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ORIGINAL RESEARCH

Gr39a, a Highly Diversified Gustatory Receptor in *Drosophila*, has a Role in Sexual Behavior

Kanako Watanabe · Gakuta Toba · Masayuki Koganezawa · Daisuke Yamamoto

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Abstract Sexual recognition among individuals is crucial for the reproduction of animals. In *Drosophila*, like in many other animals, pheromones are suggested to play an important role in conveying information about an individual, such as sex, maturity and mating status. Sex-specific cuticular hydrocarbon components are thought to be major sex pheromones in *Drosophila*, and are postulated to act through the gustatory system, since they are mostly non-volatile chemicals. However, very little is known about the molecular and neural bases of gustatory pheromone reception. So far, a few putative gustatory receptors, including Gr32a and Gr68a, have been implicated in courtship behavior. Here, we examine another putative gustatory receptor, Gr39a, which shares a cluster with both Gr32a and Gr68a in a molecular phylogeny of the gustatory receptor family, for its potential role in courtship behavior. The *Gr39a* gene produces four isoforms through alternative splicing of different 5′-most exons. A quantitative real-time PCR analysis showed that the expression levels of all four splice variants of *Gr39a* were reduced in a fly line in which a *P* element was inserted into the *Gr39a* locus. Homozygous and hemizygous males for the *P*-element insertion, as well as males in which *Gr39a* was knocked down by RNAi, all showed reduced courtship levels toward females. The courtship levels returned to normal when the *P* element was excised out. A close analysis of courtship behavior of the mutant males revealed that the average duration of a continuous courtship bout was significantly shorter in the mutants than in the wild type. The results suggest that Gr39a has a role in sustaining courtship behavior in males, possibly through the reception of a stimulating arrestant pheromone.

Keywords *Drosophila* · Pheromone · Gustatory receptor · Sexual behavior · Mutant

Introduction

In many animal species, chemical communication plays a major role in social and sexual interactions among individuals within the population (Brennan and Zufall 2006). This indeed applies to *Drosophila melanogaster*, a model organism amenable to genetic analysis of higher order functions including complex behavior and its neural basis (Ferveur 1997, 2005; Sokolowski 2010). In this species, chemical communication is especially prominent during courtship, where it depends in large part on sex pheromones for exchanging information about individuals. When a *D. melanogaster* male courts a female, it exhibits a series of stereotypical behaviors including orienting to and following the female, tapping female's body with its forelegs, singing a courtship song by extending and vibrating one of the wings, licking female genitalia with the labellum, and attempting to copulate by curling the abdomen (Greenspan 1995; Hall 1994). Pheromonal information is exchanged between the male and female during courtship through olfaction and/or gustation, and some volatile and non-volatile chemicals have been

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K. Watanabe and G. Toba contributed equally to this work.

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K. Watanabe · G. Toba · M. Koganezawa · D. Yamamoto (✉)
Division of Neurogenetics, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan
e-mail: daichan@m.tohoku.ac.jp

identified as sex pheromones that modulate sexual behavior (Ferveur 1997, 2005; Jallon 1984; Zawistowski and Richmond 1986). It is postulated that the male detects female sex pheromones by gustation during the tapping and licking steps of the courtship behavior, since cuticular hydrocarbons on the body surface have a role as sex pheromones (Ferveur 1997, 2005), and both the foreleg and labellum harbor gustatory receptor neurons (Amrein and Thorne 2005).

In *Drosophila*, a group of chemosensory receptors called the gustatory-receptor (Gr) family is mainly responsible for transducing contact chemosensory information (Vosshall and Stocker 2007). Many Grs are functionally categorized into two groups; receptors for appetitive substances such as sugar receptors, and receptors for aversive substances such as bitter receptors (Dahanukar et al. 2007; Lee et al. 2009). On the other hand, Gr68a, Gr32a, Gr33a and Gr66a are reported to play roles in pheromone perception. Knockdown of Gr68a reduces male-to-female courtship (Bray and Amrein 2003), implying its role in mediating the perception of hypothetical female aphrodisiac pheromones by males. Loss of Gr32a or Gr33a results in enhanced male-to-male courtship, suggesting their role in pheromone perception to inhibit homosexual courtship (Miyamoto and Amrein 2008; Moon et al. 2009). 7-Tricosene is a predominant cuticular hydrocarbon pheromone in males that inhibits male courtship and increases female sexual receptivity (Jallon 1984). The inhibitory effect of 7-tricosene on male-to-male courtship was markedly attenuated when *Gr66a*-expressing sensory cells were inactivated (Lacaille et al. 2007). Interestingly, Gr32a, Gr33a and Gr66a are expressed in bitter taste cells and at least the latter two mediate bitter sensation (Lee et al. 2009; Moon et al. 2006, 2009). The involvement of bitter taste receptors in inhibitory pheromone reception has also been documented by electrophysiology (Lacaille et al. 2007), which was used to record impulse discharges from bitter-sensitive receptor neurons upon stimulation of a sensory hair with 7-tricosene. Furthermore, *Gr32a*-expressing sensory cells participate in the shaping of a male courtship posture, unilateral wing extension, because, when these cells are inactivated, the male flies fail to retain one wing at the resting position during courtship, resulting in simultaneous bilateral wing extension (Koganezawa et al. 2010). These observations demonstrate that sex pheromones play multiple roles by activating multiple chemoreceptors, some of which remain to be characterized. In particular, receptors for "appetitive contact sex pheromones" such as female-specific 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND) (Jallon 1984) have not been rigorously identified although Gr68a was suggested to be a candidate (Bray and Amrein 2003).

