Short and long-lasting behavioral consequences of agonistic encounters between male *Drosophila melanogaster*

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In many animal species, learning and memory have been found to play important roles in regulating intra- and interspecific behavioral interactions in varying environments. In such contexts, aggression is commonly used to obtain desired resources. Previous defeats or victories during aggressive interactions have been shown to influence the outcome of later contests, revealing loser and winner effects. In this study, we asked whether short- and/or long-term behavioral consequences accompany victories and defeats in dyadic pairings between male *Drosophila melanogaster* and how long those effects remain. The results demonstrated that single fights induced important behavioral changes in both combatants and resulted in the formation of short-term loser and winner effects. These decayed over several hours, with the duration depending on the level of familiarity of the opponents. Repeated defeats induced a long-lasting loser effect that was dependent on de novo protein synthesis, whereas repeated victories had no long-term behavioral consequences. This suggests that separate mechanisms govern the formation of loser and winner effects. These studies aim to lay a foundation for future investigations exploring the molecular mechanisms and circuitry underlying the nervous system changes induced by winning and losing bouts during agonistic encounters.

loser/winner effects | aggression | learning and memory | behavior | *Drosophila melanogaster*

Across the animal kingdom, aggression between conspecifics often accompanies the competition for food, mates, and territory. Although an innate behavior, aggression is a highly adaptive trait as well, with animals learning from previous experience and changing their behavior in response to new challenges. In competition for rank, for example, previous fighting experience influences the outcome of subsequent contests: prior defeat decreases whereas prior victory increases the probability of winning later contests. These have been called "loser" and "winner" effects (1). Such effects have been observed in many species, including fish (2), birds (3), and mammals (4). In general, loser effects persist longer than winner effects (5). The durational asymmetry observed between loser and winner effects has been hypothesized to participate in stabilizing social hierarchies among conspecifics (6).

Fruit flies (*Drosophila melanogaster*) exhibit a variety of simple and complex social behaviors, including aggregation (7), courtship (8), and aggression (9) in which learning and memory have been demonstrated or postulated to serve important roles (10–12). Thus, characterizing the molecular basis of memory formation, retention, and retrieval is crucial to ultimately understanding the adaptability of these social behaviors. In *Drosophila*, a variety of operant and classical training paradigms have been used to subdivide memory into distinct categories. Short-term memory (STM) lasting minutes to hours is induced by a single training session, whereas long-term memory (LTM) lasting days usually requires repeated training sessions and involves de novo protein synthesis (13). A large number of studies have been carried out using olfactory, visual, social, and place memory paradigms. These have allowed the functional and molecular characterization of neuronal circuits and the identification of numerous genes underlying learning and memory (14–16). Included are mutations in *rutabaga* (*rut*, type 1 adenylyl cyclase) that interfere with learning and STM formation (17); *amnesiac* (*amn*, peptide regulator of adenylyl cyclase) that affect STM retention (18); and *crammer* (*cer*, inhibitor of a cathepsin subfamily) that prevent LTM formation (19). Whether *rut*, *amn*, and *cer* serve roles in the learning and memory that accompanies aggression remains unknown.

Male–male aggression in fruit flies was first described almost 100 y ago (20). Since then, considerable progress has been made in understanding its expression and regulation (21–26). In competition for food resources and territory, male–male agonistic encounters, composed of stereotyped behavioral patterns, usually result in the formation of dominance relationships (9). During the progression of fights, both flies modify their fighting strategies: The ultimate winners chase and lunge at their opponents to gain sole access to the resources, whereas the losers retreat from the resources after receiving such attacks (9, 10).

In second fights (30 min after first fights), losing flies display greater submissive behavior and never win against naïve or experienced opponents, revealing short-term loser effects (10). Evidence for winner effects, however, was not found (11). Recently, in olive fruit flies (*Bactrocera olea*) it was found that previous losing and winning experiences both increased the aggressiveness of the flies. This suggests that the consequences of losing or winning may vary across species (27).

Significance

Animals must adapt to constantly changing environments, defend themselves against predators, and protect mates and territories. Learning and memory are key elements to the adaptations made for survival. *Drosophila* have been favorite study animals for identifying the genes and brain regions involved in learning and memory under a variety of associative and social learning paradigms. Here, we explore an uncharacterized social learning paradigm related to aggression and the consequences of winning and losing bouts. We demonstrate that short- and long-term memories are associated with agonistic encounters, with the memory of defeat lasting longer than the memory of victory. These are first steps in examining the genetic and environmental cues that participate in the development of social memories.

NEUROSCIENCE

We previously suggested that fights between male flies function as operant learning situations in which males learn to use their most advantageous fighting strategy during fights and then continue to do so in subsequent contests (28). In an attempt to optimize the learning and memory associated with aggression, we designed new "handling-free" behavioral chambers (29). These proved to be more desirable for studying the formation of loser effects (12). By using these experimental arenas and pairing familiar opponents in second fights we previously showed that changes in fighting strategies could be developed by both winning and losing flies. This allowed us to suggest the existence of short-term winner effects along with the previously demonstrated loser effects (12). A more detailed examination of these short-term effects is presented here along with experiments attempting to measure the intrinsic changes in fighting abilities of losing and winning flies.

