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Title: Learning and memory consolidation in aggression behavior

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ABSTRACT

Aggressive behavior is important for animals for survival and reproduction as it helps in getting access to necessities like food and mates. Therefore aggressive behavior can be considered as an indicative of fitness of an organism. Such behavior can be effectively studied in *Drosophila melanogaster*, where flies put into a chamber demonstrate specific fighting strategies against opponents and forms clear dominance relationships. Moreover flies tend to learn and remember these fighting strategies over some time. Thus this paradigm offers an excellent opportunity to study short or mid-term memory in *Drosophila*. During tests we put two flies into a chamber for first fight, removed and returned to their respective vials and then reintroduce them against different opponent for their second fight. In accordance with previous results Canton-S winner and loser flies tend to progressively learn lunging and retreating respectively (3). Moreover loser flies tend to lose their subsequent fights while winner flies fought with equal probability of winning/losing (3). This suggests a role of loser mentality in these outcomes. We then did these experiments on memory mutants of *amnesiac* and *rutabaga* gene. Winner and loser *amnesiac* mutant flies progressively learnt lunging and retreating during a fight but failed to emulate these strategies in their subsequent fights. In contrast *rutabaga* mutant flies failed to learn any fighting strategies during trials which then resulted in lack of dominance relationships. Our results for the first time demonstrate prominent role for *rutabaga* gene in short term learning and *amnesiac* gene in consolidation of memories formed in context of agonistic interactions between flies.

Key words: Dominance, fighting, fruit flies, loser mentality, memory mutants

Introduction

Aggressive behavior is important for animals for survival and reproduction as it helps in getting access to necessities like food and mates (1, 2). It is also used in self-defense and protection of territory. Thus aggression can be considered as an indicative of fitness of an organism. More aggressive individual has greater probability of accessing various resources and thus is likely to have better fitness. Studying such behavior in organisms have always been challenging due to influence of various biotic and abiotic factors (3). Aggression had been demonstrated in many organisms but quantification of such behavior remained elusive. Previous studies had led to development of some robust assays in organisms, like crayfish and lobsters, where agonistic meetings are induced between organisms for studies (4). Aggression in *Drosophila melanogaster* is elaborately documented and with loads of available genetic tools it represents an ideal organism for studying such complex behavior (5).

D. melanogaster shows territoriality and aggression, which is ethologically well characterized (6). In behavioral assay a pair of socially naïve males demonstrates fighting and decision making behavior during brief spells of physical encounters against each other (5, 7). Based on such encounters some behavioral strategies have been characterized which pertains to level of aggression shown (5). Flies use a combination of these offensive/defensive strategies in every encounter which in due course leads to formation of a dominance relationship, where one fly can be designated as 'winner' and other as 'loser' (5). Formation of such dominance relationships leads to modification in behavioral strategies used by flies. Winner and loser flies increasingly employ high and low aggressive strategies respectively (3). Such observation points to the possibility of involvement of learning and memory in formation of such relationships (3).

Learning and memory in *D. melanogaster* have been widely studied through application of both operant and classical conditioning paradigms (8). These investigations had led to formulation of four distinct types of temporally divided memories (3): short term memory, midterm memory, anesthesia resistance memory and long term memory (8-10). Short term memory lasts for about an hour and mid-term memory for atleast four hours (8-10). Anesthesia resistant memory decays over four days while long term memory is retained for atleast one week (8-10). Unlike classical and operant mode, learning in social environment is influenced by many factors and thus its consolidation possibly employs multiple level controls and various neurogenetic circuits (3). Prior social experience significantly modifies behavior in flies with involvement of both short and long term memory components (11). One such example of behavior modulation is loser effect i.e. increased likelihood for a defeated animal to loss its subsequent fight, had been shown in *D. melanogaster* (3). A loser fly after experiencing a loss never win against a familiar or unfamiliar opponent (3). Moreover such experience also modifies its behavioral strategies in subsequent fights (3, 5).

Behavioral modulations associated with social defeats are well documented in *D. melanogaster* but genetic components and pertinent pathways which are involved in such developments are poorly understood. Previous studies had shown involvement of cAMP pathway in formation of operant and classical conditioned memories (8, 12). In line with it effect of various gene mutations in cAMP pathway on memory formation had been reported (8). In present study role of two such mutants of *amnesiac (amn)* and *rutabaga (rut)* genes in loser mentality is reported. The predicted gene product of *amnesiac,* AMN, is a prepronεuropeptide which had been shown to stimulate cAMP synthesis while *rutabaga* mutant flies are deficient in activity of adenylyl cyclase which catalyzes the conversion of ATP into cAMP (13-17). Studies had shown involvement of *rutabaga* and *amnesiac* genes in formation of short-term and mid-term memories respectively (8). Our results demonstrate for the first time involvement of both short/mid-term memories in formation of dominance relationships in *D. melanogaster*. Results show prominent roles for *rutabaga* gene in short term learning and *amnesiac* gene in consolidation of memories (involving mid-term memory) formed in context of agonistic interactions between flies.

Results

Stable dominance relationships are formed in CS flies

Dominant status was assigned to flies based on various fighting strategies employed in each experiment. A pair of male flies was introduced into a chamber for a period of 60 min in a trial, following which they were returned to their respective vials. Trials which demonstrated some aggressive interactions between flies were considered as valid. For CS flies, in total 93 trials were conducted out of which 52 resulted in aggressive interactions. Every fight was analyzed for a period of 30 min from the time both flies first occupy the territory. Among all recorded fighting strategies we selected lunges and retreats for assignment of dominance due to their overall higher occurrence. In a trial, a fly was assigned a status of 'winner' on using three continuous lunges (without using retreat in between) and 'loser' on using three continuous retreats (3). Fights which don't satisfy above criterion were considered as 'draw'. In CS flies 85% of valid trials produced dominance relationships while 15% ended up in a draw (Fig 1A).

Other results depicting stable dominance relationship in flies pertains to duration of encounters in a fight. An encounter depicts aggressive interaction between flies which last for at least 3 sec. Two encounters are separated by at least 2 sec, and duration of an encounter is a measure of time for which flies engage in aggressive interactions. In total 30 trials were analyzed for wild-type flies with average of 25 encounters in each fight. In CS flies duration of encounters significantly went down with successive encounters (Linear regression analysis, Y=12.77-0.382X, R^2= 0.906, ANOVA, P<0.001; n_{cs}=30) (Fig 1B). Thus tendency of engaging in continuous fights had been compromised in flies due to formation of dominance relationships.

Winners and losers progressively learn lunging and retreating respectively

Dominance shown by winners prompted us to ask whether such behavior was a result of learning in flies. We already looked at lunges and retreats as favored fighting strategies for winners and losers respectively. Previous studies had shown that over course of encounters, winner and loser flies progressively learn lunging and retreating behavior respectively (3). We began our studies with reiteration of these previously conducted experiments on CS flies ability to learn lunging and retreating and no major anomalies were observed compared to published results (3). Winner flies increasingly employ lunging behavior which renders them dominant social status in a fight (Linear regression analysis, Y=0.82+0.052X, R^2= 0.71, ANOVA, P<0.001; n_{cs}=49) (Fig 1C). On the contrary loser flies increasingly employ retreating behavior which renders them repressive social status in a fight (Linear regression analysis, Y=0.52+0.034X, R^2= 0.716, ANOVA, P<0.001; n_{cs}=49) (Fig 1D). Such progressively high usage of specific fighting strategies points toward involvement of learning in formation of dominance relationships.

Flies demonstrate robust loser mentality

Above results impelled us to test whether these dominance relationships were carried forward in second fights. Previous studies had demonstrated loser mentality in fruit flies (3). To further examine that, we kept experienced flies back under isolation for 60 min. After this rest phase these flies were re-introduced against an age and size matched naïve opponent in second fight for 60 min. Fights were analyzed for a period of 30 min and winner and losers were assigned using 3-lunge/3-retreat rule. Draws in second fight falls under three categories: 'High intensity', 'low intensity' and 'no intensity'. 'High intensity' fights comprises of experienced flies employing lunging while naïve flies retaliating (lunging back after receiving a lung) or not retreating. On the contrary in 'low intensity' fights experienced flies employ retreats but no dominance is shown by naïve flies. In 'no intensity' fights flies don't engage in dominant aggressive interactions. For both winners and losers from first fight at least 20 fights were analyzed and dominant statuses were assigned. For statistical analysis, high and low intensity draws were grouped with wins and losses respectively. Fights ending up in no intensity draws were discarded. We found that CS loser flies in their second fight always lost to a naïve opponent. In 21 analyzed second fights (6 were discarded due to no encounters), 73% were losses and 27% fells under low intensity draws (two tailed $\chi^2 = 15.00$, df= 1, $P< 0.001$; n_{cs}=15 trials) (Fig 1E). In contrast to it CS winner flies didn't show much dominance in second fight. In 20 analyzed second fights (8 were discarded due to no encounters), 33% were wins, 42% were losses and 25% fells under low intensity draws (two tailed $\chi^2 = 1.33$, df= 1, $P> 0.05$; n_{cs}=12 trials) (Fig 1E). These results recapitulate previous demonstrations of a robust loser mentality in flies. Loser flies were more susceptible to losing while winner flies won or lost at comparable frequencies in their second fights.

