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COMPARATIVE AND ONTOGENIC PHYSIOLOGY

Influence of Group Rearing on Sexual Behavior of *Drosophila melanogaster* Males

A. A. Goncharova, Yu. V. Bragina, S. A. Fedotov, and N. G. Kamyshev*

Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg, Russia
** e-mail: nkamster@gmail.com*

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Abstract—Social interactions are able to strongly influence animal physiology and behavior. As is known, social experience can lead to changes in sexual and aggressive behavior, circadian rhythms and composition of cuticular hydrocarbons in Drosophila. Previously, we have shown that housing Drosophila males in monosexual groups of 20 individuals for 3 days after eclosion leads to a strong and long-term suppression of locomotor activity as revealed at individual testing, in contrast to males kept separately. The present research addressed courtship behavior, and specifically song production, in Drosophila males reared under similar conditions. It was found that rearing males in monosexual groups leads to a suppression of courtship and song production as well as to a simultaneous increase in locomotor activity when tested with a moving female. The latter effect was due to the strong urge of males to avoid interindividual contacts that prevented triggering the courtship ritual. It was suggested that intermale aggression caused by group rearing generates a state similar to conditioned fear.

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Key words: Drosophila, social experience, group keeping, sexual behavior, song production.

INTRODUCTION

Previous social experience may strongly affect animal physiology and behavior. In rodents, it was shown that isolation in early childhood led to hyperactivity, potentiation of aggressive behavior, and suppression of social recognition [1]. These changes are caused by modulations in the hormonal system functioning, while in case of long-term behavioral modifications the epigenetic mechanisms are also involved [2, 3]. In Drosophila, the social context may affect such behaviors as biological rhythms, aggression, mating, and even the offspring genetic diversity [4]. It was shown that social isolation of males stimulated both sexual and aggressive behavior [5–8]. Potentiation of aggression due to housing in isolation was also observed in Drosophila females [9]. Intermale aggression was also influenced by preceding contacts with females (either fertilized or virgin): prior exposure to females for 10 h led to a decrease in aggression of males when they competed for a female [10]. This effect was mediated by chemoreceptors in the male leg bristles stimulated during direct physical contacts. Absolute and relative amounts of various pheromones and cuticular hydrocarbons also changed under the influence of preceding social experience [11–13].

In an earlier study [14], we have shown that locomotor behavior of Drosophila males strongly depends on previous social experience. Their housing in monosexual groups for 3 days after eclosion led to a significant suppression of locomotor activity as compared to males housed individually during the same time. This difference persists up to 5 days after isolating males from a group. The optimal group density, at which the maximal difference was observed, was 20 males per a vial (95 mm in height, 25 mm in diameter). Based on the previous works [15, 16], we have supposed that the observed behavioral modification is the consequence of operant conditioning. In a group, the vast majority of physical contacts is punished by aggressive behavior of other individuals (kicking, wing flicking) that evokes in flies two unconditioned responses. The first is running away from an approaching individual that leads to an increase in the running bout initiation frequency. The second is the cessation of running upon encounter with another individual that leads to a decrease in the running bout duration. By the trial-and-error method, flies learn to suppress their activity as manifested in a decrease in the running bout frequency, hence in the frequency of physical contacts with other individuals.

We considered it important to find out if group rearing of Drosophila males affects other forms of behavior unrelated directly to possible conditioning, which specifically suppresses locomotor activity. A discovery of the after-effect of group rearing on other forms of behavior would provide evidence against the hypothesis of operant conditioning as a cause of long-term plastic changes in the locomotor behavior. For this purpose, the male courtship behavior toward mated females, including courtship song production, was chosen as a research subject.

The courtship behavior of Drosophila males consists in the execution of the courtship ritual, which may end with successful copulation. The courtship ritual is a fixed action pattern, which includes consecutive behavioral elements: orientation toward a female, wing vibration, pursuing a female and vibrations on the run, tapping the female's abdomen and licking its genitalia, copulative attempts [7]. When vibrating by one or another wing, a male emits the courtship song composed of the pulse and sine components. The basic function of this song is to make a female ready for copulation and to ensure the species-specific recognition of a sexual partner [17]. The courtship elements may combine in various sequences and be interrupted with other actions (rest, running and preening, i.e. leg–body or leg–leg cleaning behavior).

In this study, we investigated the effect of housing Drosophila males for 3 days after eclosion in monosexual groups, consisting of 20 individuals, on the male courtship ritual, specifically on song production.