In the present study, we ask (*i*) whether a single fight can lead to the formation of loser and winner effects and how long these effects persist, (*ii*) whether flies adopt different fighting strategies in second fights depending on their opponents, (*iii*) whether longer-lasting behavioral effects result from sequential repeated defeats or victories, (*iv*) whether protein synthesis is required for the short- or long-term effects observed, and (*v*) whether mutations in genes involved in learning and memory affect aggressive behavior.

Results

Short-Term Effects Induced by Single Fights. To investigate the behavioral changes associated with losing or winning fights and examine their temporal dynamics, pairs of socially naïve male flies were introduced into arenas and allowed to interact for first fights of 20 min during which hierarchical relationships are usually developed (12). After varying periods of rest (from 10 to 240 min) after first fights, the losing and winning flies were paired with socially naïve, familiar, or unfamiliar opponents in 20-min test fights.

Loser effects. When paired with socially naïve flies, previous losers lost significantly more than 50% of their second fights (50% would be the expected value by chance) only after 10 min of rest (Fig. 1*A*, circles). An examination of which fly lunged first or the numbers of lunges displayed in fights (both indicators of higher-intensity fighting) revealed that previous losers showed greater

submissive behavior when a loser effect is present. After 10 min of rest, losers lunged first in only 20% of their test fights (Fig. 1*B*) and displayed reduced numbers of lunges compared with the naïve flies they were paired against (Fig. 1*B′*). After 60 min, losing flies lunged first in 36% of test fights (Fig. 1*B*) and delivered equal numbers of lunges as naïve flies (Fig. 1*B′*), and no significant differences were found at this time point.

Then, to ask whether the familiarity of the opponent influences the fighting strategies of the losing fly, previous losers were paired against either unfamiliar or familiar winners (Uf-Winner or F-Winner). As show in Fig. 1*A*, when paired with Uf-Winners (squares), significant loser effects were observed for up to 120 min and with F-Winners (triangles) for up to 180 min. As in the fights with naïve flies, former losers lunged first less often and showed large reductions in lunge numbers compared with Uf- and F-winners when loser effects were observed (vs. Uf-winner: Fig. 1*C* and *C′*; vs. F-Winner: Fig. S1).

Winner effects. Parallel experiments to those demonstrating loser effects were carried out to examine winner effects. Here, by pairing previous winners with naïve flies in test fights a significant winner effect was observed only in flies having 10 min of rest between fights (Fig. 2*A*). Previous winners, however, lunged first in test fights for up to 120 min between fights (Fig. 2*B*). Thus, residual aspects of aggressiveness seem to be retained for longer than 10 min but these did not contribute to the formation of clear-cut winner effects. Previous winners displayed greater numbers of lunges at 10 min compared with 60 min between fights (Fig. 2*C*). The latencies to lunge (Fig. 2*D*) and to establish dominance by prior winning flies (Fig. 2*E*) also were lower at 10 than at 60 min. These results show that winning as well as losing single fights both generated changes in aggressive behavior of fruit flies, but in opposite directions.

Most studies that report winner effects focus on pairwise interactions of animals, but do winner effects translate to fighting advantages over small groups of flies? To address this question, 60 min after first fights a naïve fly was introduced into an arena containing the previous winner and loser. Under these conditions, 78% of previous winners established dominance relationships and won second fights against both the F-Loser and naïve flies (Fig. 2*F*). Previous winners delivered comparable numbers of lunges (Fig. S2*A*) and showed short latencies to lunge and to establish dominance toward both the F-Loser and naïve flies
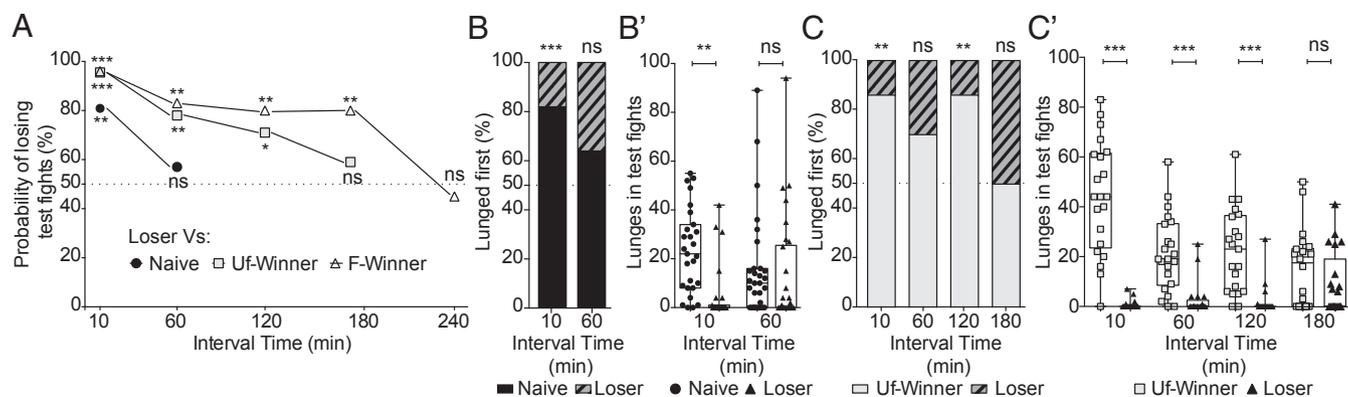