***rutabaga* mutants were unable to form dominance relationships**

As shown before CS flies were capable of forming stable dominance relationships in a fight. Similar trials were conducted in memory mutants to further corroborate our assertions pertaining to role of learning in forming dominant statuses. In *amn* mutant flies 80 trials were conducted out of which 51 resulted in aggressive interaction while in *rut* mutant flies 35 produced aggressive interactions out of 55 conducted trials. Similar to CS flies, 82% of valid trials in *amn* mutants resulted in formation of dominance relationships. Intriguingly despite comparable number of encounters no *rut* mutant trials produced dominance relationships (two tailed $\chi^2 = 35.00$, df= 1, $P< 0.001$) (Fig 2A). We then analyzed trends pertaining to duration of encounters in memory mutants. *amn* mutant flies showed significant decrease in duration of encounters as shown in wild-type flies (one-factor repeated measures ANOVA, main effect of encounter $P< 0.001$, interaction $P> 0.05$; n_{cs}, n_{amn}= 30 trials) (Fig 2B). On the contrary in *rut* mutant flies no significant decrease was observed which can be attributed to lack of dominance in them(one factor repeated measures ANOVA, main effect of encounter $P< 0.001$, interaction $P< 0.01$; n_{cs}, n_{rut}= 30 trials) (Fig 2C).

rutabaga mutants were unable to learn lunging and retreating

Above results prompted us to test whether such lack of dominance shown by *rut* mutant flies were due to their defective learning abilities. These tests comprised of analysis pertaining to trends in usage of lunging and retreating with successive encounters. More than 30 trials were analyzed in each category with average of 20 encounters in each fight. Number of lunges and retreats employed by a fly in every encounter was reported and analyzed in detail. We first tested lunging trends in winner flies. In *amn* mutants, flies learnt progressive lunging with number of encounters and shown trend was quite similar to CS flies (one factor repeated measures ANOVA, main effect of encounter $P< 0.001$, interaction $P>0.05$; $n_{cs}= 49$, $n_{amn}=41$ trials) (Fig 3A). In other set of analysis we tested ability of loser flies to learn retreating in a fight. Again *amn* mutant flies showed progressive learning of retreats with number of encounters which was similar to CS flies (one factor repeated measures ANOVA, main effect of encounter $P< 0.001$, interaction $P> 0.05$; $n_{cs}=49$, $n_{amn}=41$ trials) (Fig 3B). These results suggest that *amn* mutant flies learn perfectly well during trials.

In contrast *rut* mutant flies were unable to show any progressive trends in their lunging or retreating abilities. In 35 analyzed trials, *rut* mutants showed lunging in only 8 fights. Furthermore even such lunging was significantly different from CS flies analyzed over every encounter (Since data obtained from *rut* mutant flies was not normally distributed, for every encounter we performed nonparametric Kruskal-Wallis ANOVA, **, $P<0.01$, *, $P<0.05$; $n_{cs}=49$, $n_{rut}=8$ trials) (Fig 3C). In contrast to their lunging behavior, *rut* mutant flies showed considerably higher levels of retreats with all valid trials giving positive results. In spite of higher level of retreats these flies were unable to progressively learn retreating as it was significantly different from trends shown by CS flies (one factor repeated measures ANOVA, main effect of encounter $P< 0.01$, interaction $P< 0.001$, $n_{cs}=49$, $n_{rut}=46$ trials) (Fig 3D). Above results suggests that *rut* mutant flies are suffering from certain learning which is preventing them from emulating wild type trends.

Absence of loser mentality in *amnesiac* mutants

We had shown presence of a robust loser mentality in flies. Due to lack of dominance relationships in *rut* mutant flies in their first fights, no trials pertaining to investigation of loser mentality in them are reported here (For data pertaining *rut* mutants in second fight see supplementary figures). In contrast *amn* mutant flies had demonstrated formation of stable dominance in their first fights. Intriguingly *amn* mutant flies were unable to demonstrate any loser mentality. In 21 analyzed second fights (7 were discarded due to no encounters), 36% were wins and 43% were losses. In addition to it there were 7% low intensity and 14% high intensity draws, which differ significantly from strong loser mentality shown by CS flies (Fisher's exact test, $P< 0.01$, $n_{cs}=15$, $n_{amn}=14$ trials) (Fig 4A). These results suggest some memory defects in *amn* mutants due to which they were unable to emulate their learning from first fights.

We then tested ability of loser flies in retaining retreating behavior in second fights. For both strains, CS and *amn* mutants, we analyzed 20 trials and reported number of retreats used by each loser fly in first and second fights. We found that CS loser flies retreats significantly less in their second fights. In contrast to it usage of retreats in *amn* mutant losers were significantly different from wild type trends (two factor ANOVA, main effect of genotype $P<0.05$, main effect of fight $P<0.05$, interaction $P<0.05$, $n_{cs}=20$, $n_{amn}=20$ trials) (Fig 4B). According to loser mentality flies become more prone to a loss in their second fights. Therefore loser flies gives up in lesser number of encounters compared to their first fights and thus uses less number of retreats. In addition to it, CS flies showed no progressive learning of retreats while *amn* mutants showed some learning of retreating behavior in their second fights (Fig S1).Thus loser mentality is rendering CS flies to retreat swiftly while *amn* mutant flies were again learning to retreat in their second fights. *rut* mutant flies showed no significant changes in usage of retreats in their second fights (Fig S2A).

We then examined trends in ability of winner flies' in usage of lunging behavior in second fights. For both strains we analyzed 23 trials, reporting number of lunges used by each winner fly in first and second fights. Our results showed significant decrease in the number of lunges used by CS winner flies in second fight. Also similar decrease was observed in *amn* mutant flies (two factor ANOVA, main effect of genotype $P>0.05$, main effect of fight $P<0.001$, interaction $P>0.05$, $n_{cs}=23$, $n_{amn}=23$ trials) (Fig 4C). This decrease in lunging behavior is due to presence of losses in second fight for winners. Winner flies don't use lunging behavior during loss in second fights which renders an overall decrease in usage of lunging. In both strains winner flies that win their second fights show comparable number of lunges as in their first fights (Fig S3A). Furthermore CS and *amn* mutant flies showed some learning of lunging behavior in their second fights (Fig S3B and S3C). Thus experience of winning is not rendering both CS and *amn* mutant flies any modifications in usage of lunging behavior in second fights.

Above results suggests that due to loser mentality flies are becoming less prone to engage in aggressive interactions with naïve opponents. Thus loser flies demonstrate subdued aggression which results in swift losses in second fights. To examine it further, we tested number of attacks in which flies were engaged in first and second fights. We analyzed 17 and 24 trials in CS and *amn* mutant losers respectively, reporting number of attacks that each loser fly was engaged in first and second fight. We found that in CS loser flies number of attacks were significantly declined in second fight but decrease observed in *amn* mutant flies were significantly less compared to wild type trends (two factor ANOVA, main effect of genotype $P<0.01$, main effect of fight $P<0.001$, interaction $P<0.05$, $n_{cs}=17$, $n_{amn}=24$ trials) (Fig 4D). Decrease in number of attacks observed in *rut* mutant flies was significantly different from that observed in CS flies, due to no learning demonstrated in former's first fight (Fig S2B).

Further building upon our assertion of subdued aggression in loser flies we tested ability of such flies to retaliate (lunging after receiving lunge) in second fights. For both strains we analyzed 21 trials and reported number of trials in which flies engage in retaliatory behavior in first and second fights. We found that in 24% of first fights CS losers showed retaliatory behavior which was then turned to lack of retaliation in second fights (two tailed $\chi^2 = 6.56$, df= 1, $P < 0.01$, n=21 trials) (Fig 4E). Compare to CS losers, *amn* mutant losers showed retaliation in 29% of their second fights (Fisher exact test, $P < 0.01$, n=21 trials) (Fig 4E). These results further consolidate significance of loser mentality and its effect in subduing aggression in wild type flies. They also point towards defective memory associated with *amn* mutant flies with respect to loser mentality.

Discussion

Data presented here demonstrate that learning and memory plays a prominent role in formation of dominance relationships during aggressive interaction between flies. Moreover memory also plays a vital role in sustenance of such dominance relationships. All trials were conducted on male flies. Previous studies had shown low aggressive interactions between females which ends up in lack of dominance relationships (18). Experiments conducted on mutants, *amnesiac* and *rutabaga* mutants, further revealed genetic controls accompanying such learning and memory.