MATERIAL AND METHODS

Experimental animals. The wild-type strain Canton-S (C-S) of *Drosophila melanogaster* was used in this study. Flies were maintained on the standard medium, under a 12-h light day, at 25°C and ~65% humidity. All flies were collected during 5 hours after eclosion and reared under standard conditions in plastic vials, 95 mm in height and 25 mm in diameter. Experimental males were housed for 3 days after eclosion either individually, or in groups of 20 individuals. Females were housed in groups of 15–20 individuals. On the day before the experiment, they were combined with preselected males of the same age to be fertilized. The fertilized females become temporarily unreceptive, i.e. unable to mate repeatedly. In the experiments with decapitated females, the decapitation was executed immediately before the test under CO_2 anesthesia. All experiments were carried out under standard conditions identical to those of previous rearing, at the same time of the day.

Courtship behavior testing. Prior to testing the courtship behavior, 3-day-old experimental males (housed either individually or in groups) were subdivided into three samples. Flies from the first sample were tested immediately, while those from the second and third samples were transferred for 2.5 or 5 h, respectively, into standard vials with a circle of filter paper moistened with distilled water. For testing, a male and a fertilized female were placed into a perspex experimental chamber (15 mm in diameter, 5 mm in height) halved by a partition. A male was put into one half, a female into another, and after 45 s the partition was removed, allowing testing. An ethogram of the male courtship behavior was recorded for 300 s by fixing the onset of the courtship (orientation, pursuing, vibration coupled with rest or running, tapping, licking, copulative attempts) and non-courtship (running, preening,