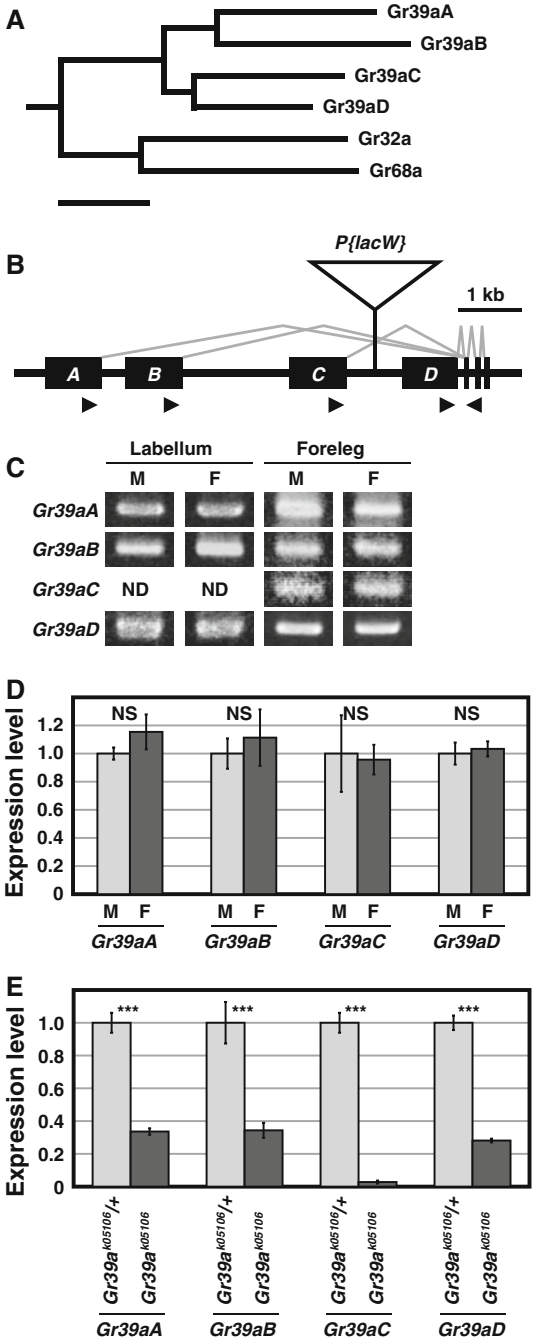
In a molecular phylogenetic tree, Gr32a is most closely related to Gr68a, the first member in Grs reported to function as a pheromone receptor (Bray and Amrein 2003), and the two Grs form a monophyletic cluster with Gr39aA, Gr39aB, Gr39aC and Gr39aD, the four isoforms derived from a single locus as a result of alternative splicing (Robertson et al. 2003; Fig. 1a, b). The gene *Gr39a* represents one of three exceptional Gr genes (out of 68 Gr genes in total) that undergo alternative splicing. Like other Grs, the Gr39a isoforms are seven-pass transmembrane proteins (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001). The seventh transmembrane domain and the C-terminus are encoded by the last three exons shared by the four isoforms, while the N-terminus and the first six transmembrane domains unique to each isoform are encoded by four alternative 5′ exons (Fig. 1b). Comparisons of genomic sequences in 12 *Drosophila* species have revealed surprising divergence in the organization of the 5′ alternative exons of *Gr39a*, i.e., extensive duplications, losses and "pseudogenizations" took place (Gardiner et al. 2008). For example, *Gr39a* in *D. mojavensis* has five active type-A exons, two inactivated type-A exons, an additional exon called E that has no counterpart in *D. melanogaster,* and no type-C exon, while the same gene in *D. sechellia* and *D. erecta* has only 3 active alternative exons B, C and D (Gardiner et al. 2008). The occurrence of such structural divergence among closely related species implies that *Gr39a* has experienced exceptional evolutionary forces such as positive selection.

