

Reviewed Preprint

v1 • October 21, 2025

Not revised

Reviewed Preprint

v2 • September 8, 2026

Revised by authors

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Competing interests: No competing interests declared

Funding: See [page 20](#)

Reviewing editor: Tihana Jovanic, Neuro-PSI, UMR-9197, CNRS, UPSaclay, France

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Selection for male aggression is associated with changes in reproductive traits, chemical signaling and lifespan in *Drosophila melanogaster*

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eLife Assessment

This **valuable** study addresses the effects of selection for aggression on fitness and life-history trade-offs in *Drosophila melanogaster*. The evidence presented is overall **solid**, however, the data as they are do not completely support the claims of increased survival of highly aggressive males at the expense of reproductive success. The main limitation is the choice to use males from only one aggressive *Drosophila* line, that do not allow disambiguation between nonaggression-related factors and aggression-related factors influencing lifespan.

<https://doi.org/10.7554/eLife.107559.2.sa3>

Abstract

Life history traits are all essential traits linked to development, survival and reproduction, directly influencing individual fitness. Limited environmental or physiological resources force organisms to balance competing demands, leading to fundamental trade-offs, particularly between survival and reproduction. Male aggression is often assumed to enhance reproductive success, yet its broader association with other fitness-related traits remains unclear. To address this, we used *Drosophila melanogaster* *Bully* lines selected for high levels of male aggression, to examine relationships between aggression, chemical signaling, and essential life-history traits including reproductive behaviors and lifespan. Our results reveal that increased male aggressiveness shifts the balance of a major life-history trade-off, favoring survival over reproductive success. *Bully* males exhibited lower mating success, shorter mating duration and less effective chemical mate-guarding. However, this was traded-off with increased lifespan, enabling more reproductive opportunities. We also uncover potential mechanistic underpinnings of the reproductive deficits. *Bully* males showed differences in their cuticular hydrocarbon (CHCs) profiles and transferred lower levels of cVA to females, a mate-guarding pheromone, thereby weakening their post-mating strategy. Overall, our findings indicate that selection for male aggression is associated with a shift in a key life-history trade-off, highlighting aggression as an evolutionary force shaping adaptation of life-history traits and providing a foundation for future genetic and mechanistic studies.

Introduction

Life history traits, such as development time, fertility, survival, and reproductive success, are key phenotypic components that directly influence individual fitness (Braendle and Paaby, 2024 [↗](#); Roff, 1992 [↗](#); Stearns, 1992 [↗](#)). These traits often face trade-offs: investing in one can compromise another (Fisher, 1930 [↗](#); Stearns, 1989 [↗](#); Williams, 1966 [↗](#)). For instance, high reproductive effort may reduce survival due to the energetic costs of gamete production or increased mortality associated with mating behaviors or competition (Flatt, 2011 [↗](#); 2020 [↗](#)). Trade-offs also emerge between pre- and post-copulatory strategies (Simmons, 2001 [↗](#); Warner et al., 1995 [↗](#)). Males prioritizing pre-copulatory investment, such as aggressive competition for mates, may have limited resources for post-copulatory investment linked to sperm competition such as mate guarding (Andersson, 1994 [↗](#); Brennan and Orbach, 2021 [↗](#)). Understanding how such trade-offs are shaped by natural selection offers insight into how organisms optimize fitness in competitive social environments (Roff, 1992 [↗](#); Stearns, 1976 [↗](#)).

The fruit fly *Drosophila melanogaster* (*D. mel.*), provides a powerful model for studying life-history trade-offs, with well-characterized developmental, behavioral, and reproductive traits (Flatt, 2020 [↗](#)). These traits are shaped by both genetic and environmental factors and can evolve under ecological pressures. Faster development, earlier reproduction and high mating rate, for instance, are advantageous in changing environments but come at the cost of reduced longevity and fecundity (Partridge and Farquhar, 1981 [↗](#); Stearns, 2000 [↗](#)). Experimental manipulations of environmental conditions such as temperature and food availability reveal how external factors shape trade-offs between survival and reproduction (Chippindale et al., 1996 [↗](#); Marshall and Sinclair, 2010 [↗](#)).

Beyond development and fecundity, *D. mel.* serves as a model to study a wide range of social behaviors, including aggregation, aggression, and courtship, that are largely mediated by chemical communication (Kim et al., 2017 [↗](#); Kohl et al., 2015 [↗](#)). Cuticular hydrocarbons (CHCs) such as some pheromones play diverse roles in social interaction: 7-Tricosene (7-T), a male-specific compound, increases female receptivity and modulates aggression (Grillet et al., 2006 [↗](#); Wang et al., 2011 [↗](#)); cis-vaccenyl acetate (cVA), a male-transferred pheromone, attracts females, repels other males, and promotes aggregation (Kurtovic et al., 2007 [↗](#); Laturney and Billeter, 2016 [↗](#)); and the female-specific hydrocarbon 7,11-heptacosadiene (7,11-HD) strongly elicits male courtship and supports species-specific mate recognition (Toda et al., 2012 [↗](#)).

Among these interactions, male-male competition plays a central role in shaping reproductive strategies. Pre-copulatory competition involves resource defense, courtship displays, and aggressive interactions to secure mating opportunities. Courtship in *D. mel.* includes stereotyped behaviors such as orientation, wing extensions (producing courtship song), tapping, licking, and attempted copulation (Yamamoto and Koganezawa, 2013 [↗](#)). Successful courtship displays lead to female acceptance, outcompeting other males that fail to meet the female's preferences (Baxter et al., 2018 [↗](#); Hindmarsh Sten et al., 2025b [↗](#)). Aggression intensifies this competition: more aggressive or dominant males often monopolize access to females (Dow and von Schilcher, 1975 [↗](#); Filice and Dukas, 2019 [↗](#); Gao et al., 2024 [↗](#); Nandy et al., 2016 [↗](#); Prunier and Trannoy, 2024 [↗](#)). Aggressive behaviors range from wing threats and lunging to high-intensity physical confrontations like tussling and boxing (Chen et al., 2002 [↗](#)). Beyond aggressive patterns, males also produce agonistic wing flicks when competing for access to females, which can interfere with a female's auditory perception of rival males and thereby influence mate choice (Hindmarsh Sten et al., 2025a [↗](#)). These behaviors promote territorial dominance and enhance mating success (Gao et al., 2024 [↗](#)), although subordinate males may also achieve reproductive success through first access to females and prolonged copulation (Filice and Dukas, 2019 [↗](#)).

Post-copulatory competition further shapes male reproductive success. After mating, males use multiple strategies to ensure paternity, including the transfer of seminal fluid proteins (SFPs) including Sex Peptide (SP) and Accessory gland proteins (Acps), which promote sperm storage, stimulate oviposition, and reduce female receptivity to further mating (Chapman et al., 2003 [↗](#); Fricke et al., 2009 [↗](#)). These factors also contribute to the formation of a mating plug that retains

sperm, acting as a passive mate-guarding strategy (Avila et al., 2011 [↗](#); Dunham and Rudolf, 2009 [↗](#)). In parallel, chemical mate guarding via pheromone transfer (e.g. cVA and 7-T) reduce female attractiveness and their likelihood of re-mating (Billeter et al., 2009 [↗](#); Gillott, 2003 [↗](#); Laturney and Billeter, 2016 [↗](#); Miyamoto and Amrein, 2008 [↗](#); Scott, 1986 [↗](#); Zawistowski and Richmond, 1986 [↗](#)). Male reproductive behavior is plastic: in the presence of rivals, males extend their mating duration (Bretman et al., 2009 [↗](#); Bretman et al., 2013 [↗](#); Kim et al., 2013 [↗](#)), increase the number of sperm in their ejaculate (Garbaczewska et al., 2013 [↗](#)), adapt seminal fluid content in their ejaculate (Hopkins et al., 2019 [↗](#)), and present higher levels of aggression toward opponents to guard their mates (Baxter et al., 2015 [↗](#); Bretman et al., 2011 [↗](#); Dore et al., 2021 [↗](#); Mazzi et al., 2009 [↗](#)). Together, this demonstrates that male-male aggression influences access to mates and reproductive outcomes through both pre- and post-copulatory competition.

While aggression can enhance male mating success in natural settings, the relationship between aggression and reproductive fitness is not always straightforward. In a previous study, Penn et al. (Penn et al., 2010 [↗](#)) generated “*Bully*” flies through 37 generations of selection for aggression starting from a *Canton-S* (Cs) population. In the first step, Cs winner males from aggressive encounters were allowed to mate with Cs females. In the resulting F1 progeny, males displaying higher levels of aggression were again selected and mated with their female siblings. The resulted hyper-aggressive males frequently escalated to boxing, the most intense aggression phenotype (Chowdhury et al., 2017 [↗](#)), but showed reduced reproductive success with virgin females compared to unselected control males (Penn et al., 2010 [↗](#)). This suggests that although aggression may enhance competitive ability, it can also incur fitness costs. This raises important questions about the role of aggression in shaping life-history trade-offs, such as: to what extent does aggression influence life history traits related to reproduction, and does it affect the balance reproduction vs survival?

To address these questions, we investigated whether *D. mel.* males from *Bully* lines exhibit changes in life-history trade-offs compared to the unselected starting wild-type flies (Cs lines). We found that hyper-aggressive *Bully* males show reduced reproductive success and shorter mating duration across their lifespan, indicating lower pre-copulatory success. Their mates present higher remating rate and increased attractiveness, suggesting that mate-guarding strategy of *Bully* males is less efficient. However, this poor pre- and post-copulatory success was traded-off with an increased lifespan, which is accompanied by late-life reproductive success. We then provided a mechanistic basis for the reduced copulatory success of *Bully* males. They showed distinct CHCs profiles compared to Cs males, which may contribute to their lower mating success. Moreover, they transferred to females lower levels of cVA, a mate-guarding pheromone, to females, potentially decreasing efficiency of their post-mating strategy. Our findings underscore the complex interplay between aggression, reproductive behavior, survival, and chemical signaling, providing insights into the evolutionary dynamics of life-history trade-offs in competitive social contexts.

Results

Males exhibiting higher aggression intensity also exhibit shorter mating durations

To determine whether selection for male aggressive traits may also be associated with changes in reproductive behaviors, we employed three male lines selected for increased aggression. In a previous study, Penn et al. (Penn et al., 2010 [↗](#)) generated the so-called “*Bully*” flies through 37 generations of artificial selection for male aggression starting from a *Canton-S* (Cs) population. Briefly, males that won aggressive encounters were allowed to mate with Cs females. In the resulting F1 progeny, males exhibiting higher levels of aggression were again selected and mated with their female siblings. Following the same experimental procedure, several independent selections were performed in parallel, giving rise to the *Bully A*, *Bully B* and *Bully C* lines. These *Bully* lines were originally derived from Cs flies and do not represent distinct populations *per se*, but rather result from male-specific selection on aggressive behavior. Males from these three lines

(*Bully A*, *B*, and *C*) were tested in standardized aggression assays (Trannoy et al., 2015b) (Figure 1A). Across same-line contests, males from all three *Bully* lines did not differ from *Cs* males in latency to lunge (Figure 1B), but displayed significant increase in the number of lunges (Figure 1C), the number of boxing events (Figure 1D), and boxing frequency (defined as the proportion of male pairs displaying at least one boxing event during the 10-min observation period) (Figure 1E) compared to *Cs* males. However, the magnitude of this phenotype differed across lines: *Bully A* and *Bully B* males exhibited higher aggression levels than *Bully C* males, which showed an intermediate phenotype (Figure 1E).

We next assessed courtship and mating behaviors by pairing males and females from the same line and scored courtship metrics. The proportion of time spent by males displaying unilateral wing extension (UWE) and latency to court did not differ significantly between lines (Figure 1F-G), while *Bully B* males exhibited a longer latency to mate compared to other pairings (Figure 1H). In contrast, mating duration (MD) was significantly reduced in *Bully A* and *Bully B* pairings compared to *Cs* (Figure 1I), while *Bully C* males again show an intermediate phenotype (Figure 1I).

These results indicate that males from the *Bully A* and *Bully B* lines, classified as hyper-aggressive in same-line competition (Figure 1B-E), also exhibit reduced mating duration when paired with same-line females, whereas *Bully C* males do not (Figure 1I). Because the *Bully A* line (hereafter named *Bully*) has been already employed in previous studies to measure differential gene expression profile (Chowdhury et al., 2017; Penn et al., 2010), we chose to conduct subsequent experiments on this line. Overall, these results support our initial hypothesis that selection for aggressive traits may have influenced other behavioral traits linked to reproductive behaviors.