Fig. 1. Prior defeat induces submissive behavior and drives short-term loser effect formation. (*A*) A significant loser effect is observed for 10 min when paired with naïve flies (black circles) (two-tailed χ^2 test, naïve: $P_{10min} = 0.001$, $n = 27$; $P_{60min} = 0.465$, $n = 30$). However, when paired with Uf-Winners (gray squares) and with F-Winners (white triangles), these effects were seen for at least 120 and 180 min, respectively (two-tailed χ^2 test, Uf-Winner: $P_{10min} < 0.0001$, $n = 21$; $P_{60min} = 0.007$, $n = 23$; $P_{120min} = 0.049$, $n = 21$; $P_{180min} = 0.394$, $n = 22$; F-Winner: $P_{10min} < 0.0001$, $n = 22$; $P_{60min} = 0.002$, $n = 23$; $P_{120min} = 0.007$, $n = 20$; $P_{180min} = 0.005$, $n = 21$; $P_{240min} = 0.655$, $n = 20$). (*B*) When paired with naïve flies in a second fight, previous losers showed reductions in lunging first for 10 min (two-tailed χ^2 test, $P_{10min} = 0.0003$; $P_{60min} = 0.144$). (*B′*) During the same time window, they lunged fewer times in test fights against naïve flies (Mann–Whitney test, $P_{10min} < 0.0001$; $P_{60min} = 0.125$). (*C*) When paired with Uf-Winners, prior losers showed reductions in initiating lunging (except at 60 min) (two-tailed χ^2 test, $P_{10min} = 0.001$; $P_{60min} = 0.061$; $P_{120min} = 0.001$; $P_{180min} = 1$) and (*C′*) in lunge number for 2 h (Mann–Whitney test, $P_{10–60 \text{ and } 120 \text{ min}} < 0.0001$; $P_{180min} = 0.28$).
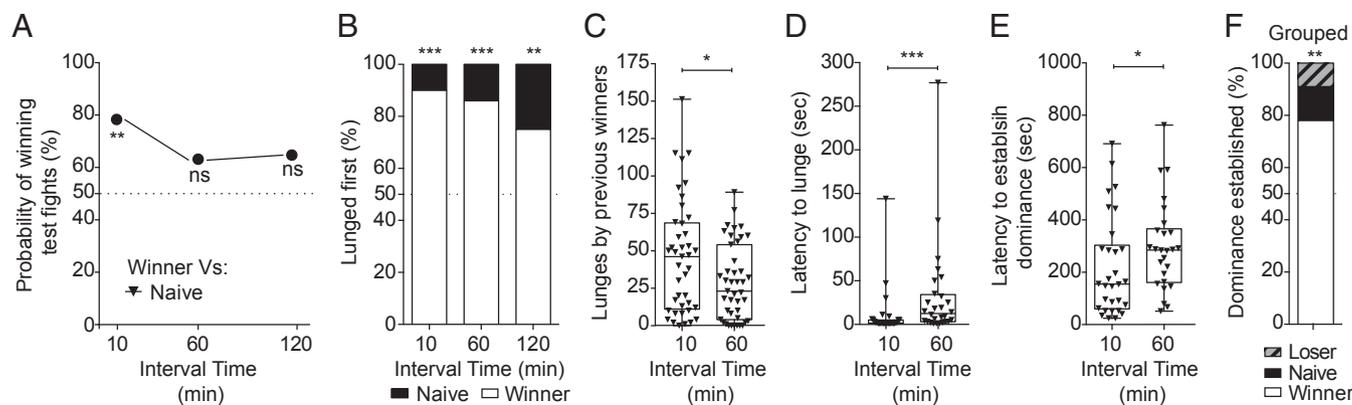
Fig. 2. Previous victory enhances aggressive behavior and promotes the formation of a short-term winner effect. (A) A significant winner effect in test fights against naïve flies was observed after only 10 min of rest (two-tailed χ^2 test, $P_{10min} = 0.001$, n = 41; $P_{60min} = 0.093$, n = 43; $P_{120min} = 0.058$, n = 40). (B) Previous winners initiated lunging in second fights against naïve opponents for up to 120 min of rest (two-tailed χ^2 test, $P_{10-60 \text{ and } 120min} < 0.0001$). (C) The total numbers of lunges displayed by winning flies was significantly higher at 10 than at 60 min (Mann–Whitney test, $P = 0.043$). (D) The latency to lunge by prior winners was significantly lower at 10 min of rest (Mann–Whitney test, $P = 0.0003$), as was (E) the latency to establish dominance (Mann–Whitney test $P = 0.04$). (F) Seventy-eight percent of previous winners won test fights against both F-Losers and naïve flies (two-tailed χ^2 test, $P = 0.007$; n = 23).

(Fig. S2 *B* and *C*). Thus, winning a single fight influences the outcome of paired and small group contests, for at least a short period.

Long-Lasting Behavioral Consequences of Repeated Defeats and Victories. The probability of losing or winning second fights did not seem to fully return to baseline values of 50% for several hours (in Figs. 1 and 2, the probabilities remain consistently above 50% but did not reach statistical significance). That raised the question of whether residual behavioral effects of single fights might persist for considerably longer times. After 1 d, prior familiar losers and winners did not show differences in initiating test fights, in the numbers of lunges delivered, or in the latency to lunge (Fig. S3). Thus, fight-experienced flies seemed to return to the same level of aggressiveness as naïve flies after 24 h of rest.