Aggression is observed in plethora of organisms ranging from insects to humans. This complex behavior is influenced by genetic, hormonal and environmental factors (19). Fruit flies present an excellent model system for studying aggression due to their tendency to engage in continuous fights (3, 5). Flies do not possess any dangerous weapons and thus are unable to cause fatal harm to its opponents (3). Therefore loser fly keep on reengaging winners in a fight but such tendencies suffer a gradual decrease due to formation of dominance relationships. Also with formation of dominance relationships, winners progressively learn more lunging while losers learn more retreating. All above trends were vividly demonstrated by CS and *amn* mutant flies. In contrast to it *rut* mutant flies were unable to demonstrate these trends. Moreover no dominance relationships were reported in them. Flies learn dominant or repressive fighting strategies and then employ them in successive encounters. *rutabaga* mutant flies had been shown to lack the ability to form short-term memories (15, 16). Such deficiency in short term memory formation renders inability to learn various fighting strategies with successive encounters which then results in lack of dominance (Fig 5). Therefore learning and memory positively affect dominance relationships. We also hypothesize active role for *rutabaga* gene in formation of dominance relationships.

Further experiments demonstrated ability of loser flies to remember their loss from first fights. Such loser mentality had been widely reported across animal kingdom and can be attributed to more cautious approach undertaken by losers in successive fights (20, 21). This behavior is also evolutionarily significant as it might facilitate long term survival in flies. In contrast to it winner flies don’t show much recollection from first fight i.e. no winner’s mentality. In all our second fights, flies fought against naïve opponents. Therefore winners winning streak is dependent on naïve flies aggressive behavior. More aggression from naïve flies will result in a loss and possible physical harm for winner flies. Therefore even though beneficial, winner’s mentality will render flies to become less cautious against strong opponents which may prove perilous. Thus our results demonstrated a robust loser effect in flies but absence of winner’s mentality. Similar studies with same or familiar opponents in second fight had also demonstrated robust loser mentality in flies (3). Trials with familiar opponents present a possibility of conspecific recognition and thus may contribute strongly towards shown loser mentality. In order to negate such effects all trials were conducted with unfamiliar opponents.

Loss in first fight renders flies to become less aggressive in their subsequent fights (22). Thus loser mentality can be attributed to subdued aggression which makes flies increasingly prone to losing. Our results revealed that *amn* mutant loser flies were unable to demonstrate loser effect. *amnesiac* mutants had been shown to lack the ability to form mid/long-term memories (8). These mutants learnt perfectly fine during their first fights but after a gap of 60 min were unable to exhibit previous experience in second fights. Thus deficiency in formation of mid/long-term memories resulted in lack of loser mentality in *amn* mutant flies (Fig 5). We also hypothesize active role for *amnesiac* gene in formation of loser mentality.

Both memory mutant genes affect cAMP synthesis through their control of adenylyl cyclase activity. *rutabaga* gene encodes a type I calcium/calmodulin dependent activated adenylate cyclase and thus exhibit strong effect on learning abilities of flies due to its direct control of cAMP synthesis (15-17). On the other hand *amnesiac* gene affect adenylyl cyclase activity through its association with G-protein coupled receptors (13, 14). Therefore it is present at higher level in cAMP pathway compared to *rutabaga* gene and has effects more pertinent to formation of memories than learning abilities.

All our experiments were conducted on normal population of flies. We considered these flies to have equal probabilities of winning/losing in first fights. Other cases where flies were inherently more/less aggressive were not applicable in our studies. This was due to presence of loser flies showing retaliatory behavior in first fights. With time losers learn to avoid winners through progressive usage of retreating behavior. Also loser flies won few of their second fights in *amn* mutants. Losers had also been shown to win against another loser fly when paired up in an experiment (3). Therefore outcomes in first fight were independent of genetic variability in flies.

We know that lunging and retreating are preferred fighting strategies of winners and losers respectively which render flies to form dominance relationships. In future we would like to see for how long such relationships can be maintained. Since both short and mid/long-term memories are required in forming such relationships, timeline can help us differentiate these forms temporally. Search for an “engram” in the brain has been a focus of neuroscience research for past 70 years (23). “Engram” represents hypothetical means through which memory traces are stored in response to external stimuli in the brain. With help of modern genetic tools, we would like to locate specific neuronal circuits which play prominent role in formation of dominance relationships. In these experiments we showed involvement of both *rut* and *amn* mutant flies in formation of dominance relationships and loser mentality respectively. In future we would like to perform spatio-temporally restricted rescue experiments in these mutants to further corroborate our hypothesis. In our quest to decipher genetic pathways involved in such memory formation, we would like to perform further experiments on other memory mutants involved in both short and mid/long-term memories. Further studies on this system can also facilitate our understanding pertaining to associations between aggression and learning and memory pathways.

Materials and Methods

Rearing of flies

Initial experiments were done on CS strain of *Drosophila melanogaster*. These were followed by experiments on *amnesiac (amn C651) and rutabaga (rut 2080)* mutants. Both these mutants are heterozygous in nature.

Flies were raised in standard bottles containing 3% fly food composed of Dry yeast (1.5%), Sucrose (8%), Agar (1%), Malt extract (3%), Corn flour (7.5%), Propionic acid (5ml), Orthophosphoric acid (1ml) and 5% p-methyl benzoate in 100% ethanol (5ml). These contents were mixed in 1000ml of water and heated in a microwave and then transferred into vials/bottles. Single larvae or1 day old fly from these bottles were transferred in standard vials containing 3% fly food. These flies were kept under social isolation (i.e. single fly per vial) for a period of 4-5 days in temperature and humidity controlled incubators (25^{o}C, 50% humidity). In order to distinguish these flies they were marked with acrylic paint. This was done by placing flies under microscope in effect of Co_2 and marking their upper thorax with white paint without harming their wings or head. Another set of wild type females (around 10 flies) were also collected. Female with chopped head was placed as an attractant for male flies in aggression assay. Yeast paste was also used as an attractant.

Inducing fights

All experiments were conducted in a 6-well plate system. It comprises of two food cups that were placed in a pair of adjacent wells which were then covered by a glass lid. A food cup (bottle cap) consists of 3% fly food with yeast paste and headless female placed on it, in order to attract male flies. A pair of flies was introduced into the chamber by gentle aspiration with the help of suction tube. A bright light was shone over this arrangement which facilitates recording and also acts as an attractant for flies towards the center of the chamber.

All fights were recorded using SONY DCR-SR47E/S handy cam. Camera was placed in front of the arrangement on a tripod and was then adjusted for focus and zooms while all other variables like brightness and exposures were kept constant. All recordings were done in MPEG format which was then analyzed separately. Continuous records of temperature and humidity had been maintained.

Learning and Memory

To investigate learning and memory, fights were induced between these flies in three phases. In the first phase pair of flies was introduced into the chamber (in a 6 well plate) for a period of 60 min. This phase is termed as a fight phase where flies were trained in fights against opponents. Depending on their aggression and various fighting strategies used (Supplementary table 1); individual fly was assigned a status of winner or loser. In other cases where flies were equally aggressive or show little aggression they were assigned a status of draw.

Following fight phase flies were placed back in their individual vials for another 60 min. This phase is termed as Rest phase. During this phase flies were again exposed to social isolation. Since these experiments were dealing with retention of short/mid-term memory in flies, this time phase was ideal as such type of memory lasts for only few hours.

Following rest phase flies (winner and loser) were put up against naïve opponents in adjacent wells in the plate. This ensures simultaneous recording of both, winner and loser, strategies against naïve opponents. This phase is termed as test phase and also lasts for 60 min. This phase facilitate analysis pertaining to memory retention of previous fight. In case of flies assigned a status of draw both are put against naïve opponents and are tested for any increase or decrease in aggression.

Data gathering and analyses

In all experiments fresh food were prepared and placed in the chamber along with two flies of same sex and age. Trials were videotaped immediately from introduction of flies. Videos were then analyzed from point where both flies first occupied food territory simultaneously. This point of time was marked as 0 min and fights were then analyzed upto 30 min. This is done due to the fact that both flies don't occupy food territory simultaneously from the beginning of trials and take some time to acclimatize in given conditions. Due to varying length of this lag phase (0-60 min) we analyzed only those fights where flies fought for atleast 30 min. This time period was also sufficient for formation of dominance relationships due to learning (3). These fights were analyzed on basis of encounters, which is defined as a period involving physical interaction (aggressive) between two flies. End of these encounters were marked by at least a 2 sec pause in aggressive interaction between flies. These encounters were recorded and analyzed for various fighting strategies and adaptations based on which flies were assigned a social status of winner/loser/draw. Such dominance statuses were assigned using 3-lunge/3-retreat rule (3). Lunge (LG) and retreat (RT) were most frequently used fighting strategies by winner and loser respectively (Fig S4). LG is an aggressive move where one fly rears up on hind legs and snaps down on the other fly. RT is a defensive move where flies run/fly away from opponent. According to 3 LG-RT rule, if one fly uses three continuous lunges (i.e. without using RT in between) and other fly uses three continuous retreats, then former fly is termed as winner and later as loser. This rule works in majority of cases because once dominance relationships are established they remain fairly stable and thus are maintained throughout that fight.