Highly aggressive males exhibit reduced mating success and have shorter mating duration

As the selection for aggression was male-specific (Penn et al., 2010), we next aimed to focus on examining male reproductive behaviors while avoiding confounding effects of female behavioral variation. Thus, all subsequent courtship and mating assays were performed by pairing *Cs* or *Bully A* males with *Cs* females, which served as a standardized female background. We first assessed mating success in competitive assays, where a *Cs* male and a *Bully* male competed for access to a single female. Under these conditions, no significant mating advantage was observed for *Bully* males (Figure 2A). We then assessed mating success in non-competitive assays by pairing each male with a virgin *Cs* female for 30 minutes. Under these conditions, *Bully* males showed a significantly reduced probability of mating compared to *Cs* males (Figure 2B), despite similar levels of courtship behavior, as measured by UWE (Figure 2C), courtship latency (Figure 2D), and mating latency among successful pairs (Figures 2E). We next examined copulation *per se* and found that MD was significantly shorter in *Bully* males, with a reduction of approximately 25% compared to *Cs* males (Figure 2F), consistent with the phenotype observed when males were paired with females from the same line (Figure 1I). Importantly, this reduction in MD persisted when *Bully* males were paired with decapitated *Cs* females (Figure 2G), ruling out female-driven termination of mating and demonstrating that the effect is male-specific.

As group-housed males have lengthened MD (Bretman et al., 2013; Kim et al., 2013), we compared MD of singly and group-housed males. We found that both *Cs* and *Bully* group-housed males exhibited significantly extended mating durations compared to single-housed males (Figure 1H). However, the MD in *Bully* males remained shorter compared to *Cs* males in group-housed condition (Figure 2H), indicating that *Cs* and *Bully* males respond equally to early social experience.

Together, these results show that *Bully* males display reduced mating success and shorter copulation duration, indicating differences in pre-copulatory outcomes and copulatory investment. This supports our main hypothesis that selecting males for aggressive traits may also had influenced one or both pre- and post-copulatory strategies.

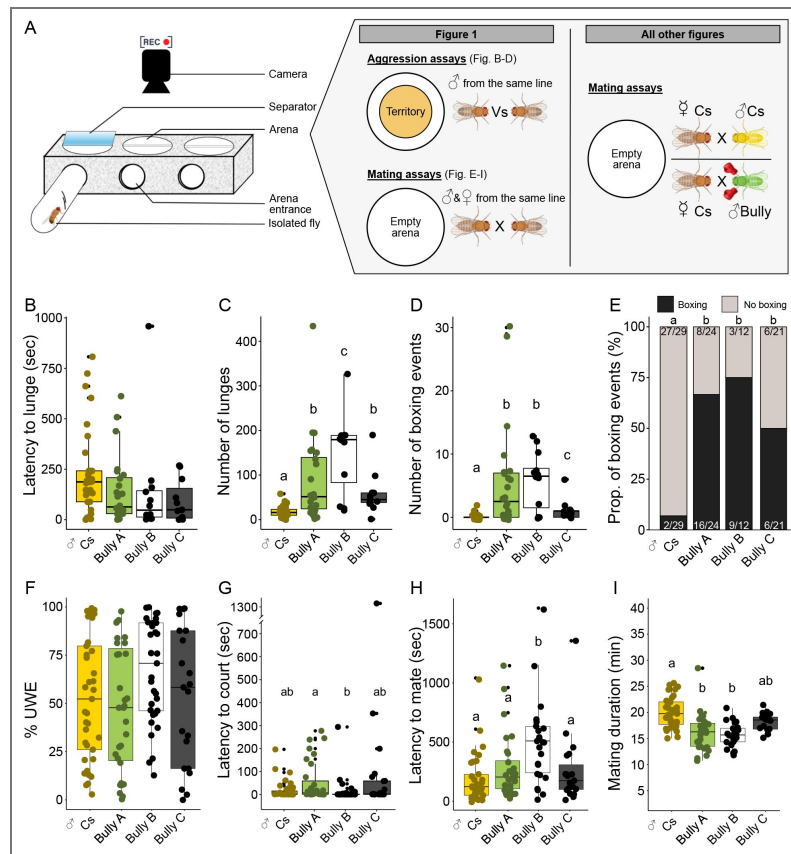


Figure 1. Courtship and aggressive behaviors in *Cs*, *Bully A*, *Bully B* and *Bully C* lines.

(A) Schema of the experimental set up to record aggressive and courtship behaviors. Couples of flies and presence of food cup are mentioned according to the figure of this article. (B) Latency to lunge of *Cs*, *Bully A*, *Bully B* and *Bully C* males in aggression assay with a territory against a male of the same line ($n_{Cs}=29$, $n_{BullyA}=24$, $n_{BullyB}=12$, $n_{BullyC}=11$, LM and permutation test (Monte-Carlo test): $P=0.136$). (C) Number of lunges of *Cs*, *Bully A*, *Bully B* and *Bully C* male pairs in aggression assays ($n_{Cs}=29$, $n_{BullyA}=24$, $n_{BullyB}=12$, $n_{BullyC}=11$, GLM quasi-Poisson and type II analysis of deviance table (Likelihood Ratio Test): $P=1.622 \times 10^{-10}$, $LR\chi^2=48.556$, post-hoc: $P_{Cs_BullyA}<0.001$, $z\text{-ratio}=-4.495$, $P_{Cs_BullyB}<0.001$, $z\text{-ratio}=-5.911$, $P_{Cs_BullyC}=0.018$, $z\text{-ratio}=-2.836$, $P_{BullyA_BullyB}=0.042$, $z\text{-ratio}=-2.308$, $P_{BullyA_BullyC}=0.232$, $z\text{-ratio}=1.195$, $P_{BullyB_BullyC}=0.018$, $z\text{-ratio}=2.797$). (D) Number of boxing events of *Cs*, *Bully A*, *Bully B* and *Bully C* male pairs in aggression assays ($n_{Cs}=29$, $n_{BullyA}=24$, $n_{BullyB}=12$, $n_{BullyC}=11$, GLM Poisson and type II analysis of deviance table (Likelihood Ratio Test): $P=2.2 \times 10^{-16}$, $LR\chi^2=2511.9$, post-hoc: $P_{Cs_BullyA}<0.001$, $z\text{-ratio}=-6.819$, $P_{Cs_BullyB}<0.001$, $z\text{-ratio}=-6.839$, $P_{Cs_BullyC}<0.001$, $z\text{-ratio}=-3.649$, $P_{BullyA_BullyB}=0.728$, $z\text{-ratio}=-0.347$, $P_{BullyA_BullyC}<0.001$, $z\text{-ratio}=5.392$, $P_{BullyB_BullyC}<0.001$, $z\text{-ratio}=5.366$). (E) Non-competitive mating success of *Cs*, *Bully A*, *Bully B* and *Bully C* males paired with a virgin *Cs* female ($n_{Cs}=37$, $n_{BullyA}=31$, $n_{BullyB}=33$, $n_{BullyC}=21$, GLM binomial and type II analysis of deviance table (Likelihood Ratio Test): $P=0.012$, $LR\chi^2=11.040$, post-hoc: $P_{Cs_BullyA}=0.934$, $z\text{-ratio}=0.728$, $P_{Cs_BullyB}=0.099$, $z\text{-ratio}=2.395$, $P_{Cs_BullyC}=0.868$, $z\text{-ratio}=1.060$, $P_{BullyA_BullyB}=0.206$, $z\text{-ratio}=2.043$, $P_{BullyA_BullyC}=0.934$, $z\text{-ratio}=0.406$, $P_{BullyB_BullyC}=0.522$, $z\text{-ratio}=-1.513$). (F) Proportion of time spent by *Cs* and *Cs*, *Bully A*, *Bully B* and *Bully C* males doing Unilateral Wing Extension (UWE) toward a *Cs* virgin female in non-competitive courtship assays ($n_{Cs}=37$, $n_{BullyA}=31$, $n_{BullyB}=33$, $n_{BullyC}=21$, LM and type II Anova table: $P=0.088$, $F=2.235$). (G) Latency to court (time difference between first interaction and first UWE) of *Cs*, *Bully A*, *Bully B* and *Bully C* males paired with a virgin *Cs* female ($n_{Cs}=37$, $n_{BullyA}=31$, $n_{BullyB}=33$, $n_{BullyC}=21$, LM and type II Anova table: $P=0.014$, $F=3.707$, post-hoc: $P_{Cs_BullyA}=0.338$, $t\text{-ratio}=-1.740$, $P_{Cs_BullyB}=0.433$, $t\text{-ratio}=1.469$, $P_{Cs_BullyC}=0.524$, $t\text{-ratio}=-1.127$, $P_{BullyA_BullyB}=0.015$, $t\text{-ratio}=3.101$, $P_{BullyA_BullyC}=0.683$, $t\text{-ratio}=0.661$, $P_{BullyB_BullyC}=0.099$, $t\text{-ratio}=-2.364$). (H) Latency to mate (time difference between first interaction and start of the mating) of *Cs*, *Bully A*, *Bully B* and *Bully C* males paired with a virgin *Cs* female ($n_{Cs}=36$, $n_{BullyA}=29$, $n_{BullyB}=24$, $n_{BullyC}=19$, LM and type II Anova table: $P=8.527 \times 10^{-4}$, $F=5.970$, post-hoc: $P_{Cs_BullyA}=0.482$, $t\text{-ratio}=-1.413$, $P_{Cs_BullyB}<0.001$, $t\text{-ratio}=-4.191$, $P_{Cs_BullyC}=0.581$, $t\text{-ratio}=-1.062$, $P_{BullyA_BullyB}=0.038$, $t\text{-ratio}=-2.725$, $P_{BullyA_BullyC}=0.863$, $t\text{-ratio}=0.173$, $P_{BullyB_BullyC}=0.041$, $t\text{-ratio}=2.615$). (I) Mating duration of *Cs*, *Bully A*, *Bully B* and *Bully C* males paired with a virgin *Cs* female ($n_{Cs}=36$, $n_{BullyA}=29$, $n_{BullyB}=24$, $n_{BullyC}=19$, LM and type II Anova table: $P=8.527 \times 10^{-4}$, $F=5.970$, post-hoc: $P_{Cs_BullyA}=0.482$, $t\text{-ratio}=-1.413$, $P_{Cs_BullyB}<0.001$, $t\text{-ratio}=-4.191$, $P_{Cs_BullyC}=0.581$, $t\text{-ratio}=-1.062$, $P_{BullyA_BullyB}=0.038$, $t\text{-ratio}=-2.725$, $P_{BullyA_BullyC}=0.863$, $t\text{-ratio}=0.173$, $P_{BullyB_BullyC}=0.041$, $t\text{-ratio}=2.615$). For all graphs, different letters mean a statistical difference. Statistical details are given in Figure 1-table supplement 1.

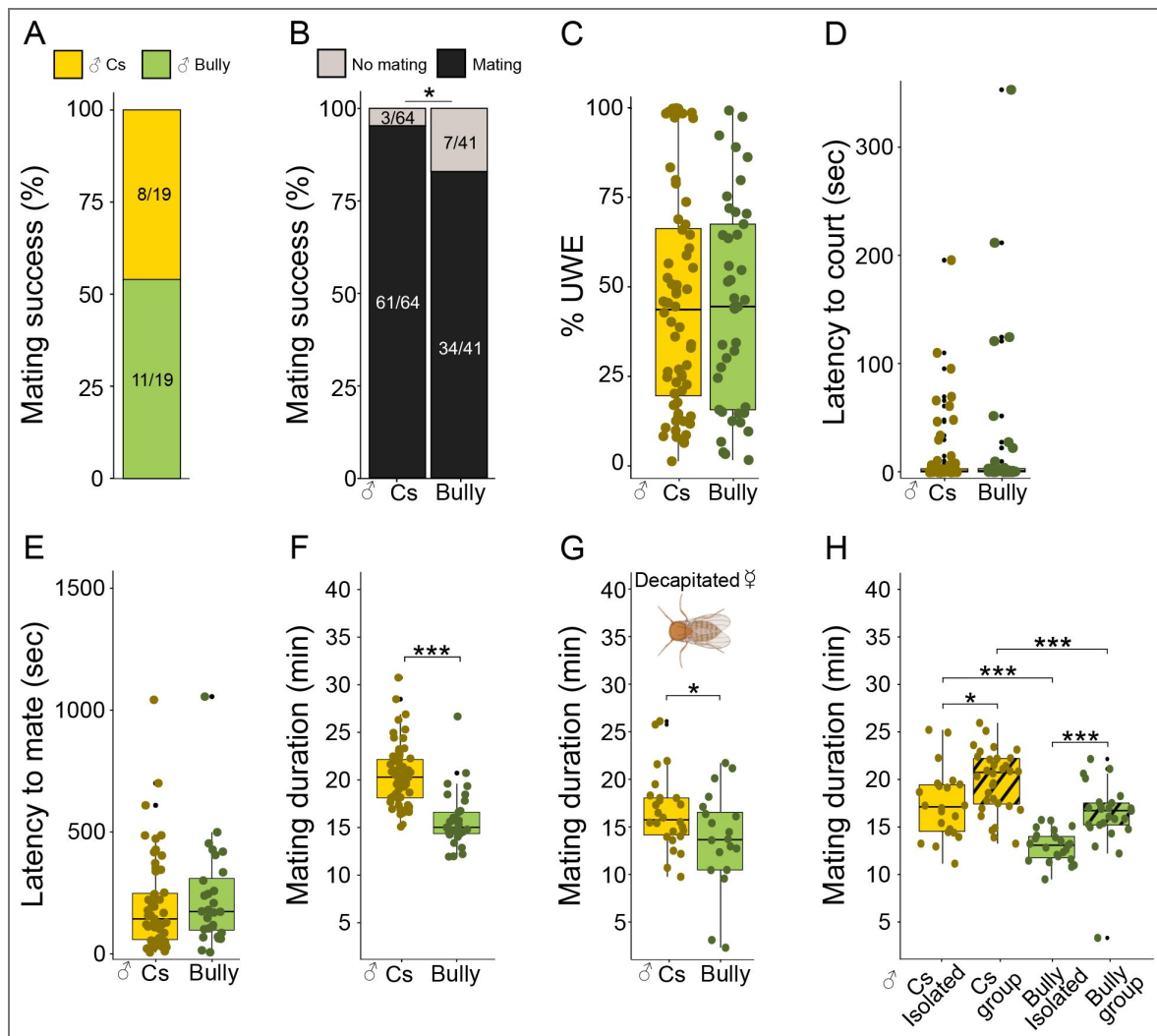


Figure 2. Bully males exhibit reduced mating success and allocate less time to mating.