Given the structural similarity of Gr39a to Gr32a and Gr68a, two Gr members known to function as sex pheromone receptors (Bray and Amrein 2003; Koganezawa et al. 2010; Miyamoto and Amrein 2008), it is conceivable that Gr39a also plays a role in a sexual context, within which positive selection often drives rapid evolution of genes (Haerty et al. 2007; Tsaur and Wu 1997; Wyckoff et al. 2000). Here we show that the disruption of Gr39a by a mutation or RNA interference (RNAi) impairs male courtship behavior, supporting the above hypothesis.

Materials and methods

Flies

Flies were raised on a cornmeal-agar-yeast medium at 25°C. The variants we used are described in the FlyBase database (http://flybase.org/). Wild-type Canton-S flies were used as the control. *l(2)k05106*^{k05106} (Bellen et al. 2004) is a strain carrying a *P{lacW}* (Bier et al. 1989) insertion at the *Gr39a* locus, and *Df(2L)DS6*) is a deficiency line lacking the entire *Gr39a* locus; both were obtained from the Bloomington Drosophila Stock Center. 8685 (w^{1118}; *P{GD3724}v8685*), 8686 (w^{1118}; *P{GD3724}v8686*) and 10172 (w^{1118}; *P{GD3479}v10172*) RNAi strains and a *UAS-Dicer-2*



◄ **Fig. 1** The *Gr39a* gene and a mutation affecting its expression. **a** Phylogenetic relationships among Gr32a, Gr68a and Gr39a isoforms. A neighbor-joining tree (Saitou and Nei 1987) of the six proteins deduced from their amino acid sequences is shown. An outgroup (Gr21a) was used to determine the root position. The branch leading to the outgroup is omitted. The branch lengths represent the number of amino-acid substitutions per site computed by the Poisson correction (Poisson correction distance *d*). The *scale bar* below represents $d = 0.25$. The six proteins form a similar monophyletic cluster in a larger phylogenetic tree of the entire seven-pass transmembrane chemoreceptor proteins in *Drosophila* (Robertson et al. 2003). **b** The exon–intron organization of the *Gr39a* locus. Exons are indicated by *boxes* and labeled with *letters* corresponding to the encoding isoforms. Splice patterns are overscored with *gray lines*. The insertion site of the *P{lacW}* element in the $l(2)k05106^{k05106}$ strain is shown by a *large open triangle*. The approximate sites and orientations of the primers used for qRT-PCR analysis are indicated with *arrowheads*. **c** The expression of *Gr39a* in the labellum and foreleg of male (*M*) and female (*F*) flies was examined by RT-PCR. A representative band of the amplified DNA fragment in an agarose gel is shown for each template-primer combination. *Gr39aA*, *Gr39aB* and *Gr39aD* transcripts were detected in the labellum and foreleg in both sexes, and the *Gr39aC* transcript was detected in the foreleg in both sexes (*ND*, not determined). **d** Comparisons of the expression levels of *Gr39a* transcripts in the labellum between male (*M*) and female (*F*) flies for four splice variants (*Gr39aA, Gr39aB, Gr39aC* and *Gr39aD*). For each transcript, relative expression levels when the mean expression level in the male was defined as 1.0 were shown. The design of this experiment did not allow direct comparisons between the expression levels of different transcripts. No significant differences were found between the sexes in any of the transcripts. The values are shown as the means ± SEM. Student's *t*-test was used to evaluate the statistical significance of differences (*NS*, not significant). **e** Comparisons of the expression levels of *Gr39a* transcripts in the labellum between the male flies heterozygous and homozygous for the $P\{lacW\}l(2)k05106^{k05106}$ insertion (here we describe as $Gr39a^{k05106}/+$ and $Gr39a^{k05106}$, respectively). The expression levels were quantified by qRT-PCR. For each transcript, relative expression levels when the mean expression level in the heterozygotes was defined as 1.0 were shown. The design of this experiment did not allow direct comparisons between the expression levels of different transcripts. All four transcripts were significantly decreased in the homozygotes compared with the heterozygotes. The most striking reduction in the expression was detected in *Gr39aC*. The values are shown as the means ± SEM. Student's *t*-test was used to evaluate the statistical significance of differences ($***P < 0.001$)