Recorded digital videos were analyzed using Sony PMB software on windows machine. Individual encounters were clipped from full length videos using windows movie maker in wmv format which were then scored for behavioral patterns.

Statistics methods

All data from wild-type male flies and heterozygous *amnesiac and rutabaga* mutants were analyzed. All significance levels of statistical tests were set to maximum of 0.05. Trends in dominance in wild-type flies were tested for significance using linear regression analysis. Learning ability of flies were compared between genotypes using one factor (genotype) repeated measures ANOVA. Ability of flies to form dominance relationships were compared between winners and losers using χ^2test where outcomes were compared with expected values of 50/50 (equal probability of wins or losses) in two tailed χ^2 analysis. This was further compared with mutants using wild type outcomes as expected in two tailed χ^2 analysis. Loser mentality in flies across fights and between genotypes were compared using two factor (genotype and fight) ANOVA which was then followed by pairwise comparisons using post hoc tests. In most cases data sets followed essential assumption of normal distribution. In other groups non-parametric tests, Kruskal-Wallis ANOVA, were performed. All data sets were analyzed using STATISTICA version 8.0. The sample size for each group is reported in figure legends.

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Figure legends

Fig 1: Learning in flies leads to formation of stable dominance relationships which is then carried in successive fights by only losers **(A)** First fight outcomes in wild type flies when paired against age and size matched flies. Dominance relationships were formed in fights with winner and losers which otherwise results in a draw. Dominant status was assigned using 3-lunge/3-retreat rule. CS: 85% fights produce dominance relationships and 15% ends up in a draw. **(B)** Duration of encounters progressively went down with successive number of encounters (Linear regression analysis, Y=12.77-0.382X, R^2= 0.906, ANOVA, P<0.001, mean$\pm$SEM, n_{cs}=30). **(C)** Over the course of encounters winner flies progressively learnt lunging behavior (Linear regression analysis, Y=0.82+0.052X, R^2= 0.71, ANOVA, P<0.001, mean$\pm$SEM, n_{cs}=49). **(D)** Over the course of encounters loser flies progressively learnt retreating behavior (Linear regression analysis, Y=0.52+0.034X, R^2= 0.716, ANOVA, P<0.001, mean$\pm$SEM, n_{cs}=49). **(E)** Outcome of second fights for losers and winners of CS strain. Loser flies: 73% losses, 27% low intensity draws (two tailed χ^2 = 15.00, df= 1, P< 0.001, n_{cs}=15 trials). Winner flies: 33% wins, 42% losses and 25% low intensity draws (two tailed χ^2 = 1.33, df= 1, P> 0.05, n_{cs}=12 trials). In analysis high and low intensity draws were grouped with wins and losses respectively. For statistical analysis, outcomes were compared with expected values of 50/50 (equal probability of wins or losses) in two tailed χ^2 analysis.

Fig 2: *rutabaga* mutant flies were unable form dominance relationships. **(A)** First fight outcomes of memory mutant flies when paired against age and size matched flies. *amn* mutant: 82% fights produce dominance relationships and 18% ends up in draw. *rut* mutant: No dominance relationships observed, ***, P< 0.001. For statistical analysis, outcomes were compared with expected value of 100% dominance relationships in a two tailed χ^2 test. **(B)** Over the course of number of encounters flies fought for lesser duration in *amn* mutants as shown in CS flies (one-factor repeated measures ANOVA, main effect of encounter P< 0.001, interaction P> 0.05, mean$\pm$SEM, n_{cs}, n_{amn}= 30 trials) **(C)** *rut* mutant flies showed no change in duration of encounter which was significantly different from CS flies (one factor repeated measures ANOVA, main effect of encounter P< 0.001, interaction P< 0.01, mean$\pm$SEM, n_{cs}, n_{rut}= 30 trials)

Fig 3: *rutabaga* mutant flies were unable to learn lunging and retreating **(A)** Winner flies in *amn* mutant strain progressively learnt to use more lunging over course of encounters as shown in CS flies (one factor repeated measures ANOVA, main effect of encounter P< 0.001, interaction P>0.05, mean$\pm$SEM; n_{cs}= 49, n_{amn}=41 trials) **(B)** Loser flies in *amn* mutant strain progressively learnt to use more retreats over course of encounters as shown in CS flies (one factor repeated measures ANOVA, main effect of encounter *P*< 0.001, interaction *P*>0.05, mean±SEM; n_{cs}= 49, n_{amn}=41 trials). **(C)** *rut* mutant flies lunged very less and it was significantly different from learning trends shown by CS flies. Due to non-normal nature of data obtained with *rut* mutant flies non parametric tests were performed which show differences in usage of lunging with every encounter (Kruskal-Wallis ANOVA, **, *P*<0.01, *, *P*<0.05; n_{cs}= 49, n_{rut}=8 trials). **(D)** *rut* mutant flies showed no progressive trends in using retreats and were significantly different from CS flies (one factor repeated measures ANOVA, main effect of encounter *P*< 0.01, interaction *P*< 0.001, mean±SEM; n_{cs}= 49, n_{rut}=46 trials)

Fig 4: Absence of loser mentality in *amnesiac* mutant flies **(A)** Outcome for second fight for losers in CS and *amn* mutant strain. CS loser flies: 73% losses, 27% low intensity draws. *amn* mutant loser flies: 36% wins, 43% losses, 7% low intensity and 14% high intensity draws (Fisher's exact test, *P*< 0.01, n_{cs}=15, n_{amn}=14 trials). **(B)** Number of retreats used by losers of both CS and *amn* mutant strains in first and second fights; CS flies use less number of retreats in second fight due to loser mentality while *amn* mutant losers differ significantly from wild type trends (two factor ANOVA, main effect of genotype *P*<0.05, main effect of fight *P*<0.05, interaction *P*<0.05, mean±95% confidence interval, n_{cs}=20, n_{amn}=20 trials) (**, *P*<0.01 in post hoc Tukey's HSD test.) **(C)** Number of lunges used by winners of both CS and *amn* mutant strains in first and second fights; No significant difference in two strains in terms of showing lunging behavior (two factor ANOVA, main effect of genotype *P*>0.05, main effect of fight *P*<0.001, interaction *P*>0.05, mean±95% confidence interval, n_{cs}=23, n_{amn}=23 trials) (***, *P*<0.001 in post hoc Tukey's HSD test.) **(D)** Number of attacks by losers of both CS and *amn* mutant strains in first and second fights; CS flies engage in less number of encounters in second fight while *amn* mutant losers differ significantly from wild type trends (two factor ANOVA, main effect of genotype *P*<0.01, main effect of fight *P*<0.001, interaction *P*<0.05, mean±95% confidence interval, n_{cs}=17, n_{amn}=24 trials) (***, *P*<0.001 in post hoc Tukey's HSD test.) **(E)** Retaliatory behavior i.e. lunging after receiving a lunge, by losers is shown in CS and *amn* mutant strains; CS losers: 24% in first fight and none in second fight (two tailed χ^2, *P*< 0.01, n_{cs}=21 trials); *amn* mutant losers: 29% retaliation in second fight (Fisher exact test, *P*< 0.01, n_{amn}=21 trials).

Fig 5: Schematic representation depicting role of *rutabaga* and *amnesiac* genes in formation of short term (STM) and mid-term memories (MTM). Short term memory renders learning ability to flies while mid-term memory contributes towards formation of memory pertaining to loser mentality.