(A) In competitive courtship assays, the mating success of Cs and Bully males was scored when paired with a single virgin Cs female ($n_{Cs}=19$, $n_{Bully}=19$, GLM binomial and type II analysis of deviance table (Likelihood Ratio): Test, $P=0.329$, $LR\chi^2=0.951$). (B) In non-competitive courtship assays, the mating success of Cs and Bully males paired with a virgin Cs female were analyzed ($n_{Cs}=64$, $n_{Bully}=41$, GLM binomial and type II analysis of deviance table (Likelihood Ratio Test): $P=0.037$, $LR\chi^2=4.347$). (C) Proportion of time spent by Cs and Bully males doing Unilateral Wing Extension (UWE) toward a Cs virgin female in non-competitive courtship assays ($n_{Cs}=64$, $n_{Bully}=41$, Fisher Pitman permutation test: $P=0.877$, $t=-0.131$). (D) Latency to court (time difference between first interaction and first UWE) of Cs and Bully males paired with a virgin Cs female ($n_{Cs}=64$, $n_{Bully}=41$, Fisher Pitman permutation test: $P=0.319$, $Z=1.043$). (E) Latency to mate (time difference between first interaction and start of the mating) of Cs and Bully males paired with a virgin Cs female ($n_{Cs}=61$, $n_{Bully}=34$, Fisher Pitman permutation test: $P=0.266$, $Z=1.170$). (F) Mating duration of Cs and Bully males paired with a virgin Cs female ($n_{Cs}=61$, $n_{Bully}=34$, T-test: $P=6.009\times 10^{-10}$, $t=-6.917$) or (G) a virgin Cs decapitated female ($n_{Cs}=26$, $n_{Bully}=20$, T-test: $P=0.022$, $t=-2.367$). (H) Mating duration of Cs and Bully males that were previously raised in group of 10 males or in social isolation ($n_{Cs-isolated}=22$, $n_{Cs-group}=32$, $n_{Bully-isolated}=23$, $n_{Bully-group}=25$, LM and type II ANOVA: F-test, $P=7.582\times 10^{-11}$, $F=21.669$, post-hoc: $P_{Cs-isolated_Cs-group}=0.013$, $F=6.583$, $P_{Bully-isolated_Bully-group}=2.532\times 10^{-4}$, $F=15.731$, $P_{Cs-isolated_Bully-isolated}=4.263\times 10^{-6}$, $F=27.697$, $P_{Cs-group_Bully-group}=1.884\times 10^{-4}$, $F=16.032$). For all graphs, stars indicate significant differences (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) and statistical details are given in Figure 1-table supplement 1.

Post-mating behaviors are affected in hyper-aggressive males

Given these differences in mating performance, we asked whether they compensate through distinct post-mating strategies. To investigate this, we first quantified courtship behavior immediately after copulation by measuring unilateral wing extension (UWE) directed toward the just-mated female. While *Cs* males performed minimal UWE towards just-mated females (Figure 3A), *Bully* males displayed significantly higher levels of post-mating courtship in this context (Figure 3A). This effect persisted when females were swapped after mating, with *Bully* males showing increased UWE towards *Cs*-mated females (Figure 3B–B'). This indicates that this phenotype reflects an intrinsic increase in courtship drive rather than a female-dependent effect. This elevated post-mating motivation led us to hypothesize that *Bully* males might achieve higher remating rates. To test this, we conducted sequential mating assays in which a single *Cs* or *Bully* male was consecutively paired with six virgin females over a 3-hour period and we scored the number of mating. Contrary to our expectation, *Bully* males did not mate with more females than *Cs* controls (Figure 3C), despite maintaining consistently shorter MD across successive mating (Figures 3D). As mating accompanies ejaculate transfer and incurs a reduction in sperm and seminal fluid reserve in *D. mel.* males (Hihara, 1981; Sirot et al., 2009), we further examined whether shorter MD in *Bully* males could reflect more efficient allocation, allowing fertilization of a greater number of females. However, analysis of progeny production revealed no significant differences between *Bully*- and *Cs*-mated females across successive matings (Figure 3E). Thus, increased post-mating courtship in *Bully* males does not translate into improved reproductive output.

We next investigated whether *Bully* males differ in their ability to modify female post-mating state. During mating, males transfer pheromonal cues, including cuticular hydrocarbons (CHCs), that reduce female attractiveness to rival males. We reasoned that the shorter MD of *Bully* males might limit the transfer of such mate-guarding signals, thereby reducing the extent of female unattractiveness. To test this independently of female behavior, we performed binary choice assays using decapitated females previously mated with either *Cs* or *Bully* males. Both *Cs* and *Bully* males spent significantly more time courting females that had mated with *Bully* males (Figure 3F, green vs yellow bars), indicating that these females remain more attractive and suggesting reduced transfer of inhibitory cues. To validate the experimental procedure and rule out potential deficits in sensory perception, we confirmed that both *Cs* and *Bully* males spent significantly more time performing UWE toward a decapitated female than toward a decapitated male (Figure 3-figure supplement 1). Finally, we assessed the functional consequences of this change in female state on remating behavior. Virgin *Cs* females were first mated with either *Cs* or *Bully* males and subsequently paired with naïve *Cs* males several days later. Females previously mated with *Bully* males, remated significantly more than females that had mated with *Cs* males (Figure 3G, green), consistent with weaker mate-guarding.

Together, these results indicate that *Bully* males fail to offset their poor pre-copulatory success with more efficient post-mating strategies. Despite increased post-mating courtship motivation, they do not achieve higher remating rates or reproductive output, and their mates remain more attractive and more likely to remate. This dissociation indicates that increased courtship motivation is not predictive of reproductive success in this context. These findings suggest that selection for aggressive traits in males appears to compromise both pre- and post-copulatory aspects of male reproductive performance.

Hyper-aggressive males present differences in their CHCs profiles and transfer lower quantities of mate-guarding pheromones to females

To investigate mechanisms underlying reduced mating success (Figure 2B) and changes in mate-guarding behaviors (Figure 3F–G) in *Bully* males, we quantified the CHCs profiles of naïve *Cs* and *Bully* males. PCA analysis revealed a clear separation between the two groups, indicating distinct

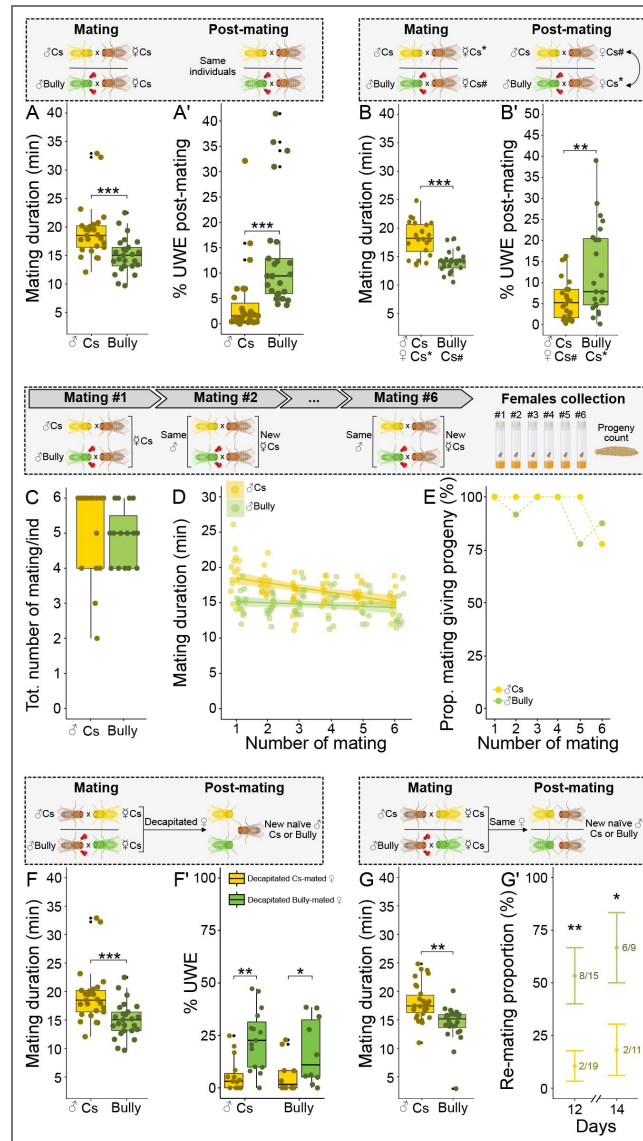


Figure 3. Hyper-aggressive males exhibit changes in mate-guarding features.

(A, B, F, G). Mating duration of *Cs* and *Bully* males paired with a virgin *Cs* female (A: $n_{Cs}=21$, $n_{Bully}=22$, T-test: $P=2.912 \times 10^{-11}$, $t=-8.759$, B: $n_{Cs}=24$, $n_{Bully}=24$, T-test: $P=5.425 \times 10^{-7}$, $t=-5.818$, F: $n_{Cs}=25$, $n_{Bully}=25$, T-test: $P=7.139 \times 10^{-4}$, $t=-3.617$, G: $n_{Cs}=30$, $n_{Bully}=24$, T-test: $P=8.743 \times 10^{-5}$, $t=-4.255$). (A') After mating ended, the proportion of time spent by each male to court performing UWE towards the just mated female was scored for 10 minutes ($n_{Cs}=21$, $n_{Bully}=22$, Fisher Pitman permutation test: $P<0.001$, $Z=3.239$). (B') The proportion of time spent by the males performing UWE an unfamiliar mated female was scored for 10 minutes ($n_{Cs}=24$, $n_{Bully}=24$, Fisher Pitman permutation test: $P=0.007$, $Z=-2.679$). (C) Total number of mating reached by *Cs* and *Bully* males over six consecutive mating with virgin *Cs* females (type II analysis of deviance table ($n_{Cs}=18^\sigma$, $n_{Bully}=18^\sigma$, Wald Chi-square test: $P=0.659$, $\chi^2=0.195$). (D) Mating duration of *Cs* and *Bully* males over six consecutive mating with virgin *Cs* females ($n_{Cs}=18^\sigma$, $n_{Bully}=18^\sigma$, LMM and Satterthwaite T-test: $P_{interaction}=0.001$, $t=-3.261$). A significant interaction between males' genotype and number of mating was found, indicating that *Cs* decreased their mating duration with the number of mating, while *Bully* males did not. (E) Proportion of mating that gave rise to progeny in consecutive mating assay ($n_{Cs}=18^\sigma$, $n_{Bully}=18^\sigma$, GLMM and Wald Chi-square test: $P_{male_genotype}=0.279$, $\chi^2=1.173$, $P_{mating}=0.847$, $\chi^2=2.019$). (F') Proportion of time spent by naïve *Cs* and *Bully* males to display UWE either toward *Cs*-mated or *Bully*-mated decapitated female over an observation period of 2 minutes ($n_{Cs-Cs-mated}=12$, $n_{Cs-Bully-mated}=15$, $n_{Bully-Cs-mated}=9$, $n_{Bully-Bully-mated}=10$, LMM and permutation test (Monte-Carlo test): $P_{male_genotype}<0.001$, $P_{mated_female}=0.457$). (G') Proportion of *Cs*-mated and *Bully*-mated females that re-mated 12 or 14 days after a first mating ($n_{Cs-12days}=19$, $n_{Bully-12days}=15$, $n_{Cs-14days}=11$, $n_{Bully-14days}=9$, GLM binomial and type II analysis of deviance table (Likelihood Ratio Test): $P_{mated_female}=3.639 \times 10^{-4}$, $LR\chi^2=12.708$, $P_{day}=0.385$, $LR\chi^2=0.756$). For graphs A, A', B, B', F, F', G and G', stars indicate significant differences (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) and statistical details are given in Figure 1-table supplement 1.