(*UAS-Dcr-2*) strain (Dietzl et al. 2007) were purchased from the Vienna Drosophila RNAi Center. $elav^{C155}$ (Lin and Goodman 1994), an enhancer-trap GAL4 driver, was used to induce *UAS-RNAi* expression in all neurons. To enhance the knockdown effect, *UAS-Dcr-2* was introduced into the flies in addition to $elav^{C155}$ and *UAS-RNAi*. $l(2)k05106^{k05106}$ used in this study were backcrossed to w^{1118} for at least 5 generations to standardize the genetic background. The *Δ2–3* (*P{Δ2–3}99B*) (Robertson et al. 1988) stock was used as a transposase source. To generate *P*-element excision lines, $l(2)k05106^{k05106}$ females were mated with *Δ2–3* males, resulting in the progeny carrying both the $P\{lacW\}l(2)k05106^{k05106}$ and *Δ2–3* insertions with mosaic eye color. White-eyed iso-male stocks established from this progeny were subjected to a screen for lines in which precise excision of the *P* element occurred by examining the genomic sequences around the insertion point. In this screen, $Gr39a^{rev}$ was obtained as a precise excision line, the X chromosome of which was subsequently substituted with the one harboring a wild-type w^+ allele to exclude a possible effect of having white-eyes on courtship behavior.

Behavioral assays

To observe courtship behavior, virgin males were collected at eclosion and placed individually in food vials for 3 days.

Each male fly was transferred to a round mating chamber (8 mm in diameter; 3 mm in height) with a wild-type (Canton-S) virgin female or male. The behavior of the fly pair was recorded using a video recorder under dim-red light. The level of male courtship activities was evaluated by the courtship index (CI). The CI was given by the proportion of time spent by a male for courtship, which included orientation, following, tapping, wing vibration, licking and attempted copulation within a 10-min observation period, or until the time of copulation initiation when the pair copulated before the end of the observation period. Male courtship activity is composed of many courtship bouts separated by non-courting periods. We defined a single courtship bout as a continuous courtship period separated from other bouts by a non-courting period lasting over 1 s. The number of courtship bouts was counted in a 10-min observation period. The cumulative time spent for courtship by a male was measured and divided by the number of bouts to calculate the average courtship duration. To measure locomotor activity of individual males, virgin males were collected and aged as described above. A single male fly was placed in the mating chamber, and allowed to behave freely for about 5 min for locomotion recording. The behavior of the male fly was recorded using a video recorder under dim-red light. The number of times the male crossed a straight reference line in the middle of the chamber within a 1-min time window was counted, and used as the index for locomotor activity.

