably because the recording conditions used in their study and ours were different. In our setup, submissive males could not escape the small bottom area of the chamber and were, therefore, frequently attacked by the dominant male. It may be that this special enclosure situation, which led to high-lunge frequency in wild-type flies, reveals the impairment of the mutant.

Because $T\beta h^{nM18}$ males have an ~10-fold increase in brain TA levels, we considered the possibility that excess TA might be the actual cause of reduced aggression. However, in $Tdc2^{RO54}$ males lacking both neuronal OA and TA, aggression

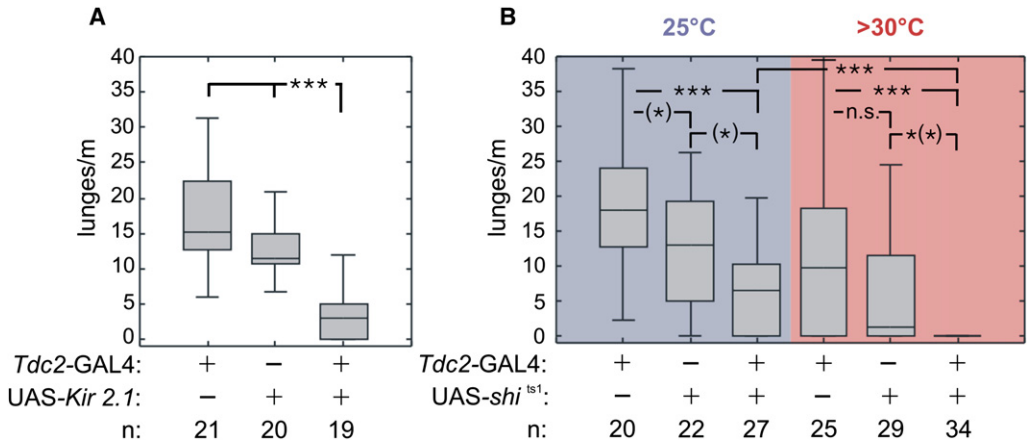


Figure 6. Impaired Signaling of Both Tyraminergic and Octopaminergic Neurons Mimicks the $T\beta h^{nM18}$ Phenotype

Plot parameters are as described for Figure 2.

(A) Males carrying both the *Tdc2*-GAL4 construct and the UAS-*Kir2.1* construct performed fewer lunges per meter than males of the driver line ($p = 0.000001$) and males of the effector line ($p = 0$).

(B) Males expressing UAS-$shi^{ts1}$ in octopaminergic and tyraminergic neurons performed fewer lunges per meter than controls carrying only either GAL4 ($w^+$; *Tdc2*-GAL4) (25°C, $p = 0.00008$; 30°C, $p = 0.000008$) or UAS ($w^+$; UAS-$shi^{ts1}$) (25°C, $p = 0.032$; 30°C, $p = 0.005$) at the permissive (25°C) and the restrictive (>30°C) temperature. $w^+$;*Tdc2*-GAL4;UAS-$shi^{ts1}$ males performed fewer lunges per meter at the restrictive temperature than at the permissive temperature ($p = 0.00001$).

was as much reduced as seen in $T\beta h^{nM18}$ males. Therefore, we attribute the aggression phenotype to low OA rather than high TA. Otherwise one would have to postulate that both high and low TA levels result in strongly reduced aggression. Immunohistochemical data indicate that in the fly's brain tyramine is not only localized in octopaminergic neurons but also in tyraminergic neurons specifically devoid of octopamine [34]. Although these would be the best candidate neurons for mediating a presumed dose-dependent biphasic effect of TA, they would not show elevated TA levels in $T\beta h^{nM18}$ mutant flies. In all, we consider it rather unlikely that TA has a major role in the suppression of aggression.

Expressing *Tβh* in all cells of adult $T\beta h^{nM18}$ males via heat shock restored aggression to a small but significant number. Also expressing UAS-*Tdc1* in *Tdc2*-neurons in $Tdc2^{RO54}$ mutant males partially rescued aggression, indicating (1) that in both cases the defects were not caused by second-site mutations and (2) that some of the octopaminergic neurons in the brain are likely to mediate the effect. The latter argument is further strengthened by the finding that aggression is suppressed if these neurons are blocked. More specific GAL4 driver lines and manipulations of the dose and dynamics of OA in these neurons are needed to further elucidate its function in the control of aggression.

### Automated Detection of Lunges

This study is based on an automated analysis of lunges, a single component of aggressive behavior in *Drosophila* males. Evaluating only a single indicator deals with aggression as if it were a unitary phenomenon and as if the various components were controlled by the same mechanism. This is unlikely to be true. As a starting point, our investigation is deliberately confined to this one aspect of aggression.