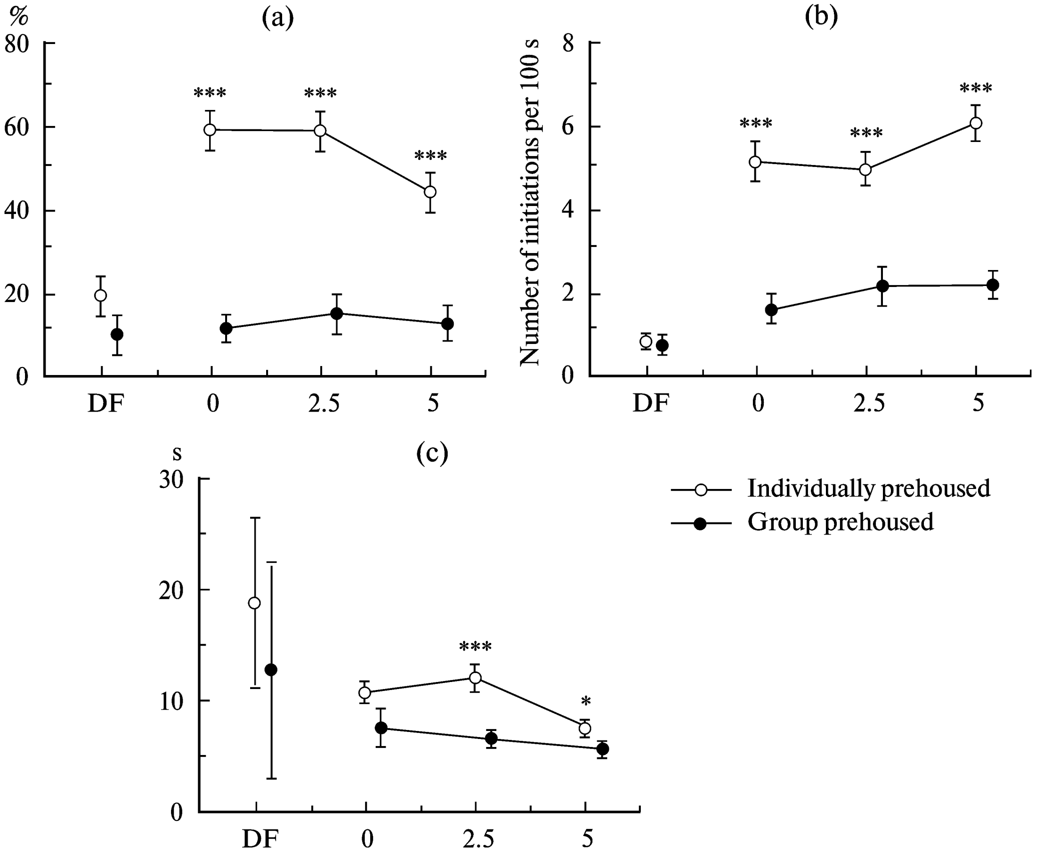

Fig. 1. The effect of group housing on sexual behavior in Drosophila males. *Abscissa*: DF—parameters of males courting fertilized decapitated female; 0, 2.5, 5—time before the onset of the experiment (h) for males courting fertilized mobile female. *Ordinate*: courtship parameters: (a)—percentage of courtship time (courtship index), (b)—courtship initiation frequency, (c)—duration of courtship bouts. Means with standard errors are shown. The difference between males housed individually and in a group is significant: *—$p \leq 0.05$, **—$p \leq 0.01$, ***—$p \leq 0.001$ (same for Figs. 2 and 4). In each sample $N = 20$.

rest) elements. This enabled further calculation of the percentage of time occupied by every behavioral element, as well as its frequency (number of initiations per 100 s) and mean duration (s).

A decoding and primary analysis of ethogram data was accomplished with the use of the "Drosophila Courtship" computer program (© Nikolai Kamyshev). The following variables were calculated: the courtship index (CI), i.e. the percent of time occupied by all courtship elements, the percent of time spent on running, preening or rest, as well as the frequency and duration of the courtship, running, preening and rest bouts.

Sound production testing. Experimental males (housed either individually or in groups) were subdivided into three samples, exactly as in case of the courtship behavior recordings. Sounds produced by a 3-day-old male courting a fertilized female in the round perspex chamber (8 mm in diameter, 4 mm in height) were recorded for 300 s. The chamber bottomed with a silicon mesh (0.6 mm hole size) was placed directly onto the membrane of a sensitive band microphone (the hardware used for sound recording was described elsewhere [17]). The four chambers with microphones were mounted inside a foam rubber box located in the soundproof room.

Sounds were recorded and analyzed using a "Drosophila Courtship Song Analysis" program (© Nikolai Kamyshev). The following three pulse song (PS) parameters were estimated:

1. pulse song index, PSI (percentage of time occupied by a pulse song during 5 minutes of recording; the time occupied by single pulses was disregarded);
2. frequency (the number of PS train initiations per 100 s);
3. the number of pulses per train.