CHCs profiles between naïve *Cs* and *Bully* males (Figure 4A). Quantification of individual compounds showed that *Bully* males exhibited significantly higher levels of alkanes, monoenes, and methyl-alkanes (Figure 4B, Table 1).

We next examined whether mating with *Bully* males induces changes in CHC profiles of females compared to those that have mated with *Cs* males. To test this hypothesis, we extracted CHCs from the cuticles of *Cs* virgin females and *Cs* females mated to either a *Cs* or *Bully* males and compared their CHCs profile. PCA analysis showed that *Cs* virgin females form a distinct cluster from mated females (Figure 4C), indicating that mating induces substantial changes in female CHC profiles. The first two principal components accounted for most of the variance, and several compounds, including C23:1(6), C25:1(6), and cVA, contributed strongly to this separation (Figure 4-figure supplement 1). Consistent with this, individual compound analysis revealed that male-specific CHCs, such as 7-tricosene (7-T), 9-tricosene (9-T), and cVA, were significantly increased in mated females compared to virgins (Figure 4D–E, Table 2). These results are consistent with previous studies showing that 7-T and 9-T are acquired by females during mating (Everaerts et al., 2010; Laturney and Billeter, 2016).

We then compared CHC profiles between females mated with *Cs* or *Bully* males. While both groups exhibited the expected increase in male-derived compounds, females mated with *Bully* males showed significantly higher levels of 5-tricosene (5-T) and 5-pentacosene (5-P) (Figure 4D, Table 2). As *Bully* males also showed higher levels of both 5-P and 5-T compared to naïve *Cs* males (Figure 4B), it is possible that the elevated levels of these compounds observed in females could result from their transfer during mating.

cVA, a male-specific compound that acts as an anti-aphrodisiac and a mate-guarding molecule, is primarily transferred to the female's genital tract during mating, with only trace amounts deposited on the cuticle (Laturney and Billeter, 2016). Females with higher cVA levels (Kurtovic et al., 2007), combined with 7-T, are less attractive to males (Laturney and Billeter, 2016). Given that remating rate of *Bully*-mated females was higher than that of *Cs*-mated females (Figure 3G), we hypothesized that *Bully*-mated females would receive less cVA in their genital tract during mating, thus decreasing their attractiveness than when mating with *Cs* males. To test this, we conducted mating assays with *Cs* females paired with either *Cs* or *Bully* males, followed by dissection of the females' genital tracts to quantify the amount of cVA transferred. We found that females mated with *Bully* males had about three times lower cVA quantities in their genital tracts compared to those mated with *Cs* males (Figure 4F). Combined with higher quantities of 5-T and 5-P, reduced cVA level in genital tract likely contribute to the increased attractiveness and higher remating rates observed in females that previously mated with *Bully* males.

Overall, our findings demonstrate that naïve *Bully* males differ from *Cs* males in their CHC profiles and transfer a distinct quantity of chemical compounds to females during mating. These observations are consistent with a model in which changes in chemical signaling in *Bully* males is associated with both reduced mating success and weaker mate-guarding-related behaviors. In particular, reduced cVA transfer, combined with elevated levels of specific hydrocarbons such as 5-T and 5-P in mated females, may contribute to the increased attractiveness and remating propensity observed in females that previously mated with *Bully* males.

Reduced copulatory investment in aggressive males is associated with extended lifespan

Based on the main life-history trade-off between reproduction and survival, we next examined whether reduced pre- and post-copulatory investment observed in *Bully* males would be accompanied by increased longevity. To this end, we conducted longevity assays and monitored lifespan in *Cs* and *Bully* males. When housed individually, *Bully* males survived significantly longer than *Cs* males (Figure 5A, green vs yellow curves). Median survival (EC50) was increased from 57 days in *Cs* males to 82 days in *Bully* males (Figure 5A), and maximum lifespan was also extended from 90 days in *Cs* males to 100 days in *Bully* (Figure 5A). To assess whether this difference could reflect hybrid vigor, we measured lifespan in heterozygous *Bully* males generated

Compounds	Mean concentration (ng)		Statistics
	Virgin males		♂ Cs vs.
	♂ Cs (n = 10)	♂ Bully (n = 10)	♂ Bully
Alkanes	252.86 ± 33.16	536.23 ± 114.02	*
nC21	38.83 ± 5.55	15.18 ± 2.96	**
nC22	21.29 ± 3.34	20.32 ± 3.96	
nC23	166.41 ± 23.18	387.19 ± 81.40	*
nC24	5.77 ± 0.81	14.83 ± 2.91	**
nC25	17.98 ± 2.31	87.98 ± 19.52	***
nC27	4.34 ± 0.20	3.91 ± 1.26	
nC29	0.76 ± 0.15	6.82 ± 2.59	**
Monoenes	758.00 ± 118.42	1927.26 ± 393.72	**
C22:1(9)	39.57 ± 13.85	47.66 ± 26.96	
C22:1(7)	10.42 ± 1.74	12.15 ± 2.36	
C23:1(9) (9-T)	56.78 ± 10.10	82.57 ± 15.02	
C23:1(7) (7-T)	473.73 ± 87.69	886.16 ± 196.86	
C23:1(5) (5-T)	17.90 ± 3.64	87.65 ± 20.23	***
C24:1(9)	13.00 ± 2.51	16.82 ± 3.10	
C24:1(7)	3.75 ± 0.79	10.27 ± 1.43	***
C24:1(5)	2.51 ± 0.46	4.70 ± 0.67	*
C25:1(9)	63.96 ± 9.86	53.13 ± 12.71	
C25:1(7) (7-P)	17.62 ± 3.10	137.17 ± 26.94	***
C25:1(5) (5-P)	54.44 ± 10.38	568.84 ± 114.33	***
C27:1(9)	/	/	
C27:1(7)	4.34 ± 0.86	20.14 ± 1.26	***
C27:1(5)	/	/	
C29:1(9)	/	/	
C29:1(7)	/	/	
Dienes			
7.11C23:2	/	/	
7.11C25:2	/	/	
9.13C27:2	/	/	
7.11C27:2 (7.11-HD)	/	/	
9.13C29:2	/	/	
7.11C29:2	/	/	
Methyl-alkanes	122.86 ± 17.76	233.55 ± 54.05	*
2MeC22	/	/	
2MeC24	5.24 ± 1.01	4.54 ± 0.68	
2MeC26	82.69 ± 12.71	132.91 ± 32.21	
2MeC28	34.94 ± 5.47	96.10 ± 21.90	**
11-cis-vaccenyl acetate (cVA)	1.91 ± 0.26	1.78 ± 0.33	
Total CHC	1135.63 ± 162.31	2698.83 ± 558.47	**

Table 1. Comparison of CHCs concentrations between naïve Cs and Bully males.

Mean ± SE concentrations are provided in ng for each compound and each group of compounds. In statistics columns, stars represent significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001). Statistical details 986 are given in [Figure 1-table supplement 1](#).

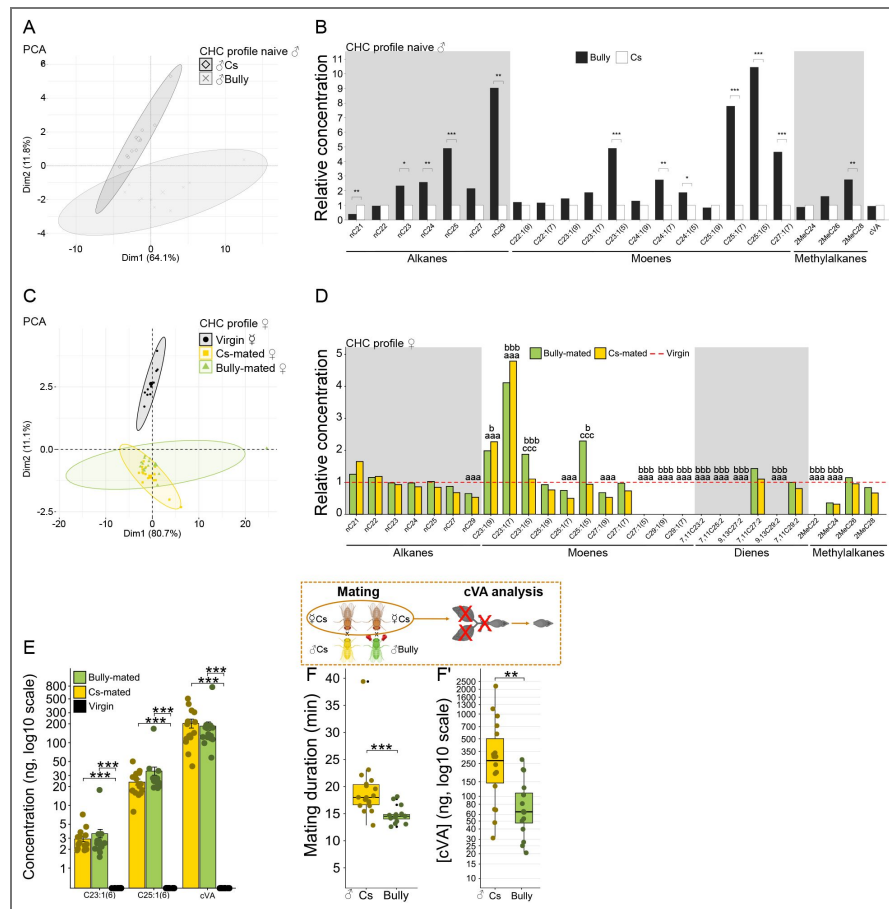


Figure 4. Hyper-aggressive males exhibit altered CHCs profiles and transfer reduced amounts of mate-guarding pheromones to females.

(A) PCA analysis on mean concentration for each Cuticular Hydrocarbon (CHCs) compound of naive Cs and *Bully*. Their CHCs profiles were found similar ($n_{Cs}=10$, $n_{Bully}=10$, PerMANOVA: $P=0.002$, $F=6.789$, $R^2=0.274$). (B) Relative concentrations of *Bully*'s CHCs were calculated with respect to those of Cs males. The CHCs levels in Cs males were normalized to 1, and *Bully*'s CHC concentrations were expressed relative to this reference ($n_{Cs}=10$, $n_{Bully}=10$, LMM and permutation test (Monte-Carlo test): $P=0.009$, post-hoc: $P_{Alkanes}=0.036$, $P_{Monoenes}=0.002$, $P_{Methyl\ alkanes}=0.048$, $P_{nC21}<0.001$, $P_{C22:1(9)}=0.892$, $P_{C22:1(7)}=0.568$, $P_{cVA}=0.731$, $P_{nC22}=0.854$, $P_{C23:1(9)}=0.175$, $P_{C23:1(7)}=0.062$, $P_{C23:1(5)}<0.001$, $P_{nC23}=0.014$, $P_{C24:1(9)}=0.345$, $P_{C24:1(7)}<0.001$, $P_{24:1(5)}=0.015$, $P_{nC24}=0.002$, $P_{2MeC24}=0.548$, $P_{C25:1(9)}=0.524$, $P_{C25:1(7)}<0.001$, $P_{C25:1(5)}<0.001$, $P_{nC25}<0.001$, $P_{2MeC26}=0.159$, $P_{C27:1(7)}<0.001$, $P_{nC27}=0.078$, $P_{2MeC28}=0.003$, $P_{nC29}=0.005$). (C) PCA analysis on mean concentration for each CHCs compound of virgin, Cs-mated and *Bully*-mated females ($n_{virgin}=10$, $n_{Cs-mated}=16$, $n_{Bully-mated}=14$, PerMANOVA: $P=0.017$, $F=2.594$, $R^2=0.123$, post-hoc: $P_{virgin_Cs-mated}=0.001$, $F=19.434$, $R^2=0.447$, $P_{virgin_Bully-mated}=0.034$, $F=2.266$, $R^2=0.093$, $P_{Cs-mated_Bully-mated}=0.746$, $F=0.428$, $R^2=0.015$). (D) Relative concentrations of CHCs compounds in mated females compared to virgin females. CHCs levels in virgin females were normalized to 1, and the concentrations in mated females were expressed relative to this baseline (LMM and permutation test ($n_{virgin}=10$, $n_{Cs-mated}=16$, $n_{Bully-mated}=14$, Monte-Carlo test: $P=0.618$). Even though the p-value was not significant, post-hoc: $P_{Alkanes}=0.705$, $P_{Monoenes}=0.294$, $P_{Dienes}=0.715$, $P_{Methyl\ alkanes}=0.750$, $P_{nC21}=0.158$, $P_{nC22}=0.927$, $P_{7,11C23:2}<0.001$, $P_{2MeC22}<0.001$, $P_{C23:1(9)}=0.023$, $P_{C23:1(7)}=0.001$, $P_{C23:1(5)}=0.016$, $P_{nC23}=0.908$, $P_{nC24}=0.899$, $P_{7,11C25:2}<0.001$, $P_{2MeC24}<0.001$, $P_{C25:1(9)}=0.832$, $P_{C25:1(7)}=0.047$, $P_{C25:1(5)}<0.001$, $P_{nC25}=0.769$, $P_{9,13C27:2}<0.001$, $P_{7,11C27:2}=0.747$, $P_{2MeC26}=0.884$, $P_{C27:1(9)}=0.016$, $P_{C27:1(7)}=0.625$, $P_{C27:1(5)}<0.001$, $P_{nC27}=0.367$, $P_{9,13C29:2}<0.001$, $P_{7,11C23:2}=0.899$, $P_{2MeC28}=0.413$, $P_{C29:1(9)}<0.001$, $P_{C29:1(7)}<0.001$, $P_{nC29}=0.022$). Significant differences are indicated by letters: a indicates a significant difference between virgin and Cs-mated females; b indicates a significant difference between virgin and *Bully*-mated females; and c indicates a significant difference between Cs- and *Bully*-mated females (a $P < 0.05$; aa $P < 0.01$; aaa $P < 0.001$). (E) Mean $\pm$ SE concentration in ng of CHCs compounds that were not present in virgin females ($n_{virgin}=10$, $n_{Cs-mated}=16$, $n_{Bully-mated}=14$, $P_{C23:1(6)}<0.001$, $P_{C25:1(6)}<0.001$, $P_{cVA}=0.001$). (F) Mating duration of Cs and *Bully* males with a Cs female ($n_{Cs-mated}=17$, $n_{Bully-mated}=14$, T-test: $P=0.003$, $t=-3.359$). (F') cVA concentration measured within Cs- and *Bully*-mated genitalia tract right after mating ($n_{Cs-mated}=17$, $n_{Bully-mated}=14$, Fisher Pitman permutation test: $P=0.001$, $Z=-2.390$). For graphs E, F and F', stars indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and statistical details are given in Figure 1-table supplement 1.