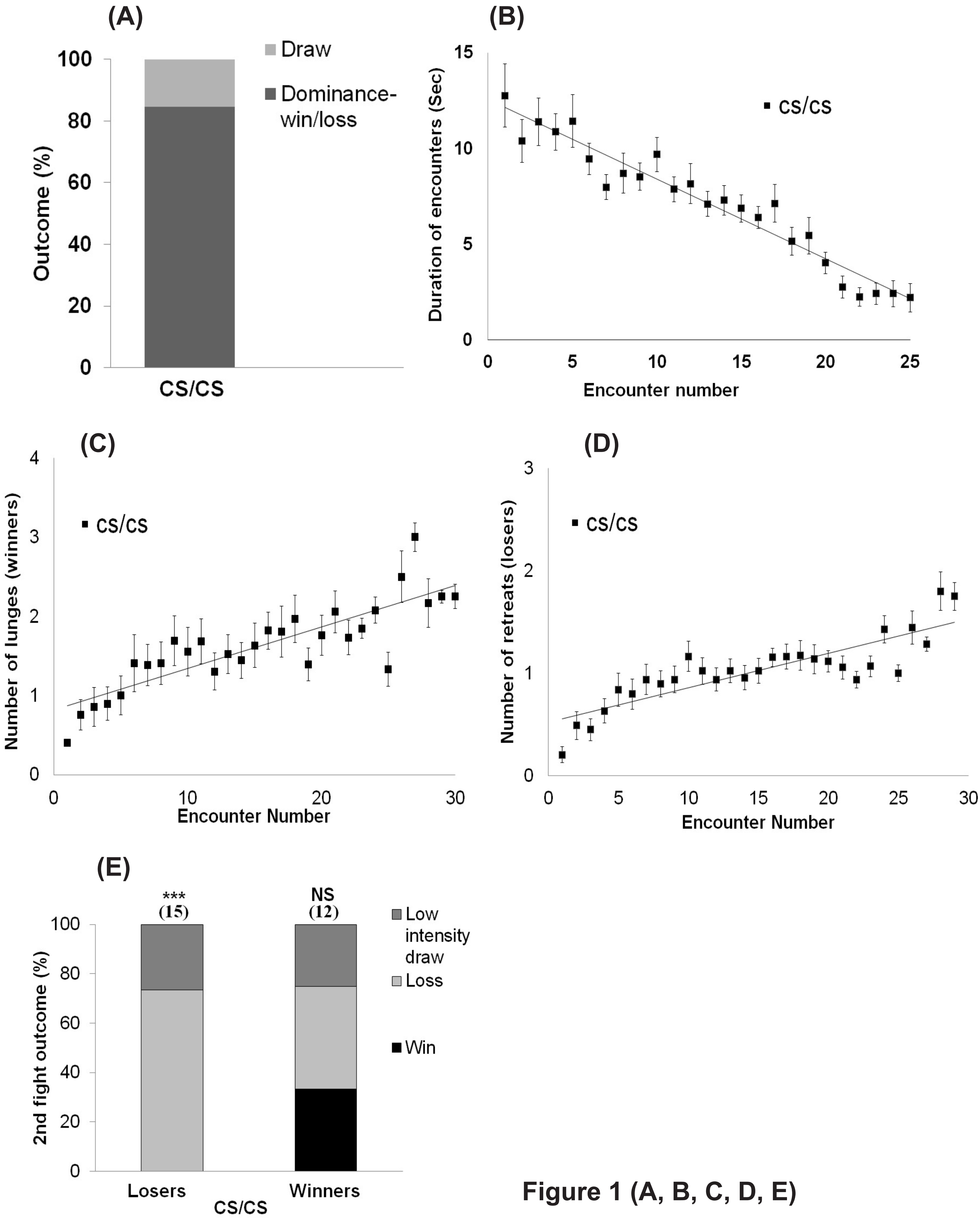

Figure 1 (A, B, C, D, E)

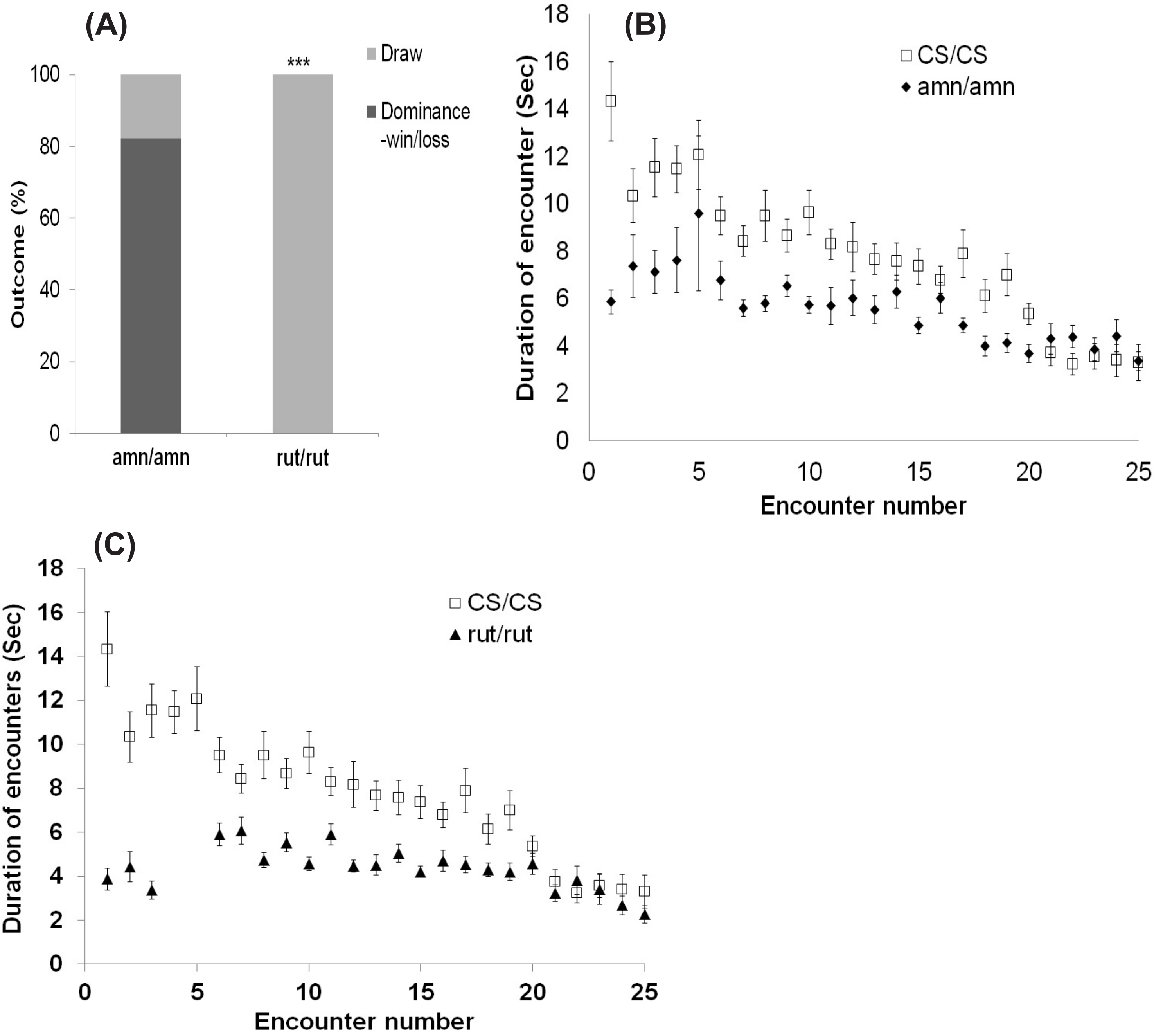
(A)
Draw
Dominance
-win/loss
***
Outcome (%)
amn/amn
rut/rut
(B)
CS/CS
amn/amn
Duration of encounter (Sec)
Encounter number
(C)
CS/CS
rut/rut
Duration of encounters (Sec)
Encounter number

Figure 2 (A, B, C)