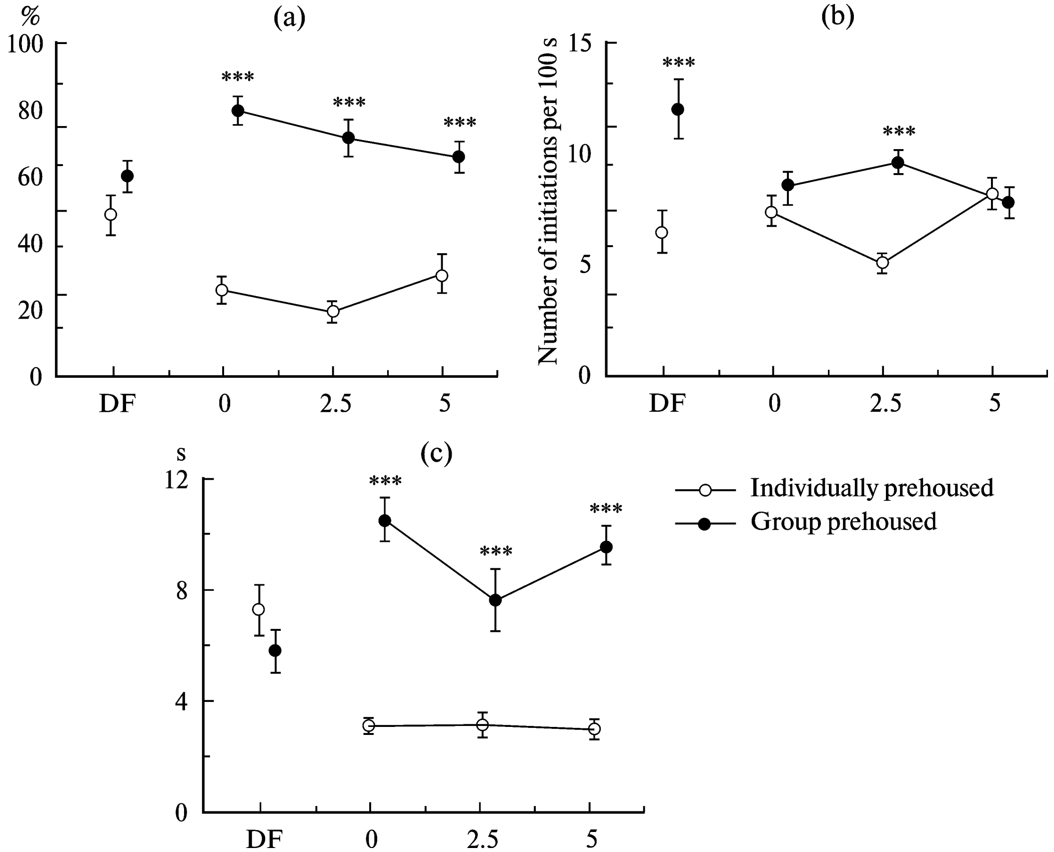

Fig. 2. The effect of group housing on locomotor activity (running) in *Drosophila* males during courtship recording. *Abscissa*: DF—parameters of males courting fertilized decapitated female; 0, 2.5, 5—time before the onset of the experiment (h) for males courting fertilized mobile female. *Ordinate*: locomotor parameters: (a)—percentage of time occupied by running, (b)—running bout initiation frequency, (c)—duration of running bouts. Means with standard errors are shown. In each sample $N = 20$.

The sine song was practically absent in males after group housing, hence its parameters were not estimated.

Statistics. Primary statistical data processing was performed with the use of the "Drosophila Courtship" and "Drosophila Courtship Song Analysis" programs. Further statistical analysis was accomplished using "Statistica 8.0" software.

Since not in all samples the distribution of variables was normal and the allowance was made for the equality of variances, the two-sided randomization test was used to perform the pairwise comparison of means [18]. To compare proportions, a z-test was applied.

RESULTS

Group housing reduces male courtship intensity. When analyzing the courtship behavior, we pooled together all the elements of the courtship ritual: orientation, pursuing a female, vibration at rest and on the run, tapping, licking and copulative attempts. Housing of Drosophila males in groups of 20 individuals during 3 days after eclosion led to a strong suppression of their courtship toward a mobile fertilized female for at least 5 h after their isolation from a group (Fig. 1a). This suppression was due mainly to a reduction in the initiation frequency of the courtship bouts (Fig. 1b). Differences in the bout duration between males with different social experience were maximal 2.5 h after transferring the group-housed males into individual vials (Fig. 1c). Small differences were also observed 5 h after the isolation from a group. When courting the fertilized decapitated female, even the control, individually housed, males showed a low courtship level (Fig. 1a, 1b). In this case, no differences in the courtship parameters were found between