Compounds	Mean concentration (ng)			Statistics		
	Virgin Cs ♀	Mated Cs ♀		♂ Cs vs.	♂ Cs vs.	Cs-mated ♀ vs.
	♂ Cs (n = 10)	Cs-mated ♀ (n = 16)	Bully-mated ♀ (n = 14)	Cs-mated ♀	Bully-mated ♀	Bully-mated ♀
Alkanes	227.22 ± 15.64	193.89 ± 19.06	220.99 ± 44.60			
nC21	4.37 ± 0.32	7.20 ± 0.74	5.47 ± 1.52			
nC22	5.20 ± 0.42	6.15 ± 0.71	6.02 ± 1.49			
nC23	80.02 ± 7.20	74.21 ± 7.40	78.56 ± 11.28			
nC24	10.29 ± 0.86	8.84 ± 0.98	10.07 ± 2.32			
nC25	72.97 ± 4.81	61.60 ± 5.88	74.89 ± 16.93			
nC27	47.91 ± 4.39	32.45 ± 3.75	41.76 ± 10.42			
nC29	6.47 ± 0.52	3.44 ± 0.30	4.21 ± 1.15	***		
Monoenes	171.43 ± 11.19	190.32 ± 19.14	250.44 ± 69.17			
C23:1(9) (9-T)	2.55 ± 0.13	5.79 ± 0.63	5.07 ± 1.16	***	*	
C23:1(7) (7-T)	12.83 ± 0.92	61.45 ± 6.92	52.76 ± 12.45	***	***	
C23:1(6)	0,00	2.91 ± 0.09	3.54 ± 1.09	***	***	
C23:1(5) (5-T)	2.86 ± 0.21	3.16 ± 0.35	5.37 ± 1.07		*	*
C25:1(9)	18.91 ± 1.92	14.40 ± 1.74	17.51 ± 5.62			
C25:1(7) (7-P)	43.11 ± 2.41	21.49 ± 2.52	32.11 ± 10.52	***		
C25:1(6)	0,00	23.48 ± 2.60	35.23 ± 10.26	***	***	
C25:1(5) (5-P)	21.85 ± 1.43	20.52 ± 2.67	50.09 ± 14.31		*	***
C27:1(9)	28,00 ± 1.94	14.78 ± 1.57	18.99 ± 4.77	***		
C27:1(7)	30.57 ± 2.34	22.35 ± 2.77	29.78 ± 8.39			
C27:1(5)	7.27 ± 5.74	Trace	Trace	***	***	
C29:1(9)	1.56 ± 0.12	Trace	Trace	***	***	
C29:1(7)	1.93 ± 0.15	Trace	Trace	***	***	
Dienes	319.86 ± 28.80	280.70 ± 22.38	358.05 ± 109.72			
7,11C23:2	4.94 ± 0.45	Trace	Trace	***	***	
7,11C25:2	8.33 ± 0.77	Trace	Trace	***	***	
9,13C27:2	8.21 ± 0.53	Trace	Trace	***	***	
7,11C27:2 (7,11-HD)	151.71 ± 17.63	167.05 ± 13.62	216.99 ± 66.11			
9,13C29:2	5.57 ± 0.62	Trace	Trace	***	***	
7,11C29:2	141.11 ± 13.85	113.65 ± 9.84	141.06 ± 43.73			
Methyl-alkanes	226.7 ± 23.87	186.84 ± 18.92	226.48 ± 59.16			
2MeC22	2.61 ± 0.17	Trace	Trace	***	***	
2MeC24	16.87 ± 1.28	5.33 ± 0.67	5.99 ± 1.89	***	***	
2MeC26	145.81 ± 18.14	138.89 ± 14.05	167.05 ± 41.77			
2MeC28	64.02 ± 6.79	42.63 ± 4.58	53.44 ± 15.58			
11-cis-vaccenyl acetate (cVA)	0,00	202.66 ± 31.99	183.55 ± 46.33	***	***	
Total CHC	947.82 ± 62.95	1054.41 ± 104.31	1239.50 ± 327.40			

Table 2. Comparison of CHCs concentrations among virgin, Cs-mated, and Bully-mated females.

Mean ± SE concentrations are provided in ng for each compound and each group of compounds. In the statistics columns, stars indicate significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001). Statistical details are given in [Figure 1-table supplement 1](#).

by crossing Cs females with *Bully* males. The survival of these heterozygous males was comparable to that of homozygous *Bully* males (Figure 5A [↗](#), red curve), with males showing an EC50 of 79 days and a maximum lifespan of 106 days. This result indicates that *Bully* background positively influence males' lifespan and argue against hybrid vigor as the main explanation for the observed phenotype. We next evaluated survival under more naturalistic social conditions by maintaining males in mixed-sex groups. Under these conditions, *Bully* males again showed increased survival compared to Cs males, with both higher median lifespan and extended maximum lifespan (Figure 5B [↗](#)). *Bully* males had a median survival of 56 days and a maximum lifespan of 71 days, compared to Cs males, which had a median survival of 43 days and all died by day 67 (Figure 5B [↗](#)).

We then assessed how reproductive performance varies across the lifespan. Individual males were paired weekly with virgin Cs females until death, and mating success and mating duration were recorded. Throughout their lifespan, *Bully* males consistently displayed shorter MD than Cs males (Figure 5C [↗](#)). While mating duration increased with age in Cs males, a pattern reported in another *Drosophila* species (Dhole and Pfennig, 2014 [↗](#)), this effect was not observed in *Bully* males, whose MD remained stable across mating and throughout their lives (Figure 5C [↗](#), green). Analysis of individual male slopes confirmed this divergence: most Cs males showed increasing MD across mating events, while *Bully* males did not (Figure 5-figure supplement 1 [↗](#)). Mating success followed a similar age-dependent pattern. *Bully* males showed reduced mating success compared to Cs males during early and mid-adulthood (until 70 days), but continued to mate at ages when Cs males had ceased reproductive activity (Figure 5D [↗](#)). While all Cs males stopped mating by day 78 and died by day 97 (Figure 5D [↗](#), yellow), *Bully* males maintained mating activity until day 98 and survived up to 119 days (Figure 5D [↗](#), green). In addition, late-life mating involving 98-days-old *Bully* males produced viable offspring (Figure 5D [↗](#), green), demonstrating that *Bully* males remain fertile at older ages.

Together, our findings demonstrate that *Bully* males exhibit both reduced copulatory investment and extended lifespan, as well as a shift in the temporal pattern of reproductive activity. These observations are consistent with a scenario in which reduced investment in mating success and mating duration is associated with extended lifespan and sustained reproductive output later in life. Rather than enhancing early-life reproductive success, *Bully* males maintain reproductive capacity over a longer time window, suggesting a redistribution of reproductive effort across the lifespan.

Discussion

Our study reveals that selection for aggressive traits in *D. mel.* males correlates with changes in key life-history trade-off, in which survival is extended while reproductive success is reduced. This pattern challenges the hypothesis that aggression necessarily enhances mating success (Andersson, 1994 [↗](#); West-Eberhard, 1983 [↗](#)), and indicates that aggressive phenotypes may incur substantial reproductive costs. Consistent phenotypes across independent *Bully* lines support a link between aggression selection and the reproductive traits described here. However, the absence of additional selected lines limits our ability to fully exclude contributions of genetic drift or line-specific effects. Because selection for aggression was applied specifically to males, we used a standardized female background (Cs) to isolate male-driven effects. While this design minimizes female variability, it does not fully disentangle male and female genotype interactions. However, the persistence of key phenotypes in *Bully* × *Bully* pairings and across independent selected lines supports a primary contribution of male genotype. Aggression therefore does not act as an isolated fitness-enhancing trait, but rather as one component of a coordinated life-history strategy in which gains in competitive ability are coupled to constraints on reproductive performance. The increased survival of highly aggressive males further raises important questions regarding the evolutionary consequences of aggression. While *Bully* males have reduced mating success compared to Cs males, their extended lifespan may allow them to sustain mating activity over longer periods and produce viable offspring at later ages, challenging the classical view that early

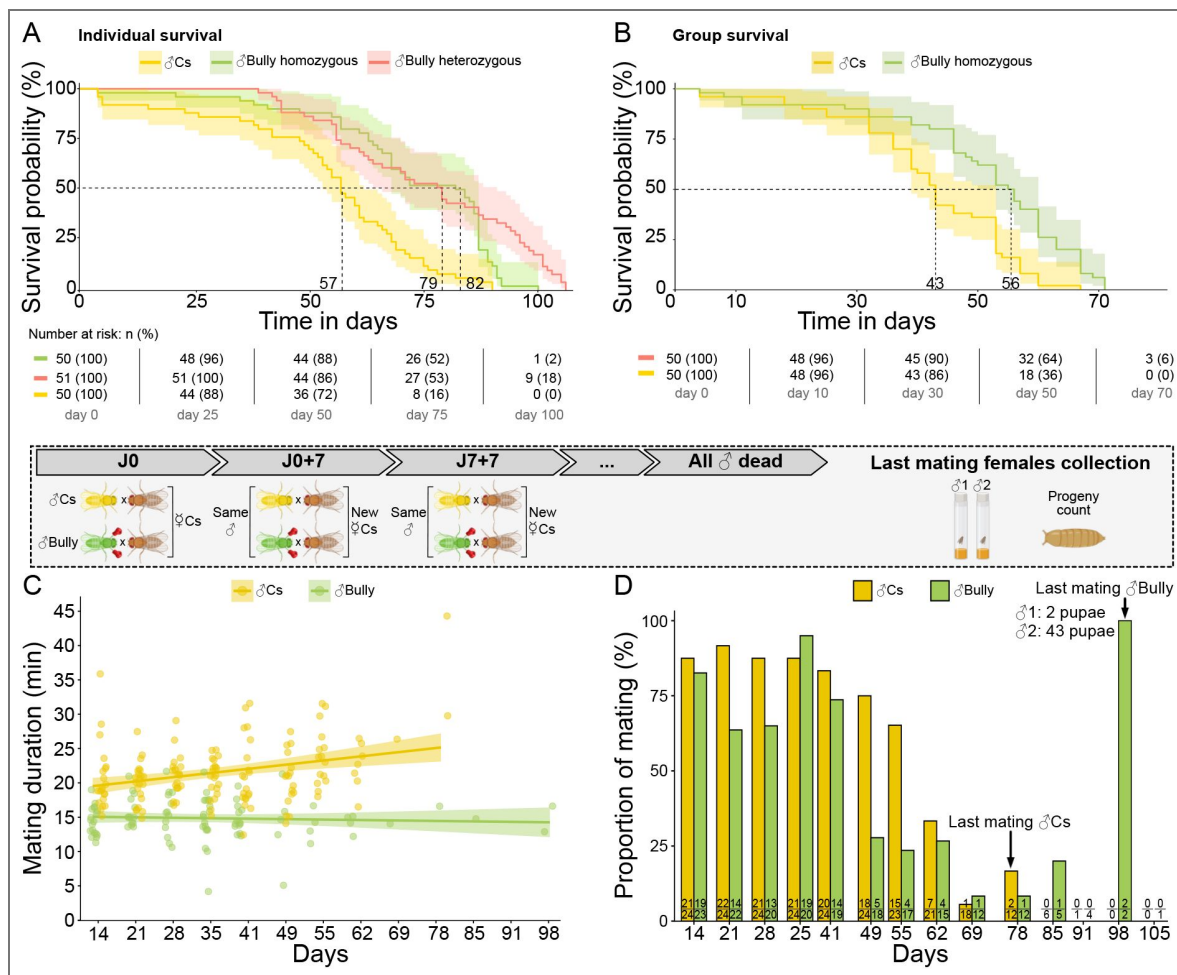


Figure 5. Aggressive males offset their reduced reproductive success with a longer lifespan and preserved reproductive success in later life.