We next asked whether repeated fight experience might yield longer-term changes in fighting ability. For this purpose, we designed a "spaced conditioning" protocol consisting of five 20-min fighting trials against the same opponent, each separated by 10 min of rest (Fig. S4A). Flies that lost or won all five fights were paired 1 or 2 d later with naive flies in test fights. During the five trials, the numbers of lunges received by losing flies significantly decreased between the first two and next three trials (Fig. 3A). In addition, the latencies to lunge by winning flies significantly decreased between the first and subsequent four trials (Fig. 3B). This suggests that repeated losses and wins reinforced the defensive or offensive behavioral patterns used by flies over the five trials.

Effect of repeated losses. One day after the spaced conditioning was implemented, a significant loser effect was observed in the previously defeated flies when these were paired against naïve flies (Fig. 3C). These flies returned to their baseline fighting abilities after 2 d. At 1 d, losing flies initiated fewer fights (Fig. 3D) and lunged significantly less than the naïve flies they were paired with (Fig. 3E), but no statistically significant differences remained at 2 d (Fig. 3 *D* and *E*). To see whether we could extend the duration of the loser effect, flies were subjected to a more extensive conditioning protocol, consisting of three 20-min fight trials separated by 10 min of rest for three consecutive days (Fig. S4B). A week later, losing flies that lost all these conditioning fight trials were paired with naïve flies for 20-min test fights. At that time losing males showed the same aggressive levels as naïve flies. They lunged first and lost test fights (Fig. S4 *C* and *D*) at a 50% level, suggesting that this protocol did not lead to the generation of extended LTM formation as seen, for example, in courtship conditioning (30).

Effect of repeated wins. In contrast to the effects observed with losing flies, winning flies trained in a spaced conditioning protocol showed no advantage over naïve opponents 1 or 2 d later (Fig. 3F). Previous winners initiated 59% and 48% of the 1- and 2-d test fights (Fig. 3G) and did not lunge significantly more than naïve flies (Fig. 3H). Thus, five repeated victories were not sufficient to induce long-term behavioral changes in winners or trigger a winner effect. It remains possible that other conditioning protocols might induce longer-lasting winner effects. However, these results are consistent with those commonly reported in the literature, in which the duration of winner effects is usually shorter than that of loser effects (5).

Formation of Long-Term Loser Effect Requires de Novo Protein Synthesis. LTM in a variety of fruit fly experimental protocols requires protein synthesis (13, 31). To ask whether this also is true in the LTM associated with losing fights, flies were fed with cycloheximide (CXM), an inhibitor of protein synthesis, for 18 h before initiating a spaced conditioning protocol, via a filter paper soaked with a solution either containing (+CXM) or lacking (−CXM) the drug (32). Ingestion of these solutions had no obvious effects on fighting or decision-making abilities of flies during the initial five trials of the spaced conditioning procedure (Fig. S5). Three and 24 h after the conditioning, + or −CXM-fed losing flies were paired against naïve flies in test fights. At the 3-h time point, both the + and −CXM-fed flies showed significant and comparable loser effects (Fig. 4A) and displayed reduced numbers of lunges compared with naïve flies (Fig. 4B). At the 24 h point, −CXM flies still showed a significant loser effect and still displayed reduced numbers of lunges in test fights (Fig. 4 *A* and *C*). In contrast, the +CXM-fed flies no longer showed a loser effect and returned close to a 50% likelihood of winning or losing test fights (Fig. 4A) and no statistically significant differences were observed in the numbers of lunges delivered between losing and naïve flies at the 24-h time point (Fig. 4C).