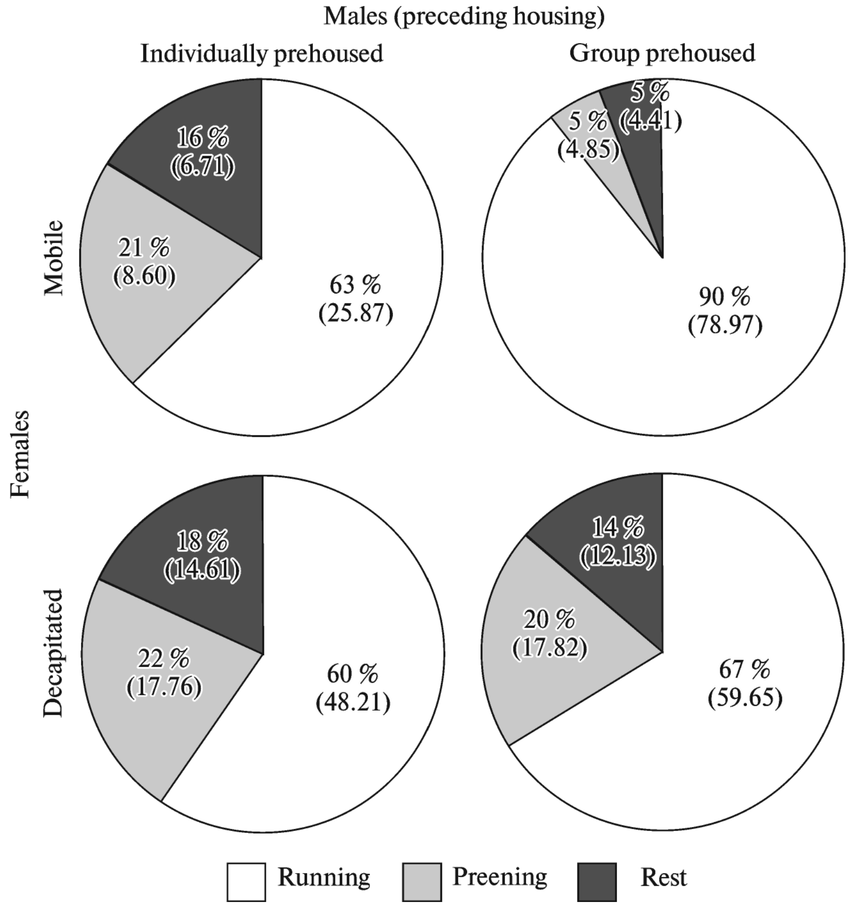

Fig. 3. The effect of preceding housing and the courtship object on non-courtship behavior in Drosophila males. The sectors show relative contribution of running, preening and rest to the total non-courtship time. The percentage of time occupied by a given behavior, relative to the whole period of observation, is shown in parentheses. In each sample $N = 20$.

males housed in groups or individually (Fig. 1).

While recording the courtship behavior, locomotor activity of males unrelated to pursuing a female (running) was also measured. In the test with a fertilized mobile female, the males housed in a group proved to be more active than males housed individually: the percentage of time spent in running rose strongly after group housing (Fig. 2a). This occurred due to an increase in the running bout duration (Fig. 2c) and, in case of males tested 2.5 hours after their isolation from a group, also due to an increase in the running bout initiation frequency (Fig. 2b). In the test with a fertilized immobile decapitated female, significant differences between males with different social experience concerned only one parameter, the running bout initiation frequency, which was higher after group housing (Fig. 2b). In terms of the proportion between the total running time and duration of separate running bouts, the tested groups of males were indistinguishable (Figs. 2a, 2c).

Figure 2 demonstrates that courting an immobile female eliminates the difference between males housed under different social conditions, as observed in case of courting a mobile female. The values of the two variables (percentage of the time spent on running and duration of running bouts) obtained in the test with courting a decapitated female take an intermediate position between the same variables obtained for males with different social experience courting a mobile female immediately after their isolation from a group. In all cases, except for the running bout frequency in individually housed males, changing the courtship object produced statistically significant ($p \leq 0.01$) shifts in all three parameters of locomotion.

If comparing the proportion between the male behavioral elements unrelated to courtship (run-