(A) Survival curves of Cs (yellow) and homozygous (green) and heterozygous (red) *Bully* males (Weibull regression model ($n_{Cs}=50$, $n_{Bully_homo}=50$, $n_{Bully_hetero}=51$, Likelihood Ratio Test): $\chi^2=23.91$, $p=2.6 \times 10^{-5}$, post-hoc: $P_{Cs - Bully_homo} < 0.001$, $t_{ratio} = 4.061$, $P_{Cs - Bully_hetero} < 0.001$, $t_{ratio} = 5.088$, $P_{Bully_homo - Bully_hetero} = 0.318$, $t_{ratio} = -1.001$). Cs males had a median survival (EC_{50}) of 57 days, with the last individual dying at day 90 (yellow), while progeny from homozygous *Bully* males showed an EC_{50} of 82 days, with the final death at day 101 (green) and heterozygous *Bully* males showed an EC_{50} of 79 days, with the final death at day 106 (red). (B) Survival curves of group of 10 Cs and 10 homozygous *Bully* males raised in group with 12 Cs females. *Bully* males had a median survival (EC_{50}) of 56 days, with the last individual dying at day 71 (red line), while Cs males showed an EC_{50} of 43 days, with the final death at day 67 (yellow line) ($n_{Cs}=50$, $n_{Bully_hetero}=50$, Cox proportional hazards regression model with stratification by group (Likelihood Ratio Test): $P=7 \times 10^{-5}$, $\chi^2=15.94$). Statistical details are given in Figure 1-table supplement 1. (C) Mating duration of Cs and *Bully* males with virgin Cs females across their lifespan ($n_{Cs-J0}=24$, $n_{Bully-J14}=23$, LMM and Satterthwaite T-test: $P_{interaction} = 3.360 \times 10^{-5}$, $t=4.227$). A significant interaction between male genotype and age was observed: *Bully* males maintained a stable mating duration over time, whereas Cs males showed an increase in mating duration with successive mating events. (D) Number of mating throughout the lifespan of Cs and *Bully* males ($n_{Cs-J0}=24$, $n_{Bully-J14}=23$, GLM binomial and Wald Z-test: $P_{interaction}=0.039$, $Z=-2.068$). Numbers in bars indicate the proportion of mating. Arrows denote the last mating event and the number of offspring produced. Statistical details are given in Figure 1-table supplement 1. Table 1: Comparison of CHCs concentrations between naïve Cs and *Bully* males. Mean $\pm$ SE concentrations are provided in ng for each compound and each group of compounds. In statistics columns, stars represent significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Statistical details are given in Figure 1-table supplement 1.

reproductive success is the primary driver of male fitness (Flatt, 2011 [↗](#)). This shift suggests that alternative reproductive strategies, even those associated with lower mating success and mate-guarding, can persist if they confer long-term survival advantages under laboratory conditions.

At the behavioral level, highly aggressive *Bully A* males exhibit changes in both pre- and post-copulatory phases. They achieve lower mating success and engage in shorter copulations than less aggressive *Cs* males, suggesting reduced efficiency in mate acquisition and potentially in sperm transfer. Despite higher courtship motivation after mating, *Bully* males do not achieve higher remating success. Instead, females previously mated with these males remate more readily, indicating reduced mate-guarding efficiency and possibly increased attractiveness. These observations point to a decoupling between courtship intensity and mating outcome, indicating that increased behavioral drive does not translate into reproductive advantage. While our study did not show a causal link, our results suggest that artificial selection applied to male aggressive traits to produce the *Bully A* line, is associated with changes in reproductive traits, enhanced survival, and increased CHCs levels.

These behavioral differences are closely associated with significant modifications in chemical signaling. Cuticular hydrocarbons (CHCs), which are crucial to mate recognition and sexual communication (Billeter and Wolfner, 2018 [↗](#); Yew and Chung, 2017 [↗](#)) differ significantly between *Bully A* and *Cs* males. We show that naïve highly aggressive individuals present distinct chemical profiles compared to unselected wild-type flies, with higher levels of most CHCs. Importantly, CHCs also contribute to physiological functions such as protection against desiccation (Nayal et al., 2024 [↗](#)), suggesting that changes in CHC composition may simultaneously influence both reproductive performances and survival. Thus, selection on aggressive traits may have been accompanied by modifications in overall chemical signaling that would impact both physiological resilience and social behaviors.

Although higher CHC level might be expected to enhance male attractiveness, *Bully* males display reduced mating success, suggesting a non-linear or dose-dependent relationship between CHC levels and female acceptance. Female mate choice relies on finely tuned blends of chemical cues, and changes in these optimal ratios may impair signal interpretation (Billeter and Wolfner, 2018 [↗](#)). In this context, excessive levels of specific CHCs could reduce courtship efficiency rather than enhance it. In addition to major compounds such as 7-tricosene, male attractiveness and courtship performance are influenced by other CHC, including palmitoleic acid, which signals through the Or47b receptor and modulates courtship behavior in an age-dependent manner (Dweck et al., 2015 [↗](#); Kohlmeier et al., 2021 [↗](#); Lin et al., 2016 [↗](#)). This pathway enables older males to initiate courtship more rapidly and with greater intensity, potentially compensating for age-related declines in competitiveness. Although palmitoleic acid levels were not measured here, change in level of such compounds could further contribute to decreasing mating success despite overall higher CHC level.

Modifications in chemical communication extend into the post-mating context, where they potentially intersect with male reproductive performance. Females mated with *Bully* males display distinct CHC profiles, with higher levels of 5-tricosene (5-T) and 5-pentacosene (5-P), together with reduced transfer of cis-vaccenyl acetate (cVA) into the female genital tract, which may fail to efficiently decrease their attractiveness, thus triggering higher remating rate. This interpretation is consistent with previous studies showing that lower cVA levels decrease the effectiveness of chemical mate guarding and accelerate the recovery of female attractiveness (Ejima, 2015 [↗](#)). At the same time, the behavioral effects of cVA depend on its interaction with other CHCs (Laturney and Billeter, 2016 [↗](#); Verschut et al., 2023 [↗](#)), suggesting that changes in the overall chemical profile may further modulate how mated females are perceived by subsequent males. These results indicate that selection on aggressive traits can indirectly shape post-copulatory sexual selection by altering female chemical signatures. While causal manipulation of CHC profiles would be required to directly test their functional role, the consistent association between CHC composition, cVA transfer, and behavioral outcomes across assays of our study identifies chemical signaling as a strong candidate mechanism.

Taken together, these observations support the idea that cuticular hydrocarbons constitute a central mechanistic substrate linking aggression, reproduction, and survival. Rather than hypothesizing selection on multiple behavioral traits, the phenotypic differences observed in our study may alternatively arise from selection acting on genes involved in chemical signaling. Because CHCs influence both social communication and physiological resilience (Nayal et al., 2024 [↗](#)), selection that have acted on CHC production or emission would be expected to generate correlated effects across social behavior, reproductive success, and survival, and physiological resilience, thereby producing the unique life-history pattern observed in *Bully* males.

At the genetic level, this integration may be supported by pleiotropic factors or by the co-selection of partially independent loci affecting multiple traits. In this study, we mainly focused on *Bully A* males, as transcriptomic analyses have identified a limited set of genes differentially expressed between *Bully A* and *Cs* males, including *CG13646*, a putative transmembrane transporter whose down-regulation increases aggressive behavior (Chowdhury et al., 2017 [↗](#)). These candidate genes provide an entry point for dissecting the molecular and genetic logic underlying the observed phenotypes, particularly in determining whether changes in aggression, CHC composition, survival and life-history traits arise from common genetic regulation.

In summary, our findings indicate that selection for male aggression is associated with a coordinated shift in reproductive investment, chemical signaling and lifespan, consistent with a redistribution of reproductive effort across the lifespan. This highlights aggression as a potential entry point into the coordinated evolution of reproductive and physiological traits.

Material and methods

Experimental model and subject details

Fly stocks

Drosophila melanogaster strains were raised at 25°C under a 12h:12h light/dark cycle (LD= 9:00a.m. – 9:00p.m.) on standard fly food (for 1l of water, 74g corn flour, 28g yeast, 40g sugar, 7.5g agar, 10ml Moldex). Canton-S (*Cs*) flies (from Edward Kravitz's laboratory, *RRID:BDSC_64349* [↗](#)) were used as wild-type. The *Bully A*, B and C lines were originally selected in Edward Kravitz's laboratory from *Cs* during 37 generations (Penn et al., 2010 [↗](#)), resulting in hyper-aggressive male flies (Garbaczewska et al., 2013 [↗](#); Hopkins et al., 2019 [↗](#)).

Experimental arena

Experimental arenas were described in details in Trannoy *et al.*, 2015 (Trannoy et al., 2015a [↗](#)). Briefly, resin blocks containing three circular behavioral arenas (dimension: 2.3 cm diameter, 1.7 cm height) were used to conduct behavioral assays. Each arena was divided in two equal sizes by a plastic divider allowing flies to acclimate without physical interactions with the second fly. Then, dividers are removed from the arenas to start the behavioral assays. All behavioral assays were performed in empty arenas, except for aggression assays and courtship assays with decapitated females, in which a food cup (1.5 cm diameter, 1 cm height) containing fresh standard fly food with a drop of fresh yeast paste on the surface was placed in the center of each arena.

Method details

Commonalities between all behavioral assays

Late-stage pupae were collected from stocks raised at 25°C. Male pupae were individually placed in 5ml vials (Dutscher, ref 390597) containing about 1ml of standard fly food while female pupae were placed in group of 15 in ~47ml vials (Dutscher, ref 789001B) containing about 15ml of standard fly food. All vials were placed at 25°C for 7 days. Seven-day-old individuals were randomly inserted in arenas by negative geotaxis, and allow to acclimate for 5 min before experiments. All behavioral assays were performed at 25°C. All tests were recorded with Basler camera Ace1970 through the StreamPix8 multi video recording software (*norpix.com* [↗](#)) and analyzed with BORIS software (Friard and Gamba, 2016 [↗](#)). After 15 min or 30 min, if pairs of flies

had not yet lunged or mated respectively, we considered that no aggression or mating occurred and arenas were not considered for analysis. No power analyses were performed prior to the experiments, as we chose to use sample sizes of approximately 20 replicates, which is considered standard practice in the field.

Aggression assays

Two days prior to the aggression assays, males were anesthetized with CO₂, and half were marked with a small white dot on the thorax before being returned to their original isolation vials. Aggression tests were conducted between ZT0 and ZT3 using size-matched male pairs. Males competed for access to a central food cup containing yeast. The following behaviors were recorded for 10 minutes following the first lunge: latency to lunge (i.e., the time between the initial encounter on the food cup and the first lunge), total number of lunges, and number of boxing events.

Mating assays

Seven-day-old individuals were tested in arenas without any food source. Latency to court and to mate, defined as the time between the first encounter and either the first Unilateral Wing Extension (UWE) or the initiation of mating, respectively, were measured. Total UWE duration was recorded over a 10-minute period starting from the first encounter. Mating proportion (proportion of fly pairs that succeeded to mate) and mating duration were also scored. In all assays, males were reared in social isolation, except for [Figure 1H](#), where they were housed in groups of 10 males for 7 days.

Mating assays with decapitated females

Females were anesthetized with CO₂ and placed under the binocular for head removal. Experiments with decapitated females were performed in arenas with food cup filled with fresh fly food but without yeast. The females' bodies were gently pressed into the food cup to ensure they remained completely immobile, as males can move their bodies during courtship. This allows to maintain a constant distance between the two decapitated females across trials, thereby improving the reproducibility of the assay. Female bodies were positioned in the food cup with the abdomen raised, allowing males to initiate and complete copulation.

Multiple consecutive mating events assays

Single naïve Cs and *Bully* males were sequentially paired with six virgin Cs females. For each male, number of mating and each mating duration were recorded. After each mating, the mated female was transferred to a 5 ml vial containing fly food to assess progeny production.