***rut²⁰⁸⁰, amn¹,* and *cerᴾ* Memory Mutants Display Decreased Aggressiveness.** In preliminary experiments, we asked whether *rutabaga, rut* (17), *amnesiac, amn* (18), or *crammer, cer* (19) mutant flies showed any alterations in either the short- or long-term consequences of losing or winning fights. Using olfactory conditioning, the first two of these mutants have previously been shown to be involved in different aspects of STM and the third in LTM formation in *Drosophila*. Unfortunately, all of these mutant lines demonstrated reduced levels of aggressiveness in first
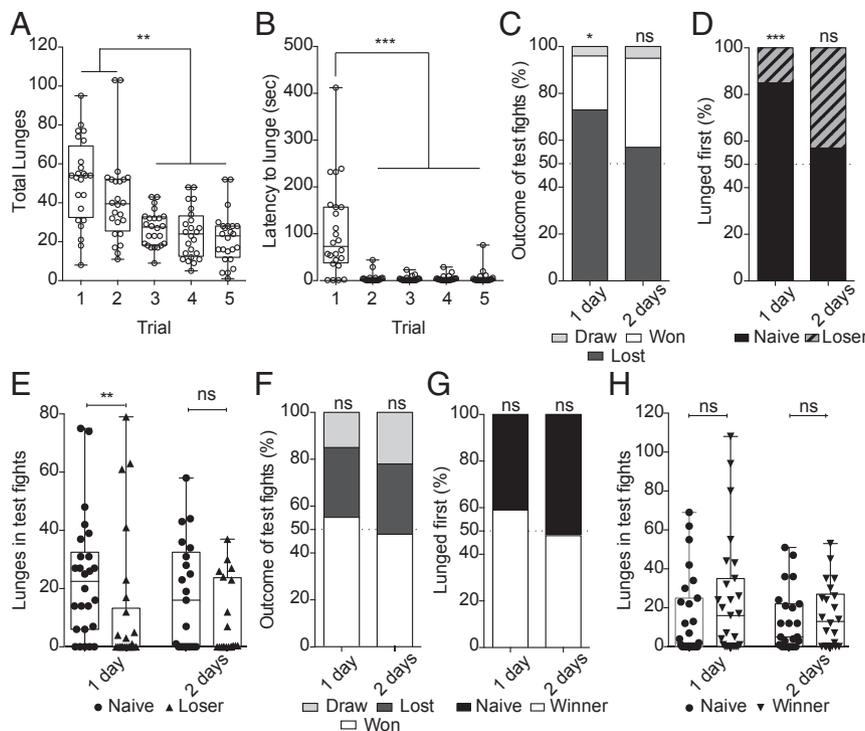
Fig. 3. Repeated defeats lead to long-lasting consequences. (A) Five sequential fights (20-min fight, 10-min rest) were performed between pairs of CS male flies. The total number of lunges was significantly decreased between the first two and next three trials (Kruskal–Wallis 37.79, Dunn's posttest, **$P < 0.001$; $n = 24$). (B) The latencies to lunge were significantly reduced between the first and subsequent four trials (Kruskal–Wallis 37.69, Dunn's posttest, **$P < 0.001$; $n = 24$). (C) A significant loser effect was observed when tested against naïve flies only 1 d later (two-tailed χ^2 test, $P_{1day} = 0.017$, $n = 26$; $P_{2days} = 0.513$, $n = 21$). (D) Previous losers showed reductions in lunging first after 1 d but not after 2 d (two-tailed χ^2 test, $P_{1day} = 0.0004$; $P_{2days} = 0.513$). (E) The losers displayed reduced numbers of lunges compared with naïve flies only after 1 d (Mann–Whitney test, $P_{1day} = 0.004$; $P_{2days} = 0.123$). (F) No significant winner effects were observed 1 and 2 d after being trained (two-tailed χ^2 test, $P_{1day} = 0.564$, $n = 27$; $P_{2days} = 0.835$, $n = 23$). (G) Previous winners did not lunge first more frequently than chance in test fights with naïve flies 1 and 2 d later (two-tailed χ^2 test, $P_{1day} = 0.336$; $P_{2days} = 0.835$). (H) Previous winners delivered the same numbers of lunges as naïve flies in the 1- and 2-d test fights (Mann–Whitney test, $P_{1day} = 0.234$; $P_{2days} = 0.728$).

fights, making comparisons between these and wild-type flies difficult. This is shown by a variety of measures used in our studies. For example, 73% of Canton-Special (CS, the wild type used in this study) 20-min fights ended in the establishment of dominance relationships, whereas only 36% (rut^{2080}), 29% (amn^1), and 52% (cer^P) of the mutant fly fights did so (Table 1, A). In addition, flies with mutations in amn and cer showed an increased latency to lunge (Table 1, B), and mutations in all three genes increased the latency to establish dominance (Table 1, C). Because an increased latency to lunge might artificially reduce the total numbers of lunges observed during a specified period, we calculated an aggression vigor index [AVI (12)] representing the total numbers of lunges 10 min after the first lunge was delivered. The AVI also was significantly decreased for all three mutant lines (Table 1, D).

With reduced level of aggressiveness, mutants might be more likely to lose contests against CS. To explore this, we paired mutant and CS flies in fights and observed that rut^{2080} and amn^1 mutant flies were more likely to lose fights against CS (Fig. S6A). This is in accord with the significantly reduced numbers of lunges they displayed compared with CS (Fig. S6B). However, cer^P mutant flies did not show fighting disadvantage against CS, even though they showed reduced numbers of lunges (Fig. S6).

Together, the results suggest that the general aggressiveness and ability to form stable dominance relationships are altered in rut^{2080}, amn^1, and cer^P mutant flies, making it difficult to examine the role of these memory-related genes in the formation of short- or long-term loser or winner effects.

Discussion

In the present study, we explored the behavioral consequences of losing and winning fights and asked how long these effects persisted. We showed that the outcome of male fruit flies' fights influenced the aggressiveness of flies in subsequent contests and led to the formation of loser and winner effects. However, the duration of those effects depended on the numbers of prior fights. After a single fight, both winning and losing flies demonstrated short-term effects lasting minutes to hours. After a sequence of closely spaced losses, the persistence of the losing memory could extend to a day. However, no long-term consequences were observed after five repeated victories. We showed further that (i) the familiarity of the opponents influenced the duration of short-term loser effects; (ii) that de novo protein synthesis was required only for the observed long-term loser effects; and (iii) that mutations in rut^{2080}, amn^1, and cer^P decreased the general aggressiveness of flies during first fights.