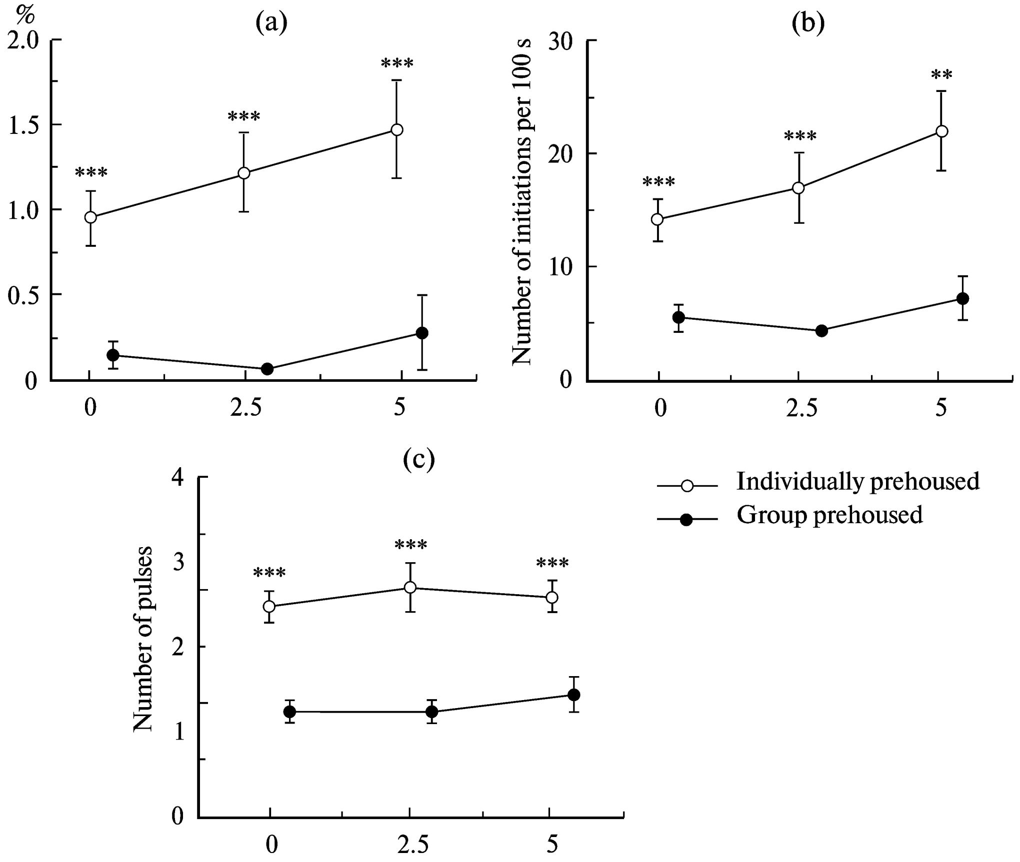

Fig. 4. The effect of group housing on sound production in Drosophila males. *Abscissa*: time before the onset of the experiment (h). *Ordinate*: pulse song parameters: (a)—percentage of pulse song time (pulse song index), (b)—pulse song initiation frequency, (c)—the number of pulses per train. Means with standard errors are shown. In each sample $N =$ 17–21.

ning, preening and rest), it can be noticed that it was affected by social experience only in case of courting a mobile female: group housing produced a significant increase in the relative portion of the running time (90 vs. 63% in control individually housed males, $p \leq 0.05$, z-test for two proportions; Fig. 3). In the test with courting an immobile decapitated female, the structure of non-courtship behavior remained intact after group housing (i.e. corresponded to the behavior of control males courting a mobile female), showing no dependence on previous experience.

Group housing of males reduces the intensity of their sound production. Our results demonstrate a decrease in the sound production by males housed in a group, as compared to males housed individually (Fig. 4a). This was due to a reduction both in the pulse song train initiation frequency and the number of pulses per train (Figs. 4b, 4c). This pattern persists at least for 5 h after isolating males from a group. These observations correlate with the above-described courtship suppression after housing males in a group, since a courtship song is one of the criteria of intensity of courtship behavior in Drosophila males.

DISCUSSION

The main purpose of this study was to elucidate whether group rearing (housing) affects other forms of behavior in addition to its effect on locomotor activity, which we have found earlier [14]. If the after-effect of group housing manifests itself in such behavioral modifications that cannot be associated with conditioning specifically suppressing locomotor activity, than the hypothesis of operant conditioning as a cause of long-term plastic changes in the locomotor behavior in Drosophila males should be rejected. To solve this issue, we have chosen a male courtship behavior, including song production, as such a behavioral form.

The results obtained are not easy to interpret. As a matter of fact, in the test with a mobile female the intensity of both behavioral forms was reduced in males previously housed in a group (Figs. 1a and 4a), arguing against the operant conditioning hypothesis. An increase in the time spent on running after group housing (Fig. 2a) might be explained simply by a decrease in the courtship intensity, since reduction in one or another behavior should be accompanied by its replacement by other behaviors. However, previous social experience led to changes in the structure of non-courtship behavior in the test with a mobile female (Fig. 3). Namely, there was a disproportional increase in runway vs. preening and rest, evidencing that a male actively avoided a mobile female. In the test with a decapitated female, this did not occur (Fig. 3), indicating that a female should move to be actively avoided by a male. Nevertheless, the running bout initiation frequency was increased in males housed in a group vs. control individuals even when they courted an immobile female (Fig. 2b).

Thus, the following behavioral pattern is emerging, being supported by straightforward visual observations. When in the chamber together with a mobile female, a male with a negative social experience tries to avoid contacts with her and runs away when they become too close to each other. Since a small size of the chamber and female's activity does not allow a male to take a position, which would exclude its possible contacts with a female, it has to be actually in a permanent movement: during the first period of observation, the time spent on running in socially experienced males is about 80% (Fig. 2a). The male's efforts to avoid physical contacts with a female prevents triggering the courtship ritual, and this may prove to be enough to explain the reduction in the courtship level of males after group housing.

Male avoidance of a female results from the previous interaction of males in a group. While the reduction in locomotor activity observed in males tested individually in the absence of a female [14] can be explained most simply by an operant conditioning due to reciprocal training of individuals in a group, as was shown for Drosophila females [15], this case looks very alike to conditioned fear. Fear conditioning is a kind of classical Pavlovian conditioning [19]. The fear-arousing object, event or action serves here as an unconditioned stimulus, while the signal (cued fear) or situation as a whole (contextual fear) associated with fear arousal serve as a conditioned stimulus. In our case, the fear-arousing objects are other aggressive males in a group. A female, undoubtedly, shares with them a number of similarities (despite multiple differences), which may serve as conditioned cues arousing fear even in the absence of the unconditioned stimulus.