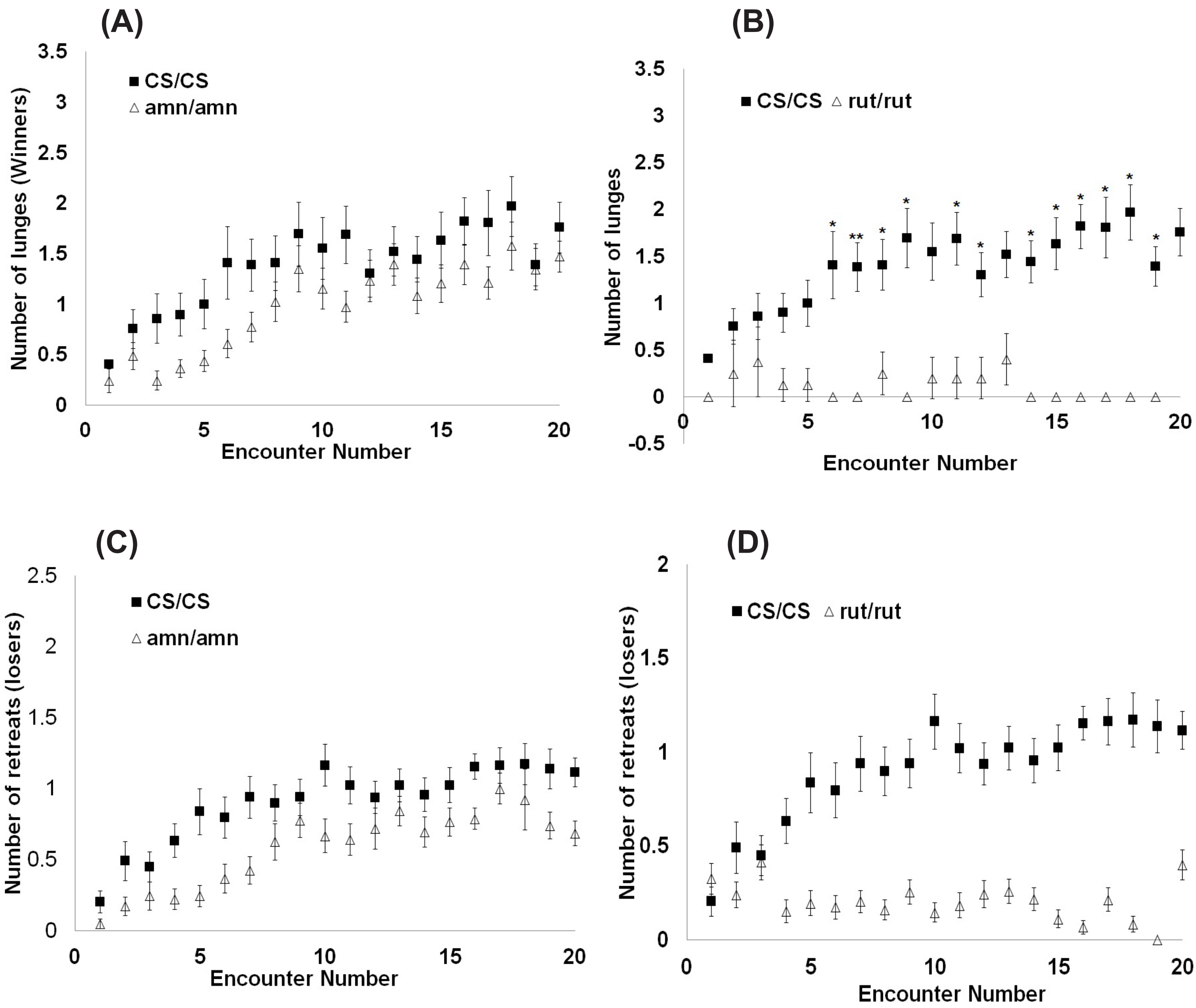

Figure 3 (A, B, C, D)

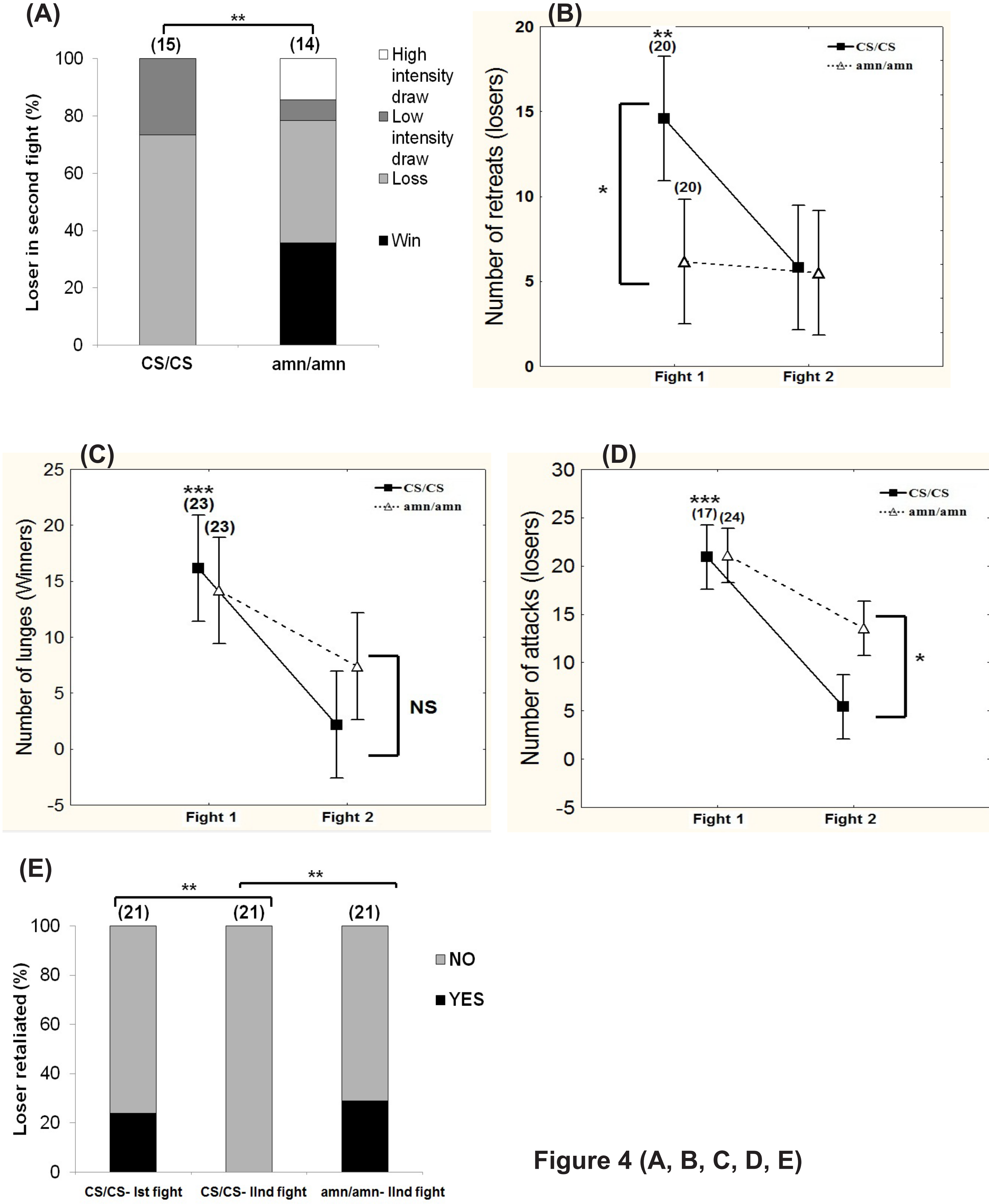

Figure 4 (A, B, C, D, E)

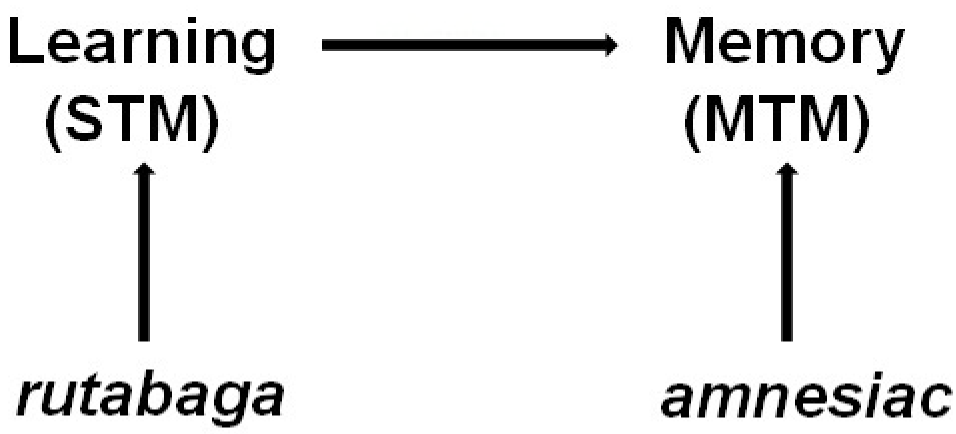

Figure 5

Supplementary figure legends

Fig S1: CS losers don't learn retreating behavior while *amn* mutants showed some learning in their second fights. **(A)** Over the course of encounters number of retreats in CS flies doesn't show any trend (Linear regression analysis, Y=0.721+.0082X, R^2=0.032, ANOVA, $P>0.05$, mean±SEM, $n_{cs}=13$). **(B)** Over the course of encounters number of retreats in *amn* mutant flies show slightly increasing trend (Linear regression analysis, Y=0.167+.0636X, R^2=0.575, ANOVA, $P<0.001$, mean±SEM, $n_{amn}=10$). **(C)** Over the course of encounters number of retreats in *rut* mutant flies doesn't show any trend (Linear regression analysis, Y=0.431-.0182X, R^2=0.138, ANOVA, $P>0.05$, mean±SEM, $n_{rut}=13$).

Fig S2: *rutabaga* mutant flies doesn't retain any memory from their experience in second fights **(A)** Number of retreats used by both CS and *rut* mutant strains in first and second fights; CS flies use less number of retreats in second fight due to loser mentality while *rut* mutant losers differ significantly from wild type trends (two factor ANOVA, main effect of genotype $P<0.001$, main effect of fight $P<0.05$, interaction $P<0.05$, mean±95% confidence interval, $n_{cs}=20$, $n_{rut}=13$ trials) (**, $P<0.01$ in post hoc Tukey's HSD test.) **(B)** Number of attacks by both CS and *rut* mutant strains in first and second fights; CS flies engage in less number of encounters in second fight while *rut* mutant losers differ significantly from wild type trends (two factor ANOVA, main effect of fight $P<0.001$, interaction $P<0.01$, mean±95% confidence interval, $n_{cs}=17$, $n_{rut}=15$ trials) (***, $P<0.001$ in post hoc Tukey's HSD test.)