Aggression, as displayed in dyadic fighting situations, is a highly complex social behavior in which the paired flies give and take cues to and from each other as they interact. Fights generally start out at lower intensity levels with limited contact between the protagonists and then transition to higher levels with more direct contact. The transitions usually are made by one fly, based on unknown decision-making mechanisms, and usually are mirrored by the opponent, at least before the fight outcomes have been decided. Quantitative analyses of the behavior have helped to explain how some decisions of these types are made. For example, the first fly to lunge is 16 times more likely to become the ultimate winner, if the opponent receiving the lunge
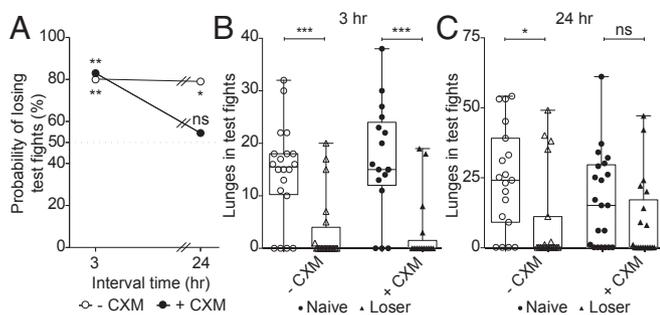
Fig. 4. The 24-h loser effect requires de novo protein synthesis. Flies were fed with control vehicle solution (−CXM) or with cycloheximide added to the solution (+CXM) before being trained in a spaced conditioning protocol and tested 3 and 24 h later. (*A*) Treatment without CXM (○) led to significant loser effects at 3 and 24 h (two-tailed χ^2 test, −CXM: $P_{3hr} = 0.0073$, $n = 20$; $P_{24hr} = 0.012$, $n = 19$). In +CXM-fed flies (●), however, the significant loser effect observed at 3 h was disrupted at 24 h (two-tailed χ^2 test, +CXM: $P_{3hr} = 0.0076$, $n = 17$; $P_{24h} = 0.513$, $n = 21$). (*B*) Losers of first bouts that were pretreated with or without CXM displayed significantly fewer lunges than naïve flies after 3 h of rest between fights (Mann–Whitney test, $P_{-CXM} = 0.0003$; $P_{+CXM} = 0.0002$). (*C*) After 24 h, the flies pretreated with the control solution still showed reduced numbers of lunges in test fights, whereas flies fed with CXM showed no reductions in numbers of lunges (Mann–Whitney test, $P_{-CXM} = 0.01$; $P_{+CXM} = 0.051$).

retreats (9). Whether flies decide to fight in the first place on meeting a conspecific is strongly influenced by cuticular hydrocarbon profiles and on how opponents "behave" (33). In studies presented here, we show that the familiarity of an opponent also can be a contributing factor to how long short-term loser effects are displayed by a former loser. Part of the extended duration of the loser effect resides in the prior experience of the winners of previous fights. Because winning flies also are more likely to win next fights against all opponents for short periods of time, their fighting strategies are likely to be different and more effective during that time period in next fights. However, individual recognition also is likely involved because the loser mentality persists for longer periods of time when former losers are paired with familiar, rather than unfamiliar, previous winners. Further work, therefore, will be required to address the issue of recognition of status between flies during aggressive interactions.

Drosophila male flies alter their fighting strategies during fights as they progress, suggesting that an operant learning paradigm may be involved (9, 10). For example, although both flies are capable of lunging at opponents at the start of a fight, by the end of the fight only winners lunge. In between these two time points a decision has been made and a hierarchical relationship has been established. Learning and memory have been shown to take place during many *Drosophila* classical and social conditioning situations

(14–16). Early and continuing studies with fruit flies have contributed fundamental information toward the construction of a large knowledge base about these essential processes. In studies presented previously (12) and here, we have shown that STM of the outcomes of fighting behavior exists, with flies retaining memory for up to several hours after they have won or lost single fights. A dramatic illustration of the strength of this short-term effect is observed in competition between males of a hyperaggressive "bully" strain of flies (11). These flies essentially always win bouts when paired with CS males, but once defeated by a second bully losers lose all competitive advantage against all other flies.

The short-term winner effects offer the obvious advantage to winning flies of sole access to desired resources for a period, but defeated flies, at least in the wild, still can leave a territory to search for new resources. The advantages of long-term loser effects are more difficult to understand. Among social species, including insects, such effects can help to stabilize social structures (6). Among solitary species, however, a longer-lasting inability to compete successfully seems to offer mainly disadvantages, other than on exerting caution before entering future contests. Nonetheless, these effects can be generated, at least in the losing flies, by short sequences of repeated losses in fights. In other LTM paradigms, such as olfactory classical conditioning or courtship conditioning in *Drosophila*, these effects can be extended for a week or longer (13, 30). However, in the present experiments, no behavioral consequences of losing in fights for longer than a day were observed, even with the conditioning trials being repeated over three consecutive days.

Using an inhibitor of protein synthesis, we presented evidence that the long-term loser effects require de novo protein synthesis. This is consistent with previous studies on learning and memory in *Drosophila* demonstrating that single training sessions usually induce memory that fades within hours whereas repeated training sessions lead to long-lasting memory persisting at least a day and depending on de novo protein synthesis (13, 30). The long-term persistence of loser effects offers the opportunity to investigate whether other physiological consequences (sleep, courtship, feeding, etc.) result from repeated defeats. This has been successfully done with social defeat models in rodents, allowing these to be used as models for depression and anxiety (34).

Monoamines such as serotonin and dopamine have been shown to play important regulatory roles in aggressive behavior and in learning and memory in most other species. These amines, along with octopamine, also have been shown to play important roles in aggression in fruit flies as well (23, 26, 35). However, any roles they serve in modulating the formation and persistence of loser and winner effects remains to be elucidated, as does the linkage between aggression and learning and memory in this complex social interaction.