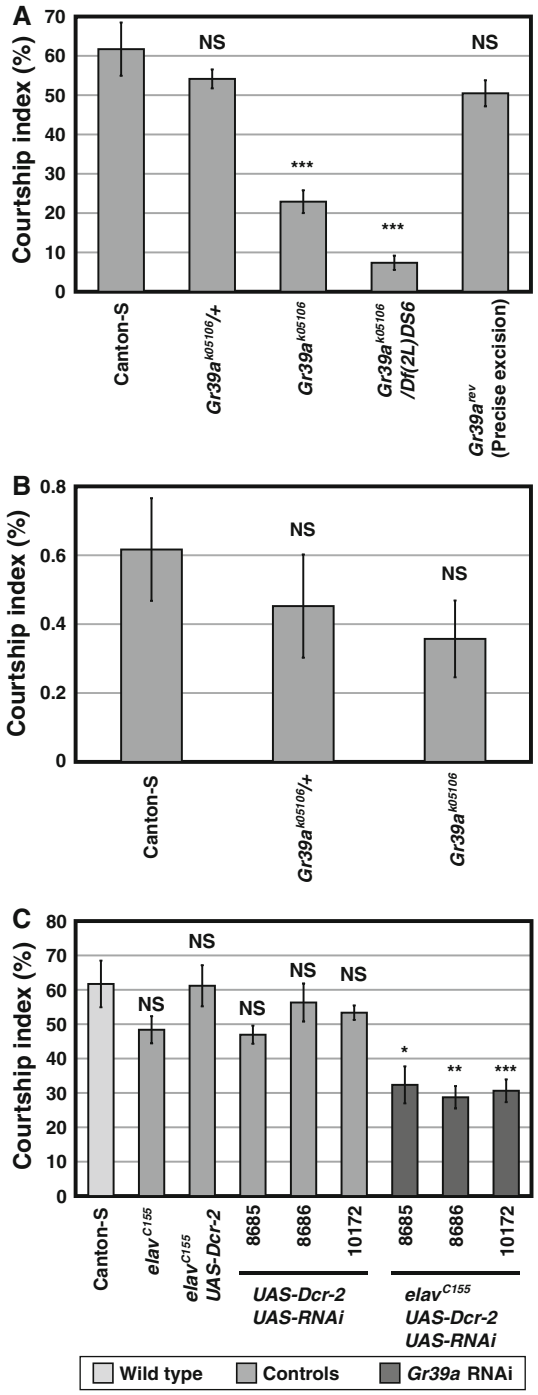
Expression analysis

For reverse transcription polymerase chain reaction (RT-PCR), total RNA was prepared from ca. 200 labella and ca. 200 forelegs of 3–5 day old Canton-S males and females using an RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA samples were reverse transcribed by the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer after DNase I treatment, and the resulting cDNAs were used as templates for PCRs. For the PCR, the AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, CA) and the primers shown in supplementary Table S1 were used. For quantitative real-time polymerase chain reaction (qRT-PCR) experiments, cDNA was prepared as described above, except that total RNA was extracted from ca. 150 labella of 3-day old adult flies of each genotype, and that DNase I treatment was omitted because of the usage of the primers with sequences at the exon–exon junctions. qRT-PCR was performed with SYBR Premix Ex Taq II (Takara, Tokyo, Japan) and the primers shown in Table S1. As an internal control for normalization, the *rp49* transcript was similarly amplified using the primers (see Table S1). qRT-PCR was conducted by using a LightCycler (Roche, Mannheim, Germany), and the data were acquired and analyzed by LightCycler Software (Roche). Three reactions were set up for each primer set, and their mean value was calculated.

Results

We examined whether *Gr39a* is expressed in the labellum and foreleg, the two taste organs that are likely to be used by *Drosophila* males to detect female sex pheromones during courtship behavior. RT-PCR analysis revealed that *Gr39aA*, *Gr39aB* and *Gr39aD* transcripts are present in both the labellum and foreleg in both sexes, and *Gr39aC* transcript exists in the foreleg in both sexes (Fig. 1c). Data for *Gr39aC* expression in the labellum were missing in our RT-PCR analysis (Fig. 1c), but the expression was confirmed in the qRT-PCR experiment (Fig. 1d). Thus, all four transcripts of *Gr39a* were expressed in the labellum and foreleg in both sexes. qRT-PCR analysis of the expression levels of the four transcripts in the labellum showed no discernible sex differences (Fig. 1d). The $l(2)k05106^{k05106}$ fly line carries the *P{lacW}* transposon in the intron intervening between exons C and D in the *Gr39a* locus (Fig. 1b). Although the name of the mutation implies it is recessive lethal, some homozygous adult escapers with no obvious morphological defects were obtained at a low rate, and backcrossing the line to the w^{1118} strain significantly improved the viability of the homozygotes (data not shown). qRT-PCR analysis of *Gr39a* mRNA levels in the labellum revealed that male flies homozygous for the $P\{lacW\}l(2)k05106^{k05106}$ insertion contain much smaller amounts of all four *Gr39a* transcripts in comparison with heterozygous males (Fig. 1e). The most severely affected mRNA species was the one encoding Gr39aC (Fig. 1e), the transcription of which is commenced in exon C, locating immediately 5′ to the *P{lacW}* insertion (Fig. 1b). This result indicates that $l(2)k05106^{k05106}$ represents a loss-of-function mutant of the *Gr39a* gene, and we refer to it as $Gr39a^{k05106}$ hereafter.

To examine the possibility that *Gr39a* is involved in courtship behavior, we measured the courtship index (CI; see Materials and Methods) of *Gr39a* mutant males. Although male flies heterozygous for $Gr39a^{k05106}$ vigorously courted female flies, $Gr39a^{k05106}$ homozygous males exhibited a remarkable reduction in courtship toward females (Fig. 2a). When $Gr39a^{k05106}$ was placed in trans to *Df(2L)DS6*, a deficiency that deletes the entire *Gr39a* locus, these hemizygous males displayed even less courtship toward females than $Gr39a^{k05106}$ homozygotes did (Fig. 2a), genetically indicating that $Gr39a^{k05106}$ is a hypomorphic allele of *Gr39a*. $Gr39a^{rev}$ is a derivative of $Gr39a^{k05106}$, resulting from the precise excision of the