Fig S3: CS and *amn* mutant winners show some learning of lunging behavior in their second fights. **(A)** There are no modifications in usage of lunging behavior in CS and *amn* mutant winner flies that won in their second fights (two factor ANOVA, main effect of genotype $P>0.05$, main effect of fight $P>0.05$, interaction $P>0.05$, mean±95% confidence interval, $n_{cs}=4$, $n_{amn}=8$ trials) (No significance showed in post hoc Tukey's HSD test.) **(B)** Over the course of encounters number of lunges in CS flies show slightly increasing trend (Linear regression analysis, Y=0.0184+.028X, R^2=0.231, ANOVA, $P<0.05$, mean±SEM, $n_{cs}=10$). **(C)** Over the course of encounters number of lunges in *amn* mutant flies show increasing trend (Linear regression analysis, Y=0.275+.0507X, R^2=0.675, ANOVA, $P<0.001$, mean±SEM, $n_{amn}=10$).

Fig S4: Lunging and retreating are most preferred fighting strategies used by winners and losers respectively. **(A)** Lunging is an aggressive move where one fly rears up on hind legs and snaps down on the other fly **(B)** Retreating is a defensive move where flies run/fly away from opponent.

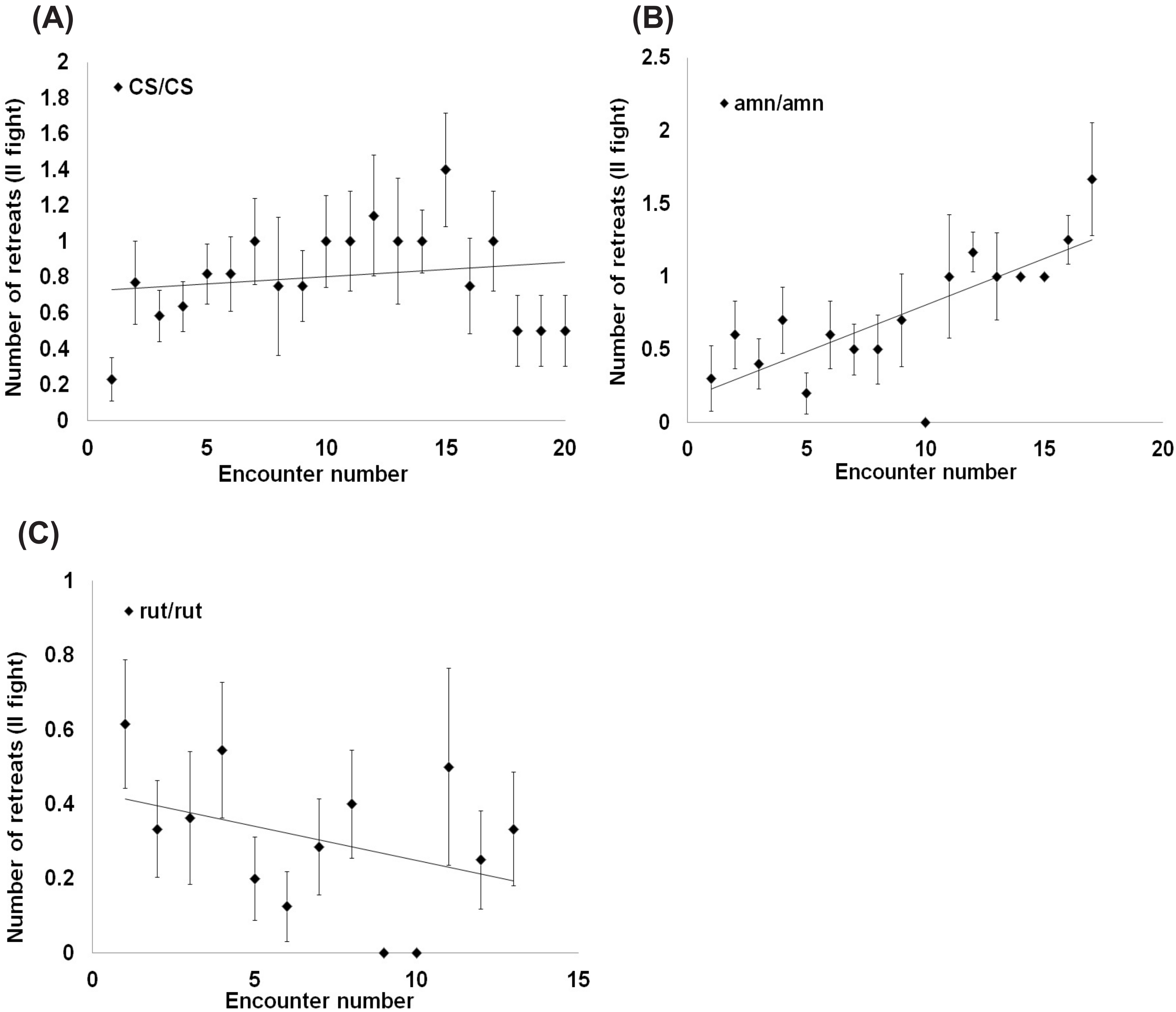

Figure S1 (A,B,C)

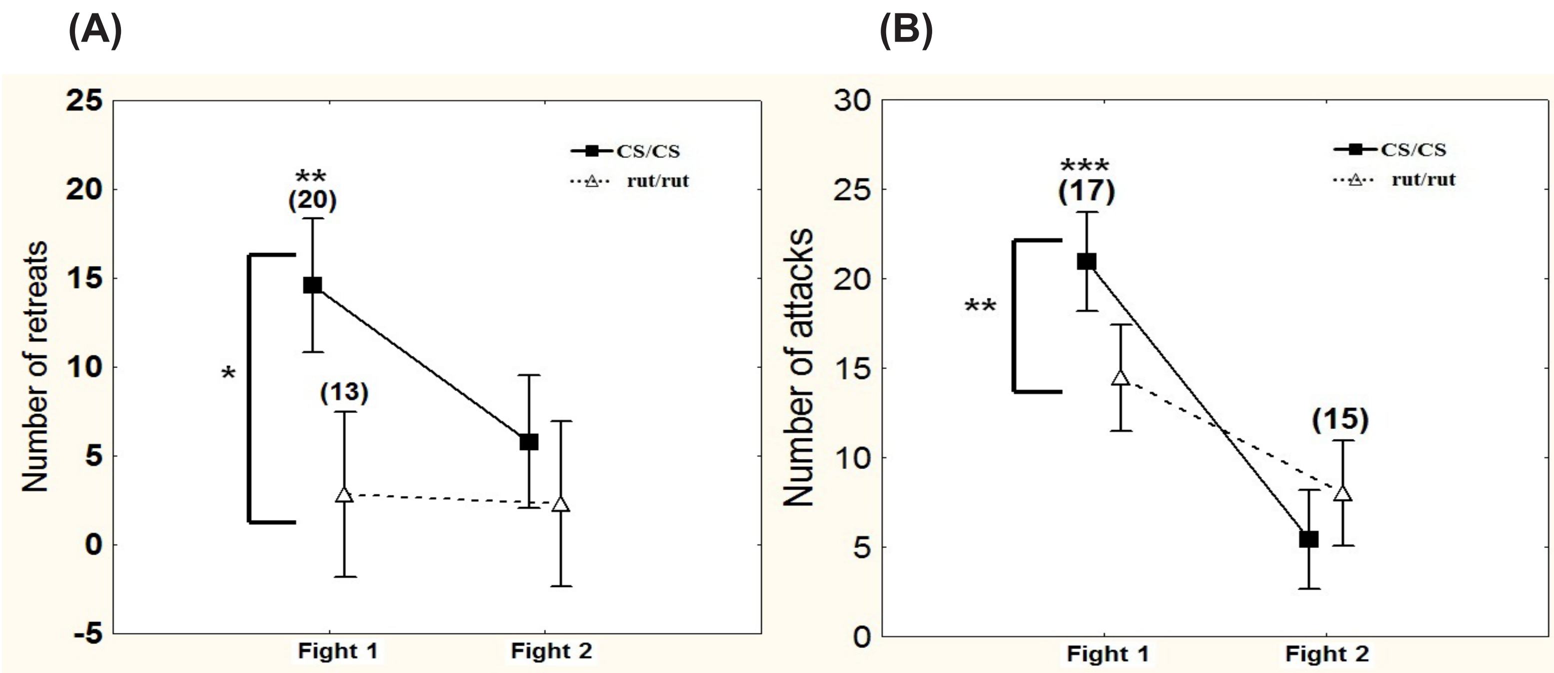
(A)
(B)
Number of retreats
Number of attacks
CS/CS
rut/rut
**
(20)
(13)
*
***
(17)
(15)
Fight 1
Fight 2
25
20
15
10
5
0
-5
30