We have not tried to bring the recording and software analysis to perfection. Rather, we decided to live with a low-tech setup and an error rate of about 11% that is mainly due to undetected lunges (tight exclusion criteria). Our study was most severely troubled by the few false positives that prevented the detection of low rescue effects in mutants. For these cases, the lunge view software was developed, which allows the investigator to first loosen the criteria for lunges and to subsequently eliminate false positives. A second problem arose in the context of tussling, a high level aggressive behavior that consists of a mixture of boxing and lunging. During tussling sequences, lunges were less precisely detected. Fortunately, during the 15$^{th}$ to the 30$^{th}$ minute tussling was rare regarding all of the genotypes under investigation.

On the positive side, the automated counting of lunges allowed us to handle large amounts of data and guaranteed standardized evaluation. Because of its variance, a quantitative assessment of *Drosophila* aggression is exceedingly time consuming. The data reported here comprise a total of 480 hr of recording and a total of over 50,000 lunges. To fully analyze a clip, i.e., regarding the number of lunges, walking activity, the fly's body size, etc.; the investigator needs to spend only ~3 min. Except for the very low end of the scale, the error rate is independent of lunge frequency. Fortunately, it is also largely independent of the genotypes used in this study.

### Conclusions

In *Drosophila*, lunge frequency is strongly reduced without OA, but OA is apparently not necessary for triggering aggressive acts because flies lacking OA occasionally execute lunges. Consistently, crickets depleted of OA and dopamine still display aggression, but fights do not escalate to the same level as in controls [5, 40], an effect that can be reversed by injecting the OA agonist CDM [5]. Likewise, injecting one of the two OA receptor antagonists, epinastine or phentolamine, depresses aggression in crickets. Interestingly, the strength of the effect is context dependent. Whereas in naive crickets, only epinastine leads to a slight reduction in escalation level, the effect is stronger and seen for both antagonists if crickets are made to fly before the fight [5]. Likewise in *Drosophila*, depletion of OA might affect aggression to varying degrees, depending on the situation. In the setup by Certel et al. [13], lack of OA results in no detectable effect, whereas it leads to a pronounced

reduction in aggression when flies, as in our setup, are forced to encounter each other at a high frequency. Thus, the strength of OA's influence on *Drosophila* aggression appears to be context dependent.

## Experimental Procedures

### Fly Stocks and Maintenance

Flies were raised at 25°C and 60% relative humidity in a 14/10 hr light/dark cycle on standard *Drosophila* medium (cornmeal, agar, molasses, yeast, and nipagin). Only flies of the UAS-RNAi-*white* experiments were raised in a 17/7 hr light/dark cycle. The UAS-RNAi-*white* stock, the $w^{1118}$ stock, the *dTdc2*-GAL4 [35] stock, and the *Appl*-GAL4 [40] stock were cantonized for at least six generations. For the outcrossing, 50 males and females were used in each generation, except for the UAS-RNAi-*white* stock, for which 20 females and males were used. The UAS-*Kir2.1* line, the UAS-$shi^{ts}$ line, and the $Tdc2^{RO54}$ line were crossed into a $w^+$ background. The $T\beta h^{nM18}$ line had already been crossed into a $w^+$ background by H. Scholz (University of Wuerzburg, Germany) and independently by S. Certel (Harvard Medical School, Boston, MA).

Newly emerged male flies were collected and individually kept for 6 days in 22 $cm^3$ vials containing food. On day 5 all flies were anesthetized by cooling down (4°C) for less than 2 min and every other male was marked with a white dot (AeroColor, color 101, Schmincke, Germany) on the thorax. Flies were put back into the food vials for at least 12 hr. On day 6, two males were aspirated into the arena.

### Behavioral Assay

The arena measures 4 cm × 5 cm × 12 cm. The floor of the arena was made of polyoxymethylen. In its center, a hollow of 1 cm × 1 cm × 0.5 cm filled with an aliquot of a mixture of 67 ml apple juice, 1.5 g agarose, and 1.7 g sucrose was surrounded by a 0.5 cm wide moat of 2% agarose. The glass walls were covered by Fluon (FluonGP1, Whitford GmbH, Germany). This tetrafluoroethylene copolymer results in a slippery layer on the glass wall to prevent the insects from sitting on the wall. The camera (Panasonic NV-GS 400; JVC GR-DVL 9800) was equipped with a +4 close-up lens (Hama, Germany) and positioned above the arena. Video recording (in progressive scan mode) started as soon as both males were in the arena and terminated after 30 min. To allow the animals to first settle down and to guarantee constant aggression, the analysis of the data was confined to the $15^{th}$ to $30^{th}$ min. All experiments were performed at 25°C and 60% humidity.

### Data Analysis: *Drosophila* Fights

#### *Walking Activity*

The lunge count software calculates per frame and per fly the shift in the center of gravity of the fly's body, i.e., the distance traveled. The distance traveled is then summed for each fly. The window for a frame to be included in the summation is 0.1–2.0 mm/frame to avoid an impact of behaviors other than walking, e.g., cleaning and flying.