Mating assays throughout the lifespan

24 Cs and *Bully* males were reared in isolation and paired with seven-day-old virgin Cs females for mating assays, conducted every seven days until all males had died. At each time point, mating duration and mating success (proportion of mating) were recorded. After all Cs males had died, females that had mated with *Bully* males were transferred to 5 ml vials containing fly food to assess progeny production.

Attractiveness assays

Measure of post-mating UWE

Mating assays were performed by pairing naïve Cs and *Bully* males with a Cs female. Right after mating end, total time that males displayed UWE for 10 minutes towards the just-mated females was scored.

Measure of post-mating UWE with switched females

Mating assays were performed with naïve Cs or *Bully* males and virgin Cs females. Immediately after mating ends, females were switched: Cs males interacted with *Bully*-mated females and *Bully* males interacted with Cs-mated females. Then total time of UWE towards the mated females was scored during 10 min.

Measure of post-mating UWE with decapitated females

Mating assays were conducted using naïve *Cs* or *Bully* males paired with virgin *Cs* females. Following copulation, mated females were decapitated. One decapitated *Cs*-mated and one *Bully*-mated female were then placed on the surface of the food cup. A new naïve *Cs* or *Bully* male was introduced and allowed to interact with both decapitated females. The duration of Unilateral Wing Extensions (UWE) directed toward each female was recorded over a 2-minute period following the first encounter. Arenas in which males interacted with only one of the two females were excluded from the analysis, as no choice could be determined.

Males' perception assays

A decapitated *Cs* male and female were placed on a food cup in presence of either a naïve *Cs* or a *Bully* male. The time spent by the focal male courting each decapitated fly was quantified over a 2-minute period following the first unilateral wing extension (UWE). Arenas in which the male interacted with only one of the two decapitated flies were excluded from the analysis, as no choice could be determined.

Re-mating assays

Mating assays were performed with naïve *Cs* or *Bully* males and virgin *Cs* females. Mated-females were then individually placed in 5ml vials containing about 1ml of standard fly food and kept at 25°C. 12 or 14 days after the initial mating, mated-females were paired with a new naïve *Cs* male in empty arena and the proportion of re-mating during 30 min was scored.

Survival assays

Survival with isolated males

Cs, homozygous and heterozygous *Bully* late-stage pupae were individually isolated in 5 ml vials containing 1ml of standard fly food and kept at 25°C until adult emergence. The number of dead individuals was recorded daily until all flies had died. To maintain food quality, flies were transferred weekly to fresh vials containing approximately 1 ml of new fly food.

Survival with group-raised males

Late-stage *Cs* and homozygous *Bully* male pupae were placed in group with *Cs* female pupae in ≈47ml vials containing 15mL standard fly food and kept at 25°C until adult emergence. Groups were composed of 10 males (either *Cs* or *Bully*) and 12 *Cs* females. The number of dead males was recorded daily until they all had died. To maintain food quality, flies were transferred twice a week to fresh vials containing approximately 15mL of new fly food.

CHCs analysis

Extraction and analysis of cuticular hydrocarbons (CHCs) of naïve *Cs* and *Bully* males, as well as virgin *Cs*, *Cs*- and *Bully*-mated females were performed in Jean-Christophe Billeter's lab, Groningen, Netherlands, with a Gas Chromatography coupled with Flame Ionization Detection (GC-FID) as already described (Laturney and Billeter, 2016 [\[2\]](#)). Briefly, single whole flies were put in a 2ml glass vial (Supelco certified vial kit, 2ml clear glass vial 12×32mm and Supelco inserts) containing 50µl of nC18+nC26 10 ng/µl standard solution (made in Billeter's lab) diluted in n-Hexane 99%+ (Acrös Organic®). Vials were then vortexed at the lowest speed for 2min, afterward flies' body were gently removed from vials using a "J-shape" paper clip sliced at the end, taking care not to damage the body to prevent from hemolymph contamination. Finally, samples were placed in GC-FID for apolar compounds analysis only.

cVA quantification into genital tract

Mating assays were performed with naïve *Cs* or *Bully* males and virgin *Cs* female. Right after mating, females were transferred into a 5ml vial previously put in ice in -80°C fridge to make them fall asleep quickly and prevent from sperm ejection. Mated-females' genital tract was dissected in PBS with precision forceps under a binocular loupe. Protocol was already employed in (Verschut et al., 2022 [\[2\]](#)). Briefly, abdomen was torn to make ovaries and genital tract out and

visible, then ovaries, oviduct, spermathecae, parovaria and cuticula were removed to keep only the uterus, taking care not to injure it to prevent from sperm leak. Each uterus was inserted in a 2ml glass vial (Supelco certified vial kit, 2ml clear glass vial 12×32mm and Supelco inserts) containing 50µl of n-Hexane 99%+ (Acrös Organic) for 1h at ambient temperature. Hexane solution was then evaporated under helium flow to prevent from cVA oxidation with dioxygen. Samples were sent to Jean-Christophe Billeter's lab for GC-FID analysis of cVA, as describe above in CHCs analysis method part.

Quantification and statistical analysis

No data have been removed from the statistical analysis (except arenas where no choice was observed within the males' perception assays, as described below). Some experiments were independently replicated by different authors to reduce potential bias. All statistical analyses were performed on R software version 4.0. (RC Team, 2020 [\[1\]](#)) with packages (Fox and Weisberg, 2019 [\[2\]](#)), coin (Hothorn et al., 2006 [\[3\]](#)), dplyr (Wickham et al., 2023 [\[4\]](#)), factoextra (Kassambara and Mundt, 2020 [\[5\]](#)), FactoMineR (Lê et al., 2008 [\[6\]](#)), lme4 (Bates et al., 2015 [\[7\]](#)), lmerTest (Kuznetsova et al., 2017 [\[8\]](#)), MASS (Venables and Ripley, 2002 [\[9\]](#)), MuMIn (Bartoń, 2023 [\[10\]](#)), nlme (Pinheiro et al., 2022 [\[11\]](#)), performance (Lüdtke et al., 2021 [\[12\]](#)), pgirmess (Giraudeau, P, 2018 [\[13\]](#)), predictmeans (Luo et al., 2021 [\[14\]](#)), survival (Therneau, 2022 [\[15\]](#)), vegan (Oksanen J, 2022 [\[16\]](#)).

Comparisons of means ($k = 2$) were performed with a T-test when possible (*i.e.* mating duration) otherwise a Fisher Pitman permutation test was used (*i.e.* UWE, latency to court and to mate). For comparisons with more than 2 conditions, a linear model (LM) was used and tested either with a type II ANOVA when possible (*i.e.* mating duration) or a permutation test instead (*i.e.* latency to lunge and CHC concentrations). For comparison of proportions and counting events, a type II analysis of deviance table with a Likelihood Ratio Test (*i.e.* mating success, proportion of female re-mating, number of lunge and boxing events) or a Wald Chi-square test (*i.e.* number of consecutive mating events) was performed. A Principal Component Analysis (PCA) was performed to see different CHC profiles and tested with a PerMANOVA and a SIMPER analysis. Survival data were analysed with a Weibull regression model and a Likelihood Ratio Test.

When paired data, dependency was considered thanks to linear mixed model (LMM) with the source of dependency as random factor. Data following a distribution other than a normal distribution (*e.g.* binomial or Poisson distribution) were computed in a generalized linear model (GLM) (*i.e.* mating success, number of consecutive mating events, number of mating giving progeny, proportion of females re-mating, number of lunges and boxing events). When necessary, interactions were considered within models and tested.

All post-hoc comparisons were performed with a sequential Bonferroni correction on the alpha level using either Holm method (Holm, 1979 [\[17\]](#)) when few numbers of comparisons or Benjamini and Hochberg method (Benjamini and Hochberg, 1995 [\[18\]](#)) when more comparisons. All statistical details are given in Figure 1-table supplement 1 [\[19\]](#).

Data availability

All data generated or analyzed during this study are included in the manuscript and its supporting files. Additional information is available upon request from the lead contact, Séverine Trannoy (severine.trannoy@univ-tlse3.fr or severine.trannoy@utoulouse.fr).

Acknowledgements

We thank the members of EXPLAIN and IVEP team at the CRCA for their helpful discussion and support on this study. We thank Kravitz's lab for sharing the *Bully* lines. We also thank all the member of Billeter's lab to host A. Defert and train him to GC-FID methodology. We acknowledge support from the French Research National Agency (ANR) (ANR-19-CE37-0018-01 to S.T.), the Fondation Fyssen (190573 to S.T.), and the Doctoral Mobility Campaign of Toulouse III University in 2023 (to A.D.).

Funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Additional information

Resource availability

Further information and requests for resources and reagents are accessible from the lead contact, Séverine Trannoy (severine.trannoy@univ-tlse3.fr or severine.trannoy@utoulouse.fr).

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Funding

Funder	Grant reference number	Author
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Additional files

Supp Figure 1  Cs and Bully males discrimination between males and females CHC profiles. Time spent by Cs and Bully males to court either a naïve decapitated Cs male or a virgin Cs decapitated female (LMM and Analysis of Deviance Table (Type II Wald chisquare tests): $P_{\text{male_genotype}}=0.327$, $\chi^2=0.960$, $P_{\text{mated_female}}=5.518 \times 10^{-8}$, $\chi^2=29.626$, post-hoc: $P_{\text{Cs_male}}=2.064 \times 10^{-4}$, $\chi^2=13.772$, $P_{\text{Bully_male}}=6.308 \times 10^{-5}$, $\chi^2=16.008$). Stars represent significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Statistical details are given in [Figure 1-table supplement 1](#) . $n_{\text{Cs}}=10$, $n_{\text{Bully}}=13$.

Supp Figure 2  Details on PCA analysis for virgin and mated females. (A) Eigenvectors and associated $\cos^2$ for each CHCs compounds. Contribution of each CHCs compound in the difference between (B) virgin and Cs-mated, (C) virgin and Bully-mated and (D) Cs-mated and Bully-mated CHCs profiles. Compounds contributing significantly to the difference between CHCs profiles are represented in red. Numbers above bars refer to the mean contribution of each compound in the difference between CHCs profiles. Statistical details are given in [Figure 1-table supplement 1](#) . $n_{\text{virgin}}=10$, $n_{\text{Cs-mated}}=16$, $n_{\text{Bully-mated}}=14$.

Supp Figure 3  Interindividual variability across multiple mating events throughout the flies' lifespan. Interindividual variability in mating duration for each (A) Cs and (B) Bully male when mated with a virgin Cs female across their lifespan. Each panel represents the mating durations (in minutes) for a single individual, with each dot corresponding to a single mating event. Statistical details are given in [Figure 1-table supplement 1](#) . $n_{\text{Cs}}=24$, $n_{\text{Bully}}=23$.

Supp Table 1  Statistical details of all analyses. This table gathers all statistical analyses performed in all the Figures. The first column refers to the panels of each Figure, the second one gives the statistical model used to analyze data and the last one provides the names of statistical tests and corrections performed as well as all p-values and statistical values.

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Peer reviews

Reviewer #1 (Public review):

Summary:

This study asks how selection for male aggressiveness affects life-history and reproductive fitness traits in *Drosophila melanogaster* males.

Strengths:

Multiple comprehensive assays are used to address the question.

Weaknesses:

- (1) The flies used for comparisons are inadequate. Behavioral assays compare Bully males mated to non-coevolved Cs females with Cs males mated to coevolved Cs females.
- (2) Lifespan analysis is done on male progeny of Cs females mated to either genetically more distant Bully or co-evolved Cs males, the longer lifespan and performance on the former is interpreted as trade-off with aggressiveness, rather than a simple explanation of hybrid vigor.
- (3) Differences in CHCs between Bully and Cs males and Cs females mated to those males are not shown to cause difference in measured behavioral outcomes.

Comments on revised version.

I appreciate authors responding to reviewer's comments. The inclusion of additional Bully lines in behavioral analysis, and Bully homozygous male progeny in lifespan analysis gives more strength to the authors' conclusions. It does not exclude other possible explanations for the observed results, but now authors note genetic drift as an alternative explanation for some of their results.

I do want to point to a potential misunderstanding of male-female co-evolution by authors. The authors state that "The Bully lines used in our work were derived from Canton-S flies and thus did co-evolve with Cs". This statement is incorrect if the process of selection and line maintenance in this study was the following:

In my understanding to create Bully lines the most aggressive males were first chosen from an ancestral Cs line and their most aggressive male progeny were mated to their sibling females, repeating the process for 37 generations. Therefore, Bully females were co-evolving with Bully males during selection process of over 37 generations, while Cs females were staying co-evolved with their own males, since they mated within the line. Moreover, after aggressive lines were created, they were kept separate from each other, and from Cs line since about the year 2010, until the experiments described in the paper were performed (which must over 10 years?). Over 10 years, a significant genetic drift can happen, that changes allele frequencies, and may results in differences in male-female co-evolved traits and in lifespan that are unrelated to selection for aggression.

Also, decapitating females does not completely prevent female influence over mating process, but just removes central brain control over it. In *Drosophila*, however, the main control over copulation process for males and female is not central. Therefore, you do not completely remove the effect of coevolved or non-coevolved female traits over copulatory and post-copulatory processes.

