Neuroscience

Learning and memory during aggression in *Drosophila*: handling affects aggression and the formation of a "loser" effect

Severine Trannoy[1], Edward A. Kravitz.

Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA

Aggressive behavior in *Drosophila melanogaster* serves to acquire or defense vital resources such as food, territory or access to mates. Flies learn from previous fighting experience and modify and adapt their behavior to new situations, suggesting that learning and memory play a major role in agonistic encounters. Prior fighting experience influences the outcome of later contests: losing a fight increases the probability of losing second contests, revealing the formation of a "loser" effect. In a recent publication, we developed a new behavioral arena that eliminates handling of flies prior to, during and after fights to study the learning and memory associated with aggression. We compared two handling procedures commonly used in laboratories to study aggression with the new chambers and demonstrated that handling negatively influences aggression and prevents "loser" effect formation. In addition, we observed new aspects of behavior such as the formation of robust winner effects. *Journal of Nature and Science, 1(3):e56, 2015.*

aggression | *Drosophila melanogaster* | loser and winner effects

Aggression is commonly used across the animal kingdom to acquire resources, including food, mates and territory. Usually this involves the establishment of social hierarchies. For males, rank in social hierarchies influences much of their daily life including improving overall health and increasing the ability to acquire mates, reproduce and raise viable offspring [1]. In competition for rank, previous fighting experience influences the outcome of later contests. In general, prior losing experience increases, and winning experience decreases, the probability of losing later contests (called "loser" and "winner" effects [2]). "Loser" effects have been reported in a wide range of species, and evidence exists that these can last for several days [2-4]. "Winner" effects also have been observed, but these are less common, not as strong, and last for shorter periods of time than "loser" effects [2, 5]. In studies with cichlid fish, Oliveira et al [6] have shown that treatment with anti-androgens blocks the formation of "winner" effects without altering the establishment of "loser" effects. While this suggests an important involvement of androgens in the formation of "winner" effects, the molecular mechanisms and neural circuits concerned in the formation of the "loser" and "winner effects remain unknown.

Fruit flies (*Drosophila melanogaster*) offer an attractive and powerful model system for the study of behavior. These animals display a wide variety of innate and learned simple and complex behaviors. Most importantly, they offer the distinct advantage that sophisticated genetic tools are readily available allowing investigators to manipulate genes, neurons and neuronal circuits in awake behaving animals. The Benzer laboratory pioneered the use of fruit flies to explore the genetics of behavior, making important discoveries about clock mechanisms [7] and learning and memory [8, 9]. Learning and memory continues to be an important area of study in *Drosophila* with demonstrations that: (i) fruit flies utilize olfactory, visual and place memory capabilities in classical and operant modes in their decision-making; and (ii) distinct brain regions, humoral substances, neurons and neural circuits involved have been identified and characterized [10, 11]. Such studies represent particularly elegant and successful examples of the creative use of powerful genetic tools in a fruit fly model system to unravel brain circuitry underlying a complex behavioral process.

Although aggression between fruit flies has been known about for close to 100 years [12-14], only recently *D. melanogaster* has emerged as an important model system for the study of aggression [15]. Male and female fruit flies fight in same sex pairings: some patterns displayed in these fights are male specific, some are female specific and some are shared by both sexes [16]. A single gene, *fruitless* of the sex-determination pathway of genes, determines whether fruit flies fight like males or females [17]. Male fights go to high intensity levels, result in the formation of hierarchical relationships, and end with the formation of clear « winner » and « loser » flies. Female fights are less intense and end up with flies sharing resources [16]. Although strains of *Drosophila melanogaster* used in the laboratory have been inbred for thousands of generations, considerable heterogeneity is observed between individuals in any single generation in the levels of aggression displayed.

Flies learn from previous fighting experience and modify and adapt their behavior to new situations, suggesting that learning and memory accompany and are consequences of agonistic encounters [18]. Using wild-type Canton-S flies, it was shown that fighting strategies slowly change in fights in which hierarchical relationships are established: by the end of 30 min fights winners lunge more and retreat less while losers show opposite patterns of behavior. Fights appear to function as operant learning situations in which flies try a behavioral strategy and if it works (the opponent runs away after a lunge, for example) they use that strategy more and more during subsequent meetings. In 2nd fights, after a 30-minute separation period, losers fight differently depending on their opponents: against familiar winners they rarely lunge, while with unfamiliar winners of previous fights or socially naïve flies, they sometimes lunge. In all cases, however, flies that lose 1st fights are highly likely to lose 2nd fights. If two losers are paired in 2nd fights, however, a winner can emerge in a small percentage of cases, if that fly lunges at an opponent [18].

The Kravitz laboratory next took advantage of the heterogeneity in the intensities of aggression displayed in fly populations to build a hyperaggressive fly line, called « bullies » [19]. This was done by selecting and breeding wild-type winners of fights over 35 generations (see also [20]). Male bullies go to high levels of aggression faster and win essentially all fights against the parent population. When two socially naïve bullies are paired, however, winners and losers are generated. Defeated bullies lose 90% of 2nd fights against socially naïve bullies and lose all competitive advantage against the parent strain for a time, thereby displaying clear loser effects. These results represent a clear case of nurture overwhelming nature. Hyperaggressive flies, however, are less effective than the parent Canton-S line in competing for copulation with females, raising the possibility that there is a price to pay for enhanced aggression [19].