Table 1. rut^{2080}, amn^1, and cer^P memory mutants show low levels of aggressive behavior

Genotype	A. Dominance relationship established, %	B. Latency to lunge, s	C. Latency to dominance, s	D. AVI
CS	73	84.9 ± 18.3	233.2 ± 35.9	25.6 ± 3.8
rut^{2080}	36	204.6 ± 60.2	597.9 ± 72.1	7.4 ± 1.7
amn^1	29	214.8 ± 49.9	597.1 ± 102.1	11.2 ± 2.9
cer^P	52	220.1 ± 49.3	521.7 ± 78.7	16.2 ± 3.4

(A) In assays with same genotype, 73% of the CS fights ended up in the formation of dominance relationships. With the memory mutant flies, dominance relationships were established in only 36% for rut^{2080}, 29% for amn^1, and 52% for cer^P. The fight outcomes were statistically different from CS to mutant flies (two-tailed χ^2 test, $P_{CS-rut}^{2080} < 0.0001$; $n = 22$; $P_{CS-amn}^1 < 0.0001$, $n = 23$; $P_{CS-cer}^P < 0.0001$, $n = 29$). (B) The latencies to lunge by amn^1 and cer^P flies were statistically increased compared with CS, but not for rut^{2080} (Mann–Whitney test, $P_{CS-rut}^{2080} = 0.063$, $n > 18$; $P_{CS-amn}^1 = 0.047$, $n > 19$ $P_{CS-cer}^P = 0.012$, $n > 23$). (C) The latencies to establish dominance relationships were significantly increased for the three mutant lines compared with CS (nonparametric Mann–Whitney, $P_{CS-rut}^{2080} = 0.0002$, $n > 8$; $P_{CS-amn}^1 = 0.0002$, $n > 7$; $P_{CS-cer}^P = 0.0003$, $n > 14$). (D) The AVI (numbers of lunges after the first lunge) was significantly lower in the three mutant lines compared with CS (Mann–Whitney test, $P_{CS-rut}^{2080} = 0.0001$, $n > 22$; $P_{CS-amn}^1 = 0.004$, $n > 23$; $P_{CS-cer}^P = 0.027$, $n = 29$).

In summary, by demonstrating and quantifying the short- and long-term consequences of losing and winning in fights, the present studies attempt to lay a foundation on which future investigations can be built aimed at unraveling the circuitry and molecular mechanisms involved.

Experimental Procedures

Fly Stocks. The *D. melanogaster* CS strain was used as the wild-type for this study. Homozygous *rut²⁰⁸⁰*, *amn¹*, and *cerᴾ* mutants, in CS genetic background, were used for aggression assays. Flies were raised on standard food medium at 25 °C and 50% relative humidity under a 12-h:12-h light: dark cycle.

Aggression Assays. The experimental procedure was recently described (12, 29). Briefly, male flies were reared in social isolation from the time of eclosion. Two days before the assays, a dot of acrylic paint was applied on the dorsal thorax for identification purposes. Aggression assays were performed with pairs of age- and size-matched 7-d-old males at 25 °C.

In the short-term loser and winner effect experiments, CS males were paired and allowed to interact for 20 min. After varying periods of time, prior losers and winners were paired for 20-min test fights against different opponents.

For small-group experiments, 60 min after first fights single naïve males were introduced into arenas containing a prior winner and loser for a 20-min test fight.

In the spaced conditioning, five fighting trials of 20 min separated by 10 min of rest were performed with the same males. Flies that won or lost all five bouts were placed into new isolation vials and paired with naïve flies 1 or 2 d later, for test fights.

CXM (10 g/L) in vehicle solution (mineral water with 50 g/L sucrose) was used as an inhibitor of protein synthesis as previously described (32). Flies were fed with or without CXM for 18 h before being transferred to new isolation vials 30 min before spaced conditioning. Flies that lost all five trials

were paired with naïve flies 3 or 24 h later, in test fights. Naïve flies were treated under the same experimental condition as the losing flies.

In extensive conditioning trials, three 20-min fights separated by 10 min of rest were performed over three consecutive days with hyperaggressive male flies (11). Flies that lost all nine bouts were kept in social isolation and paired with naïve flies a week later in test fights.

With the mutant lines, experiments were conducted with same genotype pairings, or mutants were paired against CS for 20 min.

Further detailed descriptions of the aggression assays are provided in *SI Experimental Procedures*.

Behavioral Analysis. For all fights, behavioral parameters were scored manually. Only aggressive patterns displayed on the food cup were included in the analyses. Losers are defined as the fly retreating three times from the food cup after receiving lunges. Fights in which fewer than five lunges were observed were usually removed or sex scored as "no fighting" in Fig. 4. Fights in which flies displayed more than five lunges but did not result in three retreats by either fly after receiving lunges were considered as "draws." Loser and winner effects represent the percentages of previous losing or winning flies that lost or win their test fights. More detailed descriptions of the behavioral parameters analyzed are provided in *SI Experimental Procedures*.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism 6. All data were subjected to a Grubb's test (alpha = 0.05) to determine whether extreme values were outliers. Because most of the dataset did not pass a Shapiro–Wilk normality test, nonparametric statistical tests were used for data analysis. Further details of the statistical tests used are provided in *SI Experimental Procedures*.