Aggression in Drosophila was the subject of many studies [20]. Competing for territory, food resources and mating partners, Drosophila males enter into the struggle which may lead to the establishment of hierarchical relationships. Males which lose the battle have a lesser chance to win the next one even with quite a new foe (loser effect), indicating the involvement of learning and memory in changing their social status [21, 22].

In vertebrates, the emotional reaction of fear manifests itself as freezing, tachypnea and palpitation, release of stress hormones, and lowering the threshold of the startle response [19]. If there is something like emotions in insects is a hard and virtually unstudied question. However, recently an attempt has been undertaken to prove the reality of fear emotion in Drosophila by examining the so-called emotional primitives [23]. When observing behavioral responses to repeated threatening visual stimuli (e.g. shadows flickered over the fly), the authors inferred the existence in Drosophila of the internal state, urging the defense responses (running away, jumps, freezing), analogous to fear in mammals.

Male fights are accompanied by stress. It was shown that in insects stress induces hormonal changes [10, 24, 25] analogous to those in vertebrate animals [1, 26]. Whether this contributes to long-term behavioral changes that we observed in males after their prior housing in a group still remains unclear and requires special examination.

CONCLUSION

The results presented in this paper demonstrate the dependence of sexual activity and sound production in Drosophila males on conditions of their preceding housing. Housing males after eclosion in groups of 20 individuals for 3 days reduces the intensity of their courtship toward an unreceptive fertilized female in contrast to control males housed individually. This occurs mainly due to a decrease in the courtship ritual initiation frequency. A decrease in the sound production, observed at the same time, is caused by a reduction in the frequency of the pulse song train initiation and in the number of pulses within a train. These changes are accompanied by an increase in male's locomotor activity due to its active avoidance of a moving female; this, in turn, leads to the observed reduction in the courtship initiation frequency.