◀**Fig. 2** Effects of *Gr39a* disruption on the male courtship behavior. **a** The CI toward the wild-type Canton-S females was compared among the male flies of Canton-S, $Gr39a^{k05106}$/+, $Gr39a^{k05106}$, $Gr39a^{k05106}$/*Df(2L)DS6* and $Gr39a^{rev}$. While the courtship activities of $Gr39a^{k05106}$ heterozygous males were not significantly different from those of the wild-type Canton-S males, flies homozygous and hemizygous for $Gr39a^{k05106}$ showed a significant decline in male courtship activities compared with the Canton-S flies. The CI returned to the wild-type level when the *P* element was excised out ($Gr39a^{rev}$). Student's *t*-test was used to evaluate the statistical significance of differences from the values for Canton-S flies. The numbers of males examined were as follows: Canton-S (n = 13), $Gr39a^{k05106}$/+ (n = 17), $Gr39a^{k05106}$ (n = 14), $Gr39a^{k05106}$/*Df(2L)DS6* (n = 20) and $Gr39a^{rev}$ (n = 31). **b** The CI toward Canton-S males (male-to-male courtship) was compared among the Canton-S, $Gr39a^{k05106}$/+ and $Gr39a^{k05106}$ male flies. No significant difference was detected in the CI between the Canton-S wild-type and $Gr39a^{k05106}$ heterozygous or homozygous males by the Student's *t*-test. The numbers of males examined were as follows: Canton-S (n = 10), $Gr39a^{k05106}$/+ (n = 14) and $Gr39a^{k05106}$ (n = 14). **c** The CI toward Canton-S females was measured for the male flies in which *Gr39a* was knocked down by RNAi, and compared with that of control flies. Genetically encoded RNAi (*UAS-Gr39a RNAi*) constructs were expressed under the control of a pan-neural driver $elav^{C155}$. In the *UAS-Gr39a RNAi*-expressing flies, *UAS-Dcr-2* was co-expressed to enhance the effectiveness of RNAi. Three *UAS-Gr39a RNAi* lines, 8685, 8686 and 10172, were examined. The control fly strains used were Canton-S, $elav^{C155}$ without any *UAS* construct ($elav^{C155}$), $elav^{C155}$ with *UAS-Dcr-2* ($elav^{C155}$ *UAS-Dcr-2*), and *UAS-Dcr-2* and *UAS-RNAi* without $elav^{C155}$ (*UAS-Dcr-2 UAS-RNAi*). All three RNAi lines similarly suppressed male courtship activities only when expressed by $elav^{C155}$. Student's *t*-test was employed to evaluate the statistical significance of differences. The CI of each control line was compared with that of Canton-S flies, and the values of *Gr39a* RNAi flies were compared with those of the respective *UAS-Dcr-2 UAS-RNAi* controls. The complete genotypes (shown in *square brackets*) and numbers of males examined were as follows: Canton-S [+] (n = 13), $elav^{C155}$ [w^{1118} $elav^{C155}$] (n = 14), $elav^{C155}$ *UAS-Dcr-2* [w^{1118} $elav^{C155}$; *UAS-Dcr-2*/+] (n = 17), 8685 (*UAS-Dcr-2 UAS-RNAi*) [w^{1118}; *UAS-Dcr-2*/+; *P{GD3724}v8685*/+] (n = 12), 8686 (*UAS-Dcr-2 UAS-RNAi*) [w^{1118}; *UAS-Dcr-2*/+; *P{GD3724}v8686*/+] (n = 13), 10172 (*UAS-Dcr-2 UAS-RNAi*) [w^{1118}; *UAS-Dcr-2 P{GD3479}v10172*/+] (n = 14), 8685 ($elav^{C155}$ *UAS-Dcr-2 UAS-RNAi*) [w^{1118} $elav^{C155}$; *UAS-Dcr-2*/+; *P{GD3724}v8685*/+] (n = 12), 8686 ($elav^{C155}$ *UAS-Dcr-2 UAS-RNAi*) [w^{1118} $elav^{C155}$; *UAS-Dcr-2*/+; *P{GD3724}v8686*/+] (n = 10) and 10172 ($elav^{C155}$ *UAS-Dcr-2 UAS-RNAi*) [w^{1118} $elav^{C155}$; *UAS-Dcr-2 P{GD3479}v10172*/+] (n = 10). The same data for Canton-S males were repeatedly shown in graphs **a** and **c**. In all graphs, each *bar* represents the mean of all CI values obtained for the particular male-genotype. The *error bars* show ± SEM (*NS* not significant; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$)