1. Hsu Y, Earley RL, Wolf LL (2006) Modulation of aggressive behaviour by fighting experience: Mechanisms and contest outcomes. *Biol Rev Camb Philos Soc* 81(1):33–74.
2. Oliveira RF, Silva JF, Simões JM (2011) Fighting zebrafish: Characterization of aggressive behavior and winner-loser effects. *Zebrafish* 8(2):73–81.
3. Drummond H, Canales C (1998) Dominance between booby nestlings involves winner and loser effects. *Anim Behav* 55(6):1669–1676.
4. Kasumovic MM, Elias DO, Sivalinghem S, Mason AC, Andrade MC (2010) Examination of prior contest experience and the retention of winner and loser effects. *Behav Ecol* 21(2):404–409.
5. Rutte C, Taborsky M, Brinkhof MW (2006) What sets the odds of winning and losing? *Trends Ecol Evol* 21(1):16–21.
6. Dugatkin LA, Druen M (2004) The social implications of winner and loser effects. *Proc Biol Sci* 271(Suppl 6):S488–S489.
7. Simon AF, et al. (2012) A simple assay to study social behavior in Drosophila: Measurement of social space within a group. *Genes Brain Behav* 11(2):243–252.
8. Pavlou HJ, Goodwin SF (2013) Courtship behavior in Drosophila melanogaster: Towards a 'courtship connectome'. *Curr Opin Neurobiol* 23(1):76–83.
9. Chen S, Lee AY, Bowens NM, Huber R, Kravitz EA (2002) Fighting fruit flies: A model system for the study of aggression. *Proc Natl Acad Sci USA* 99(8):5664–5668.
10. Yurkovic A, Wang O, Basu AC, Kravitz EA (2006) Learning and memory associated with aggression in Drosophila melanogaster. *Proc Natl Acad Sci USA* 103(46):17519–17524.
11. Penn JK, Zito MF, Kravitz EA (2010) A single social defeat reduces aggression in a highly aggressive strain of Drosophila. *Proc Natl Acad Sci USA* 107(28):12682–12686.
12. Trannoy S, Chowdhury B, Kravitz EA (2015) Handling alters aggression and "loser" effect formation in Drosophila melanogaster. *Learn Mem* 22(2):64–68.
13. Tully T, Preat T, Boynton SC, Del Vecchio M (1994) Genetic dissection of consolidated memory in Drosophila. *Cell* 79(1):35–47.
14. Kahsai L, Zars T (2011) Learning and memory in Drosophila: Behavior, genetics, and neural systems. *Int Rev Neurobiol* 99:139–167.
15. Guven-Ozkan T, Davis RL (2014) Functional neuroanatomy of Drosophila olfactory memory formation. *Learn Mem* 21(10):519–526.
16. Griffith LC, Ejima A (2009) Courtship learning in Drosophila melanogaster: Diverse plasticity of a reproductive behavior. *Learn Mem* 16(12):743–750.
17. Livingstone MS, Sziber PP, Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. *Cell* 37(1):205–215.
18. Keene AC, Krashes MJ, Leung B, Bernard JA, Waddell S (2006) Drosophila dorsal paired medial neurons provide a general mechanism for memory consolidation. *Curr Biol* 16(15):1524–1530.

19. Comas D, Petit F, Preat T (2004) Drosophila long-term memory formation involves regulation of cathepsin activity. *Nature* 430(6998):460–463.
20. Sturtevant AH (1915) Experiments on sex recognition and the problem of sexual selection in Drosophila. *J Anim Behav* 5:351–366.
21. Vrontou E, Nilsen SP, Demir E, Kravitz EA, Dickson BJ (2006) fruitless regulates aggression and dominance in Drosophila. *Nat Neurosci* 9(12):1469–1471.
22. Kravitz EA, Fernandez MdeL (2015) Aggression in Drosophila. *Behav Neurosci* 129(5):549–563.
23. Andrews JC, et al. (2014) Octopamine neuromodulation regulates Gr32a-linked aggression and courtship pathways in Drosophila males. *PLoS Genet* 10(5):e1004356.
24. Asahina K, et al. (2014) Tachykinin-expressing neurons control male-specific aggressive arousal in Drosophila. *Cell* 156(1-2):221–235.
25. Kayser MS, Mainwaring B, Yue Z, Sehgal A (2015) Sleep deprivation suppresses aggression in Drosophila. *eLife* 4:e07643.
26. Alekseyenko OV, et al. (2014) Single serotonergic neurons that modulate aggression in Drosophila. *Curr Biol* 24(22):2700–2707.
27. Benelli G, et al. (2015) Contest experience enhances aggressive behaviour in a fly: When losers learn to win. *Sci Rep* 5:9347.
28. Trannoy S, Kravitz EA (2015) Learning and memory during aggression in Drosophila: Handling affects aggression and the formation of a "loser" effect. *J Nat Sci* 1(3):e56.
29. Trannoy S, Chowdhury B, Kravitz EA (2015) A new approach that eliminates handling for studying aggression and the "loser" effect in Drosophila melanogaster. *J Vis Exp* Dec 30(106):e53395.
30. McBride SM, et al. (1999) Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. *Neuron* 24(4):967–977.
31. Krashes MJ, Waddell S (2008) Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in Drosophila. *J Neurosci* 28(12):3103–3113.
32. Plaçais PY, et al. (2012) Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in Drosophila. *Nat Neurosci* 15(4):592–599.
33. Fernández MP, et al. (2010) Pheromonal and behavioral cues trigger male-to-female aggression in Drosophila. *PLoS Biol* 8(11):e1000541.
34. Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B (1997) The temporal dynamics of the stress response. *Neurosci Biobehav Rev* 21(6):775–782.
35. Alekseyenko OV, Chan YB, Li R, Kravitz EA (2013) Single dopaminergic neurons that modulate aggression in Drosophila. *Proc Natl Acad Sci USA* 110(15):6151–6156.