At present, many laboratories have begun studying aggression using the *Drosophila* model [21-24]. Laboratories have designed different chambers and experimental protocols for these studies, but essentially all involve handling and manipulation of flies to

Conflict of interest: No conflicts declared.
[1]Corresponding author. Email: Severine_Trannoy@hms.harvard.edu

introduce them to fight arenas. Gentle aspiration is the commonest way to manipulate flies [18-24], but cold or CO2 anesthesia are also used [25, 26], even though several studies have reported that the latter procedures cause deleterious effects on behavior [27, 28]. We were concerned about the possible stress associated with handling animals immediately before behavioral experiments. Therefore we felt it important to examine and compare the different experimental protocols used to transfer animals during aggression assays and ask if negative consequences might accompany the use of any of the procedures. Towards that goal, we designed a novel behavioral chamber that eliminates handling of flies entirely, and instead, used negative geotaxis to introduce flies into fight arenas. With these chambers, we tested three different sets of experimental conditions and compared the fight dynamics and outcomes of 20-min fights between pairs of male flies. We also measured locomotion and courtship behavior. The conditions used were: (i) *non-handled*, in which flies use negative geotaxis to enter behavioral chambers; (ii) *aspiration*, in which gentle aspiration is used to introduce flies to and from chambers; and (iii) *anesthesia*, in which brief ice-bath anesthesia is used prior to introducing flies to chambers [29].

In aggression assays with male flies, we observed that both types of handling decreased the total numbers of lunges observed in fights (lunging is an indicator of higher-intensity fighting behavior) but did not reduce the total numbers of encounters (brief meetings) between the flies. Cold *anesthesia*, but not *aspiration*, affected the time between the first encounter and the first lunge (latency to lunge) and the time between the first encounter and the establishment of a dominance relationship (latency to dominance). In courtship assays, we examined the latencies to court and to copulate and calculated a courtship vigor index with *non-handled* and the *aspiration* groups of flies and observed no significant differences between the two experimental conditions. Thus of the two social behaviors examined, handling by *aspiration* appears to have small effects on aggression but does not affect the ability to court or copulate. However, handling by *anesthesia* has highly significant negative effects on social behavior.

We also asked whether handling influences the locomotion of flies. In these experiments we placed single flies into behavioral chambers, and counted the numbers of midline crossings over a 20 min period. With both handling procedures, we observed significant reductions of locomotion compared to non-handled flies. If instead of single flies, we used small groups of flies, however, the locomotion deficit was overcome when *aspiration* was used, but remained in the cold *anesthesia* protocol. Together, the results demonstrate that *aspiration* has effects on aggression and locomotion but not on courtship behavior, while *anesthesia* has more important effects of all behaviors measured.

More important and more significant negative effects of handling were observed, however, on the learning and memory that accompanies aggression. In these studies, we introduced pairs of male flies into fighting chambers by negative geotaxis (*non-handled*) or by gentle aspiration (*handled*) and allowed them to interact for 20 min. Then, in the *non-handled* condition, flies were separated by inserting a thin opaque plastic divider into the fighting chamber. This was removed 10 min later, and the flies were allowed to interact for a further 20-mins. In the *handled* condition, flies were returned to their original vials by gentle aspiration between the two fights and reintroduced to the fight arenas again using the same procedure (this parallels the experimental protocol used in the original demonstration of a loser effect [18, 19]).

Using this protocol, we showed a robust and highly reliable loser effect only in the *non-handled* condition. No significant loser effect was observed in the *handled* condition. By comparing a variety of behavioral patterns between 1st and 2nd fights of both groups of flies, further significant differences were observed relating to handling. To illustrate, *non-handled* losers of 1st fights displayed highly submissive behavior and far fewer lunges in 2nd fights, while *handled* flies, if anything, showed higher levels of aggression and more lunges in 2nd fights. Previous winners in the *non-handled* group, were more aggressive in 2nd fights than in the *handled* group, always initiated the first encounter by lunging and initiated encounters earlier in the 2nd fights. Thus handling of animals using the most commonly utilized procedure, gentle aspiration, has particularly strong effects on the learning and memory that accompanies fights but also can have lesser effects on other aspects of the normal behavior of flies.

In summary of this recent work [29], we have developed a new experimental chamber that eliminates the handling of flies prior to, during and after experiments useful for studying social behavior. We compared the usefulness of these new chambers to two types of handling routinely used in laboratories to study *Drosophila* behavior, gentle aspiration and cold-anesthesia. We found that cold-anesthesia profoundly reduces locomotion and the general aggressiveness of flies and suggest that this is not an effective way to handle flies prior to behavioral studies. Gentle aspiration has smaller effects on movement and aggression and appears to have no effect on courtship behavior. Aspiration does, however, strongly influence the learning and memory that accompanies aggression.

The new chambers also have allowed improvements in experimental protocols and new information to be gathered about the consequences of aggressive interactions in fruit flies. For example, only two previous studies have reported the formation of loser effects in *Drosophila melanogaster* [18, 19]. The earlier studies used larger chambers than the ones used in our studies, the animals were handled by aspiration to move flies in and out of fight arenas, and longer fight times and interval times between fights were necessary to generate clear cut effects. Elimination of handling in our studies allows a clear establishment of dominance relationships after only 20 mins, a period of time sufficient to induce profound modifications in fight strategies during 1st fights and to observe strong loser effects in 2nd fights. In addition, for the first time with fruit flies clear short-term winner effects also were observable during 2nd fights.

In most laboratory animal behavior studies, the animals are handled to introduce or to remove them from experimental arenas. Considerable care usually is reported as taken by investigators to be gentle in such handling, but this procedure still may be stressful for animals. Here we have shown that gentle handling can significantly impact on the results of behavioral experiments with fruit flies. Perhaps further efforts should be made to generally reduce the handling of animals prior to behavioral experimentation.

Acknowledgments

This research was supported by grants from the National Institute of General Medical Sciences (GM0067645 and GM074675) to E.A.K. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank the people from our machine shop at Harvard Medical School for the design of the behavioral chambers (contact: http://mikesmachine.com).

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