P{lacW} insertion. Male flies homozygous for $Gr39a^{rev}$ showed a high level of courtship activities toward females that was indistinguishable from that of wild-type males (Fig. 2a), supporting the hypothesis that reduced courtship activities in $Gr39a^{k05106}$ resulted from a malfunctioning of the *Gr39a* gene due to the *P{lacW}* insertion. Importantly, no discernible difference was detected in the level of male-to-male courtship among Canton-S wild-type, $Gr39a^{k05106}$ heterozygous and homozygous males (Fig. 2b), in contrast to *Gr32a* and *Gr33a* mutants (Miyamoto and Amrein 2008; Moon et al. 2009). To evaluate the possibility that the reduced courtship activity of $Gr39a^{k05106}$ males toward females is due to the general motor activity defect of the mutant, we examined the relationship between locomotor activity and CI of individual males for Canton-S and $Gr39a^{k0510}$. Although $Gr39a^{k0510}$ mutant males did exhibit significantly lower locomotor activity than Canton-S males, no correlation between the two traits was detected in either genotype (supplementary Figure S1). The result suggests that the observed reduction in courtship activity of

Gr39a^{k0510} mutant males was not a consequence of their reduced locomotor activity.

We also employed the RNAi method to knock down the *Gr39a* gene. We used a pan-neural *GAL4* driver, *elav*^{C155}, to induce *UAS-Gr39a RNAi* expression, on the assumption that *Gr39a* is expressed in sensory neurons as other *Gr* genes are. We used three independent RNAi lines against *Gr39a*, i.e., 8685, 8686 and 10172. 8685 and 8686 carry the same RNAi construct that targets a sequence in exon A, while 10172 has a different construct that targets exon D. *UAS-Dcr-2* was co-expressed with the *UAS-Gr39a RNAi* constructs to enhance the efficacy of RNAi (Dietzl et al. 2007). All the RNAi lines diminished the male courtship activities toward females when co-expressed with *UAS-Dcr-2* under the control of *elav*^{C155}, while none of the controls did so (Fig. 2c). This observation further strengthens the notion that *Gr39a* functions in male courtship toward females.

In *D. melanogaster*, the male typically performs the courtship behavior as multiple bouts of a continuous action towards the female separated by non-courting periods. In each courtship bout, the male shows one or more of the behavioral elements in the courtship ritual such as following, tapping or wing vibration, whereas it shows behaviors unrelated to courtship such as grooming or walking, or no apparent movement in each non-courting period. Since *Gr39a*^{k05106} mutant males appeared to show shorter courtship bouts, we compared the number and duration of courtship bouts between the wild-type and mutant males. Indeed, the average duration of courtship bouts was much shorter in *Gr39a*^{k05106} mutants than wild-type males (Fig. 3a). On the other hand, *Gr39a*^{k05106} mutant males initiated courtship bouts more often than the wild-type males did in unit time (Fig. 3b), probably as a result of the intermittent courtship. These results suggest that *Gr39a* functions are required for persistent courtship bouts by males.

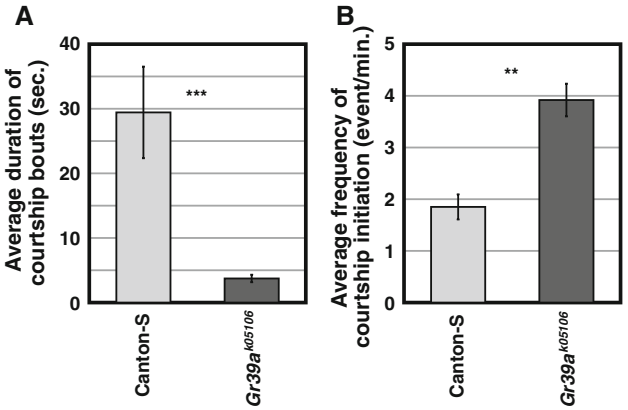


Fig. 3 Duration and frequency of courtship bouts in wild-type and *Gr39a*-mutant males. **a** The average duration of courtship bouts was lower in *Gr39a*^{k05106} mutant males compared with Canton-S males. **b** The frequency of courtship bouts was higher in *Gr39a*^{k05106} mutant males than in Canton-S males. Values are shown as the means ± SEM. Student's *t*-test was used to evaluate the statistical significance of differences (**$P < 0.01$; ***$P < 0.001$). The numbers of males examined were as follows: Canton-S (n = 13), *Gr39a*^{k05106} (n = 14)