#### *Size*

Size measurements are based on the two-dimensional area of the fly from top view. For each fly the average size is calculated out of 200 (at least 30) frames. Each frame has to meet four criteria: (1) the fly's distance to the glass wall is greater than 2 mm (control for body posture), (2) the two flies have to be more than 4 mm apart from each other (control for body posture), (3) the aspect ratio of body width to body length is 0.42 ± 0.07 (control for body posture), and (4) the fly is not on the food patch (control for constant contrast).

### Data Analysis: High-Speed Clips

To capture high-speed movies of lunges, the arena was reduced to 2 cm × 3 cm × 12 cm. Clips were recorded at 500 frames/s with a Redlake MotionPro 2000 digital high-speed camera equipped with a Sigma Macro lens. For CS males, videos were made from lateral and dorsal views, whereas for $T\beta h^{nM18}$ males only videos in dorsal view were captured. Anatomical landmarks were digitized frame by frame with Didge (version 2.2.0, Alistair Cullum, Creighton University, Omaha, NB). For videos recorded from lateral view, only the tip of the head and the tip of the abdomen were digitized. With these two digitized points, maximal head velocity and maximal displacement of the body angle were calculated for each lunge. To analyze clips recorded in dorsal view, 18 points were digitized to track movements of the body, the legs, and the position of the legs relative to each other (Figure S8A). Based on the 18 points, 13 variables describing the displacements of body and limb segments were calculated (Figure S8B). Displacement profiles were smoothed with a fourth-order zero-phase-shift butterworth filter with the user-defined cut-off frequency set at 150 Hz [41]. Velocities were then calculated by differentiation of the displacement profiles. Although displacements were calculated for both limb pairs, only the greater of the two was retained for statistical analysis to reduce the number of variables.

### OA/TA-Enriched Food

$TDC2^{RO54}$ flies received food supplemented with 0.3 mg/ml tyramine hydrochloride (T2879; Sigma) and 3 mg/ml octopamine hydrochloride (O0250; Sigma) for the time period between eclosion and test. Food was melted in a microwave. Shortly before it solidified, TA and/or OA was mixed into the food. Food for $T\beta h^{nM18}$ flies was supplemented with 5 mg/ml OA. Here, in one group, the eggs were already laid on OA-enriched food; in the other group, treatment started after eclosion and ended with the start of the recording period.

### Heat-Shock Protocol

Males were treated as described in [33]. Males were heat shocked at 37°C for 30 min 18 hr and 12 hr before the recording period. For the heat shock, males were aspirated into a new vial containing only a moist filter paper. These vials had been preheated at 37°C for 30 min. After each heat-shock, males were aspirated back into the original vial. Females were treated as described in [32]. Females were heat shocked twice for 60 min with a 3 hr break in between. This heat-shock regime was not applied for males, as it led to a high mortality rate in males. Otherwise, the treatment was the same as that of males.

### *shibire*$^{ts}$ Experiments

Flies were raised at 25°C. The arena was preheated for 30 min before the first pair of males of the day was set up. Flies were directly aspirated into the arena without prior heating. Three different sets of experiments were performed at 33°C, 31.5°C, or 30.5°C, in order to find a temperature that affected aggression of control males the least. However, each genotype was affected to the same extent independent of how much the temperature was elevated; therefore, data were pooled for each genotype.

### Statistical Analyses

When not otherwise stated, Kruskal-Wallis-ANOVA was applied to detect overall differences among several unpaired groups. When differences between groups occurred, the significantly different groups were filtered out by pairwise comparisons by using Mann-Whitney U tests. Differences between two genotypes concerning the percentage of egg-laying females were determined with Fisher's Exact test. In all figures, one, two, and three asterisks indicate an $\alpha$-level of 0.05, 0.01, and 0.001, respectively. For all multiple comparisons, Bonferroni correction was applied. However, even those differences are indicated that failed to pass the significance criterion after Bonferroni correction but were significant without it. In these cases asterisks are given in parentheses.

To compare the kinematics lunges performed by CS males with lunges executed by $T\beta h^{nM18}$ males, 12 variables were calculated. As $T\beta h^{nM18}$ males were significantly smaller than CS males, a MANCOVA was applied including all 12 variables. Because only the front leg variables correlated with size, an ANCOVA was run for each of them. For each of the other variables, a t test was used.

Statistical Analyses were performed with STATISTICA, version 7.1 (StatSoft, Tulsa, OK) and JMP IN software, version 4.02 (SAS Institute, Cary, NC).

### Supplemental Data

Eight figures and four movies are available at http://www.current-biology.com/cgi/content/full/18/3/159/DC1/.

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