Figure S2 (A, B)

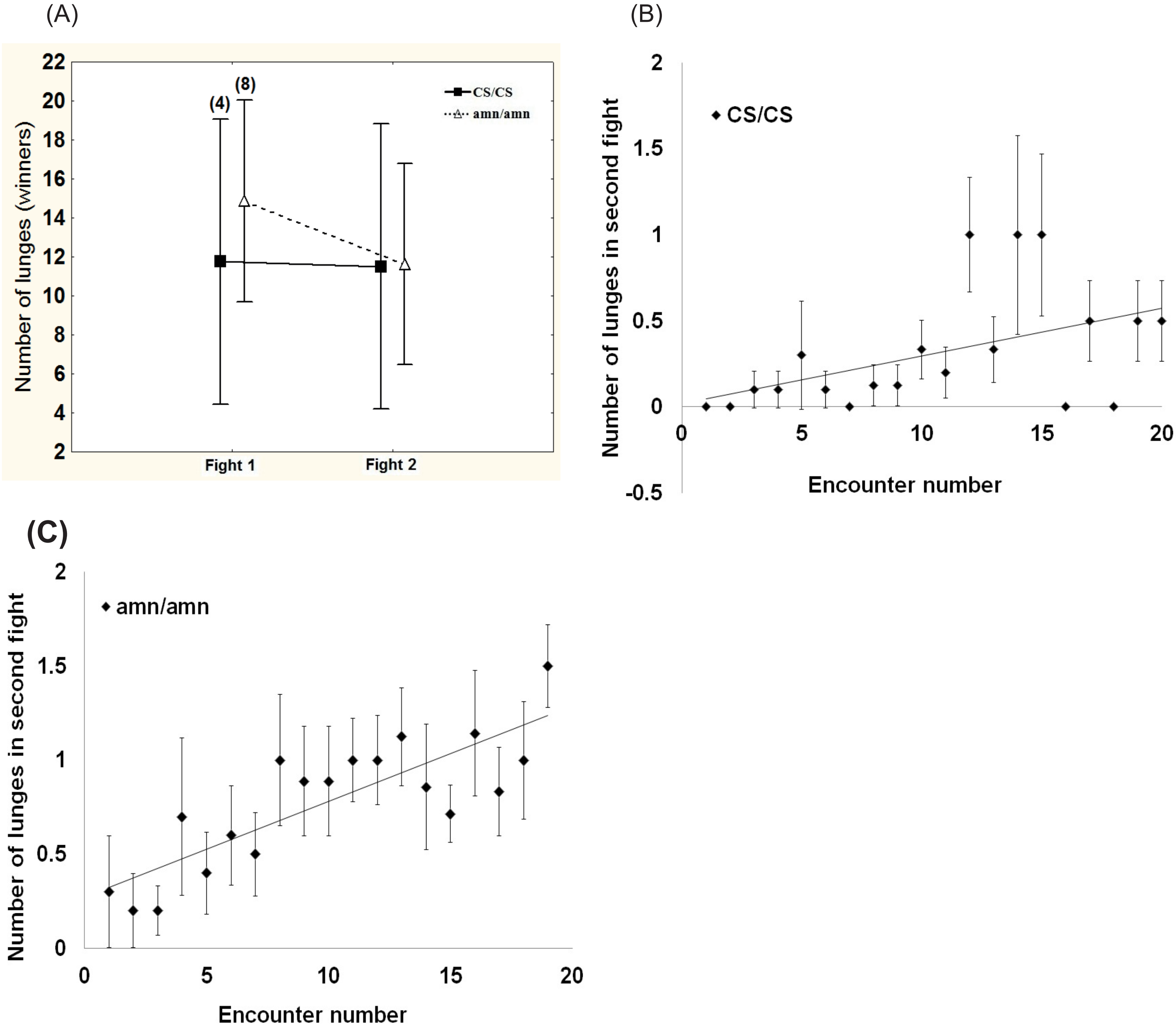

Figure S3 (A, B, C)

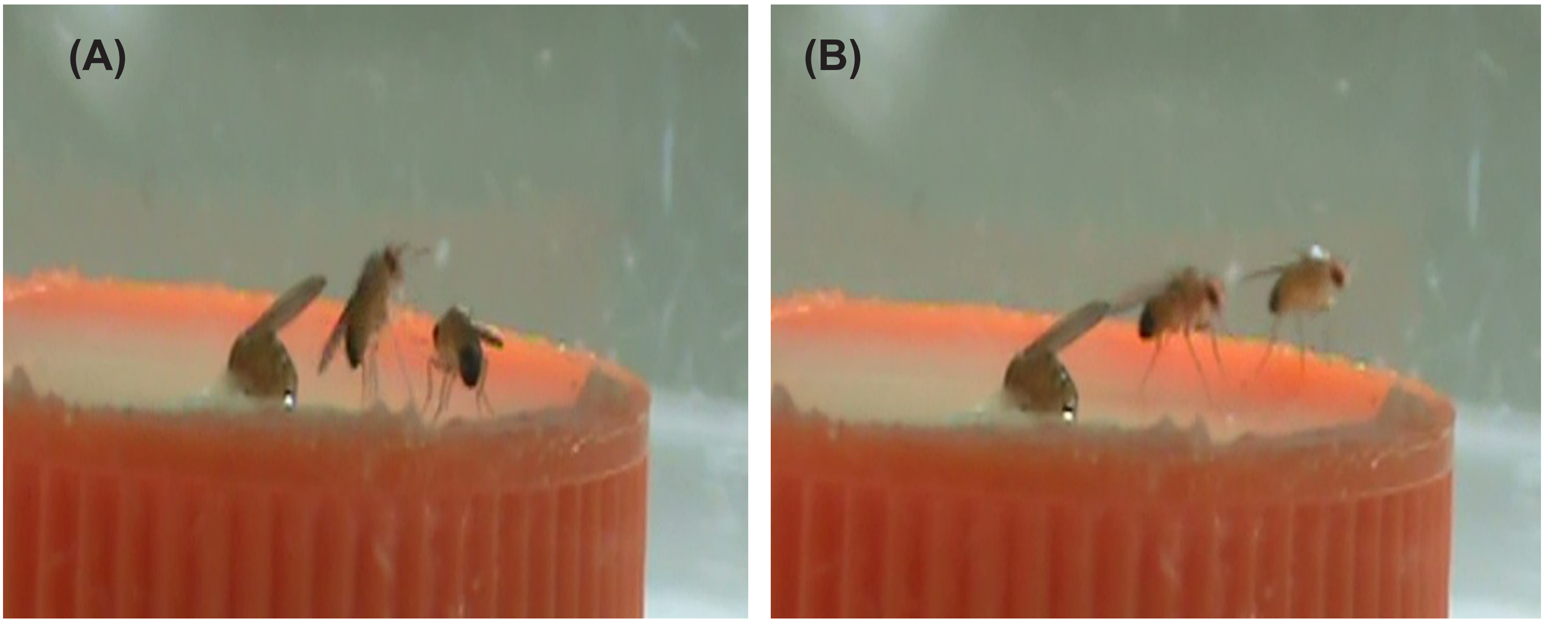

Figure S4 (A, B)

Supplementary table 1: Various offensive and defensive actions used by male flies during aggressive interaction with other opponents (5).

Offensive actions		
AP	Approach	One fly lowers body, then advances in the direction of the other
LLF	Low-Level fencing	Both flies extend one leg and tap opponent's leg
OWT	Wing threat	One fly quickly raises both wings to a 45° angle towards opponent
HLF	High-level fencing	One or both flies face each other, extend leg forward and push opponent
RDD/A	Round attack	Moving round around opponent defense/attack
BKS	Backstab	Attacking from behind
CS	Chasing	One fly runs after the other
LG	Lunging	One fly rears up on hind legs and snaps down on the other
HD	Holding	One fly grasps the opponent with forelegs and tries to immobilize
BX	Boxing	Both flies rear up on hind legs and strike the opponent with forelegs
TS	Tussling	Both flies tumble over each other, sometimes leaving food surface
FL	Fall	Fall from cup during fight
Defensive actions		
MW	Walk away	Loser turns and retreats slowly from advance of winner
DWT	Defensive wing threat	Loser flicks wings at 45° angle while facing away from opponent
RT	Retreat	Fly run/fly away from opponent
RW	Run away	Loser run away from opponent leaving food surface
FW	Fly away	Loser flies off food surface