Discussion

In this work, we showed that male flies with decreased Gr39a functions curtail individual courtship bouts, which results in the reduced over-all courtship activities of the males. This observation tempts us to postulate that Gr39a functions to maintain the excitatory state of the central nervous system (CNS), which in turn activates motor outputs for a sustained courtship bout (Kanzaki et al. 1994). Our RT-PCR analysis revealed the existence of *Gr39a* transcripts in the labellum and foreleg. A previous RT-PCR analysis has shown that *Gr39a* transcripts also exist in the thorax, abdomen and wing, although the expression in the leg was not detected (Clyne et al. 2000). Because the largest population of Grs consists of receptors for contact chemicals, an attractive hypothesis is that Gr39a acts as a receptor for a non-volatile female sex pheromone that induces tonic activation of a courtship center in the male CNS. The tissues with *Gr39a* expression mentioned above are compatible with this idea: Grs (e.g., Gr66a) in labellar sensory neurons are likely activated during licking (Lacaille et al. 2007) and those in the foreleg tarsi during tapping (Koganezawa et al. 2010) in male courtship. Although the identity of the sex pheromone that acts through Gr39a to sustain male courtship is unknown, likely candidates would be species- and/or sex-specific cuticular hydrocarbons such as 7,11-HD and 7,11-ND in *D. melanogaster* females, both of which have been shown to stimulate male courtship (Antony et al. 1985; Ferveur and Sureau 1996; Jallon 1984). Cuticular hydrocarbon profiles widely vary among *Drosophila* species, and it has been shown that males use the species-specific profile for mate discrimination (Cobb and Jallon 1990; Coyne et al. 1994). The multiple alternative exons and the high degree of inter-specific variability of the *Gr39a* gene might reflect the evolutionary history of the gene in which adaptation to the newly established species-specific pheromonal profile has driven the generation and elimination of additional exons. Another possible group of ligands for Gr39a is the basal attractive pheromones called *ur*-pheromones that make the flies of both sexes sexually attractive to males even in the absence of sex-specific hydrocarbons (Billeter et al. 2009; Savarit et al. 1999). The fact that neither *ur*-pheromones nor *Gr39a* expression is sex-specific raises an intriguing possibility that *ur*-pheromones function as stimulating arrestant pheromones also in females.

However, other scenarios are similarly possible. For example, Gr39a may mediate the transduction of sensory modalities other than gustation, because vision, audition, gustation and olfaction all strongly affect male courtship depending on the context (Krstic et al. 2009). It is of particular interest in this context to note that a significant portion of courtship defects in males with inactivated Gr68a-expressing cells are ascribable to mechanosensory/auditory deficits in these males (Ejima and Griffith 2008), although Gr68a was originally identified as a chemosensory pheromone receptor (Bray and Amrein 2003). In fact, the *Gr68a* reporter is expressed in many mechanosensory cells in addition to chemosensory cells. Some *Gr68a*-reporter expressing cells project to the antennal mechanosensory and motor center in the brain, and some to the thoracic ganglia, and these characteristics are typical of mechanosensory neurons but not of gustatory neurons (Ejima and Griffith 2008; Koganezawa et al. 2010). Because *Gr39a* is closely related to *Gr68a* in its phylogeny, careful examinations of its anatomical localization, phenotypic analysis and physiological characterization are needed to deduce its molecular functions.

Although we have shown that *Gr39a* is required in male courtship behavior, it is possible that *Gr39a* also contributes to other non-sexual behaviors. Indeed, we found that *Gr39a* was expressed in both sexes. Several lines of evidence indicate that bitter-sensitive Grs respond to a wide variety of organic compounds with little structural similarity (e.g., Moon et al. 2009), ranging from plant chemicals that function as feeding repellents for phytophagous insects (Park et al. 2000) to cuticular hydrocarbons with pheromonal functions (Lacaille et al. 2007). In the transduction of pheromonal signals, Gr32a is assisted by an odorant-binding protein OBP57d (Koganezawa et al. 2010), which, together with the sister protein OBP57e, determines the host plant-specificity of some *Drosophila* species (Matsuo et al. 2007). Intriguingly, the *Obp57d* and *Obp57e* genes exhibit extensive diversification among species, with several duplications, losses and "pseudogenizations" of exons (Matsuo 2008), reminiscent of the divergence pattern found in *Gr39a* (Gardiner et al. 2008). It is tempting to speculate that Gr39a is a multifunctional receptor set, and alternative splicing is the mechanism for the generation of molecular forms adapted to different biological functions, such as pheromonal reception and host plant selection. Further characterization of the expression and function of *Gr39a* will be needed to unravel the significance of this gene in the biology and evolution of *Drosophila*.

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