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REFERENCES

1. Lupien, S.J., McEwen, B.S., Gunnar, M.R., and Heim, C., Effects of stress throughout the lifespan on the brain, behaviour and cognition, *Nat. Rev. Neurosci.*, 2009, vol. 10, no. 6, pp. 434–445.
2. Lee, H.J., Caldwell, H.K., Macbeth, A.H., Tolu, S.G., and Young, W.S. 3rd, A conditional knockout mouse line of the oxytocin receptor, *Endocrinology*, 2008, vol. 149, no. 7, pp. 3256–3263.
3. Curley, J.P., Jensen, C.L., Mashoodh, R., and Champagne, F.A., Social influences on neurobiology and behavior: epigenetic effects during development, *Psychoneuroendocrinology*, 2011, vol. 36, no. 3, pp. 352–371.
4. Billeter, J.C., Jagadeesh, S., Stepek, N., Azanchi, R., and Levine, J.D., *Drosophila melanogaster* females change mating behaviour and offspring production based on Social context, *Proc. Biol. Sci.*, 2012, vol. 279, no. 1737, pp. 2417–2425.
5. Maynard Smith, J., Fertility, mating behavior and sexual selection in *Drosophila subobscura*, *J. Genet.*, 1956, vol. 54, pp. 261–279.
6. Sene, F., Effect of social isolation on behavior of *Drosophila silvestris* from Hawaii, *Proc. Hawaii. Entomol. Soc.*, 1977, vol. 22, pp. 469–474.
7. Bastock, M. and Manning, A., The courtship of *Drosophila melanogaster*, *Behav.*, 1955, vol. 8, pp. 85–111.
8. Ellis, L.B. and Kessler, S., Differential posteclosion housing experiences and reproduction in Drosophila, *Anim. Behav.*, 1975, vol. 23, no. 4, pp. 949–952.
9. Ueda, A. and Kidokoro, Y., Aggressive behaviours of female *Drosophila melanogaster* are influenced by their social experience and food resources, *Physiol. Entomol.*, 2002, vol. 27, pp. 21–28.
10. Yuan, Q., Song, Y., Yang, C.H., Jan, L.Y., and Jan, Y.N., Female contact modulates male aggression via a sexually dimorphic GABAergic circuit in Drosophila, *Nat. Neurosci.*, 2014, vol. 17, no. 1, pp. 81–88.
11. Kent, C., Azanchi, R., Smith, B., Formosa, A., and Levine, J.D., Social context influences chemical communication in *D. melanogaster* Males, *Curr. Biol.*, 2008, vol. 18, pp. 1384–1389.
12. Krupp, J.J., Kent, C., Billeter, J.-C., Azanchi, R., So, A.K.-C., Schonfeld, J.A., Smith, B.P., Lucas, C., and Levine, J.D., Social experience modifies pheromone expression and mating behavior in male *Drosophila melanogaster*, *Curr. Biol.*, 2008, vol. 18, pp. 1373–1383.
13. Farine, J.-P., Ferveur, J.-F., and Everaerts, C., Volatile *Drosophila* cuticular pheromones are affected by social but not sexual experience, *PLoS ONE*, 2012, vol. 7, no. 7, e40396.
14. Panova, A.A., Bragina, J.V., Danilenkova, L.V., Besedina, N.G., Kamysheva, E.A., Fedotov, S.A., and Kamyshev, N.G., Group rearing leads to long-term changes in locomotor activity of Drosophila males, *Open J. Anim. Sci.*, 2013, vol. 3, no. 4B, pp. 31–35.
15. Kamyshev, N.G., Kamysheva, E.A., Smirnova, G.P., and Parfeniuk, I.V., The reciprocal training of Drosophila individuals in a group situation by the trial-and-error method, *Zh. Obshch. Biol.*, 1994, vol. 55, no. 6, pp. 737–748. See author's translation at https://www.researchgate.net/publication/15334948_The_reciprocal_training_of_Drosophila_individuals_in_a_group_situation_by_the_trial-and-error_method
16. Kamyshev, N.G., Smirnova, G.P., Kamysheva, E.A., Nikiforov, O.N., Parafenyuk, I.V., and Ponomarenko, V.V., Plasticity of social behavior in Drosophila, *Neurosci. Behav. Physiol.*, 2002, vol. 32, no. 4, pp. 401–408.
17. Popov, A.V., Savvateeva-Popova, E.V., and Kamyshev, N.G., Peculiarities of acoustic communication in the fruit flies *Drosophila melanogaster*, *Sens. Systemy*, 2000, vol. 14, no. 1, pp. 60–74.
18. Kamyshev, N.G., Iliadi, K.G., and Bragina, J.V., Drosophila conditioned courtship: two ways of testing memory, *Learn. Mem.*, 1999, vol. 6, no. 1, pp. 1–20.
19. Anagnostaras, S.G., Sage, J.R., and Carmack, S.A., Pavlovian fear conditioning, *Encyclopedia of Psychopharmacology*, Stolerman, I.P. and Price, L.H., Eds., Berlin, 2014, pp. 1–4.
20. Zwarts, L., Versteven, M., and Callaerts, P., Ge-

netics and neurobiology of aggression in Drosophila, *Fly*, 2012, vol. 6, no. 1, pp. 35–48.

21. Yurkovic, A., Wang, O., Basu, A.C., and Kravitz, E.A., Learning and mMemory associated with aggression in *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA*, 2006, vol. 103, no. 46, pp. 17 519–17 524.
22. Trannoy, S., Chowdhury, B., and Kravitz, E.A., Handling alters aggression and “loser” effect formation in *Drosophila melanogaster*, *Learn. Mem.*, 2015, vol. 22, pp. 64–68.
23. Gibson, W.T., Gonzalez, C.R., Fernandez, C., Ramasamy, L., Tabachnik, T., Du, R.R., Felsen, P.D., Maire, M.R., Perona, P., and Anderson, D.J., Behavioral responses to a repetitive visual threat stimulus express a persistent state of defensive arousal in *Drosophila*, *Curr. Biol.*, 2015, vol. 25, no. 11, pp. 1401–1415.
24. Gruntenko, N.E., Khlebodarova, T.M., Vasenkova, I.A., Sukhanova, M.Zh., Kaidanov, L.Z., and Raushenbakh, I.Yu., Selection for sex behavior changes the metabolism of juvenile hormone in *Drosophila melanogaster*, *Genetika*, 1998, vol. 34, no. 4, pp. 480–485.
25. Neckameyer, W.S. and Matsuo, H., Distinct neural circuits reflect sex, sexual maturity, and reproductive status in response to stress in *Drosophila melanogaster*, *Neuroscience*, 2008, vol. 156, pp. 841–856.
26. Dalla, C., Pitychoutis, P.M., Kokras, N., and Papadopoulou-Daifoti, Z., Sex differences in response to stress and expression of depressive-like behaviours in the rat, *Curr. Top. Behav. Neurosci.*, 2011, vol. 8, pp. 97–118.