<https://doi.org/10.7554/eLife.107559.2.sa2>

Reviewer #2 (Public review):

Summary:

The authors compare "Bully" lines, selected for male aggression, to Canton-S controls and find that Bully males have lower mating success, shorter mating durations, and remate sooner. Chemical analyses show Bully males have distinct cuticular hydrocarbon (CHC) signatures and transfer markedly less cVA to females, offering a plausible mechanistic link to weaker mate-guarding. Paradoxically, Bully males live longer and remain fertile at older ages when Cs males no longer mate, indicating a shift in the reproduction-survival trade-off in aggression-selected populations. Importantly, the work sheds light on proximate mechanisms, demonstrating that shifts in CHCs and pheromone transfer co-occur with changes in fitness traits.

Strengths:

The manuscript's strengths lie in its comprehensive and integrative approach framed within an evolutionary context. By combining behavioral assays, chemical profiling, and lifespan measurements, the authors reveal a coherent pattern linking aggression selection to life-history trade-offs. The direct quantification of cVA in the female reproductive tract after mating provides a particularly compelling mechanistic correlate, strengthening the link between behavior and chemical signaling. Findings on altered 5-T and 5-P levels further highlight how chemical communication shapes mating and mate-guarding strategies.

Analytical approaches are largely rigorous, and the results provide valuable insights into the pleiotropic effects of selection on socially relevant traits.

The revision responds directly to the main concerns raised previously. The addition of a third, independently selected line (Bully C), together with the Bully $\times$ Bully data, considerably reduces the concern that the behavioral phenotypes reflect line-specific drift or founder effects rather than a correlated response to selection. The reorganized survival figure (Figure 5) is a clear improvement over the previous version, with isolated and group-housed males in separate panels and a heterozygous Bully condition added, so the longevity claim can be evaluated more directly. The isolated-male data are especially useful here, since those flies never mate, and a longevity difference under that condition argues that the effect is not simply a consequence of Bully males mating less often. The behavioral schematic, corrected symbols, and reported sample sizes also help, as does the reinterpretation of the post-mating courtship data in terms of courtship motivation rather than a refractory-period effect once no latency difference was found.

Weaknesses:

Most of the remaining weaknesses are ones I raised in the first round, and the revision has narrowed them. The links between the altered CHC profiles, the reduced cVA transfer, and the behavioral outcomes remain correlative. The causal experiments that would establish them (for example, perfuming or cVA-equalization) are acknowledged by the authors as future directions, which is reasonable, but it means the mechanistic claims should be read as candidate explanations rather than demonstrated ones. It is also worth noting that the CHC differences and the behavioral differences may both be downstream of a common selection target (for instance, genes affecting oenocyte function or CHC biosynthesis) rather than one causing the other; the Discussion would be more balanced if this alternative were stated explicitly.

My main remaining concern is with the lifespan data. The behavioral phenotypes are replicated across Bully A, B, and C, but the survival assays were done on Bully A only, so a line-specific contribution to the longevity result, including drift, cannot be excluded, even though this has been addressed for the behavioral traits. This matters because the title and abstract present the survival-reproduction trade-off as a general consequence of selection for aggression, whereas the survival evidence rests on a single line. The authors can either run the lifespan assays on a second line, or calibrate the text, title, and abstract so that the strength of the survival claim matches the single-line evidence behind it, with second-line lifespan data noted as a future step.

The Bully C line is currently underused. Its intermediate aggression, together with the absence of a significant reduction in mating duration, points to a graded rather than binary relationship between aggression intensity and mating duration. This is one of the more interesting features of the expanded dataset, and it deserves more than its present role as a justification for focusing on Bully A.

The authors have appropriately softened causal language in the title, subheadings, and much of the Discussion. A few residual passages still imply causation or directional transfer and would benefit from the same treatment.

<https://doi.org/10.7554/eLife.107559.2.sa1>

Author response:

The following is the authors' response to the original reviews.

eLife Assessment:

*This valuable study addresses the effects of selection on aggression on fitness and life-history trade-offs in *Drosophila melanogaster*. However, the evidence presented is incomplete and does not support the claims proposed in the study of increased survival of highly aggressive males at the expense of reproductive success and shorter mating duration. The main limitation of the study is the choice to use males from only one aggressive *Drosophila* line in combination with Canton-S females, that do not allow disambiguation between nonaggression-related factors, such as hybrid vigor and aggression-related factors influencing mating and lifespan.*

We would like to clarify the points raised in the eLife assessment.

The report states that we relied on a single line of hyper-aggressive males tested with Canton-S females, and implies that Bully and Cs have not co-evolved. This is a misunderstanding: Bully flies were derived from Cs population. Thus, Bully and Cs have co-evolved. In addition to the Bully A line presented in the main figures of the manuscript, we replicated several of our findings with a second independent selected line, Bully B. Results from courtship assays involving both Bully A and Bully B couples males and females were presented in Figure Supp1. We apologize for not having made this more explicit in the original manuscript, which we will correct. These experiments should alleviate the concerns from the reviewers; they demonstrate that our conclusions are supported by two independent hyper-aggressive lines, and these include assays with selected male and female flies.

Public Reviews:

Reviewer #1 (Public review):

Summary:

*This study asks how selection for male aggressiveness affects life-history and reproductive fitness traits in *Drosophila melanogaster* males.*

Strengths:

Multiple comprehensive assays are used to address the question.

We thank the reviewer for recognizing these strengths.

Weaknesses:

(1) The flies used for comparisons are inadequate. Behavioral assays compare Bully males mated to non-coevolved Cs females with Cs males mated to coevolved Cs females.

We thank the reviewer for this comment, which made us realize that we had not sufficiently highlighted some of our experiments. The Bully lines used in our work were derived from Canton-S flies and thus did co-evolve with Cs. As originally described by Penn et al. (2010), highly aggressive “Bully” lines were generated through selective breeding from Canton-S males that consistently won aggressive encounters. After 34–37 generations, stable Bully lines were established. Thus, 1) Bully and Cs flies have co-evolved and 2) the selection applied was male-specific. Independent selection replicates produced distinct lines, including Bully A and Bully B. Previous studies only characterized Bully A (Penn et al., 2010; Chowdhury et al., 2017), but our work includes both Bully A and Bully B (Fig. S1).

The rationale for pairing Bully or Cs males with Cs females (with which both male types co-evolved) follows the approach used by Dierick et al. (2006), who investigated how the male-specific selection for aggression affected courtship and mating behaviors by testing them with standard Canton-S females. This design allows to isolate the effects of male genotype and

behavior on courtship and mating outcomes, avoiding confounding effects from female behavioral changes.

We initially compared selected Bully pairs (Bully males $\times$ Bully females) (Fig. S1) with Cs pairs and observed similarly shortened mating durations in both Bully $\times$ Bully and Bully $\times$ Cs matings (Fig. S1, Fig. 1F and G). Thus, the reduction in mating duration arises specifically from Bully males. We therefore chose to use Cs females as a standard background to assess the consequences of male-specific selection for aggression on reproductive behaviors.

(2) Lifespan analysis is done on male progeny of Cs females mated to either genetically more distant Bully or co-evolved Cs males; the longer lifespan and performance on the former is interpreted as a trade-off with aggressiveness, rather than a simple explanation of hybrid vigor.

We appreciate this comment, which again stems from a poor explanation from our part about the origin of the Bully line in the original manuscript. The Bully flies were derived from the same original population as the Cs line. Hybrid vigor typically arises when crossing individuals from distinct populations, which is not the case here as both Bully and CS come from the same population.

To further support our conclusions, we conducted additional experiments using progeny from within-line crosses (Bully males $\times$ Bully females) and results revealed the same phenotype: the progeny of these flies also exhibited significantly longer lifespans than Cs males $\times$ Cs females progeny. This finding argues against hybrid vigor as the main explanation for the observed phenotype, since both the Bully and Cs crosses result in inbreeding, yet give longer lifespan in Bully. We will include these additional longevity data (currently not included in the manuscript) to strengthen our results and reinforce our interpretation.

(3) Differences in CHCs between Bully and Cs males and Cs females mated to those males are not shown to cause differences in measured behavioral outcomes.

We thank the reviewer for raising this important point regarding causality. One way to establish a causal link between differences in CHCs observed in Bully and Cs flies and the corresponding behavioral outcomes would be to experimentally manipulate CHC profiles. For instance, one could perfume oenocyte-less males with the compounds found in higher abundance in Bully flies, then perform behavioral assays to assess causality. We agree that such experiments would be highly informative in determining the functional roles of specific CHCs elevated in Bully males. However, this approach is technically challenging, as the perfuming technique must be optimized to transfer precise amounts of each compound. For example, this method can be used to gradually perfume flies to assess dose–response behavioral effects, whereas matching exactly the natural concentrations found in individuals, especially given inter-individual variability, remains difficult.

We considered conducting such experiments during our study but did not pursue them for these technical reasons. Nevertheless, we can include a statement in the Discussion acknowledging this as an important future direction to test the causal relationship between CHC variation and behavior.

Reviewer #2 (Public review):

Summary:

The authors compare "Bully" lines, selected for male aggression, to Canton-S controls and find that Bully males have lower mating success, shorter mating durations, and remate sooner. Chemical analyses show Bully males have distinct cuticular hydrocarbons (CHC) signatures and transfer markedly less cVA to females, offering a plausible mechanistic link to weaker mate-guarding.

Paradoxically, Bully males live longer and remain fertile at older ages when CS males no longer mate, indicating a shift in the reproduction-survival trade-off in aggression-selected populations.

Importantly, the work sheds light on proximate mechanisms, demonstrating that shifts in CHCs and pheromone transfer co-occur with changes in fitness traits, thus offering new entry points for understanding life-history evolution.

We thank the reviewer for this positive summary of our work.

Strengths:

The manuscript's strengths lie in its comprehensive and integrative approach framed within an evolutionary context. By combining behavioral assays, chemical profiling, and lifespan measurements, the authors reveal a coherent pattern linking aggression selection to life-history trade-offs. The direct quantification of cVA in female reproductive tracts after mating provides a particularly compelling mechanistic correlate, strengthening the link between behavior and chemical signaling. Findings on altered 5-T and 5-P levels further highlight how chemical communication shapes mating and mate-guarding strategies. Analytical approaches are largely rigorous, and the results provide valuable insights into the pleiotropic effects of selection on socially relevant traits. The study will be of interest to Drosophila biologists working on sexual selection, behavioral evolution, and aging.

We thank the reviewer for recognizing the integrative design and mechanistic contributions of our study.

Weaknesses:

The weaknesses are primarily conceptual rather than procedural. The generality of the findings is uncertain, as selection appears to be represented by only one (and a second closely related) Bully line, limiting conclusions about selection responses versus line-specific drift or founder effects. The causal link between aggression selection and increased longevity is not established: the data show a correlated shift but do not identify mechanisms underlying lifespan extension. In several places, the manuscript uses causal language (e.g., that selection 'influences' longevity or mating strategy) where association would be more accurate; this should be toned down to avoid overstatement. Ecological relevance is also not addressed, since laboratory conditions may bias the balance between costs and benefits of aggression compared with variable natural environments. Addressing these points would strengthen both the impact and clarity of the study.

(1) Generality of findings and potential line effects

We agree that our results presented in the main figures of the manuscript relied mainly on one Bully line (Bully A). To address potential line-specific effects, we replicated key courtship experiments with another independent line, Bully B, selected in parallel from the same Canton-S stock but through distinct selection replicates. The results obtained from Bully B closely matched those from Bully A, suggesting that the observed phenotypes are consistent consequences of aggression selection rather than random drift or founder effects.

(2) Causality versus correlation

We concur that some sentences in the manuscript could overstate causal interpretations. We will revise the text to clearly distinguish correlation from causation and to avoid implying direct causal relationships where data only support association.

(3) Ecological relevance

We appreciate this point. Our experiments were performed under controlled laboratory conditions, which may not fully capture the ecological contexts shaping the costs and benefits of aggression. We will acknowledge this limitation and expand the Discussion to consider how environmental variability could modulate the fitness trade-offs associated with aggression in natural populations.

We thank both reviewers for their constructive feedback, which will help us strengthen the rigor and clarity of the manuscript. We believe that the additional results and revisions will satisfactorily address their concerns.

Recommendations for the authors:

Reviewing Editor Comments:

The major weaknesses raised by the reviewers, namely the flies used in the study (CsxBully compared to CsxCs) where the effect on lifespan could be explained by hybrid vigor and the use of only one Bully and one Cs line that does not allow to link unambiguously the observed effect to the selection for aggression, should be addressed by a different experimental design and additional lines to exclude the effect of non-aggression related factors.

We thank the Reviewing Editor for these comments.

(i) Experimental design and hybrid vigor:

Hybrid vigor typically arises from crosses between genetically divergent populations. In our study, Bully lines were derived from Canton-S background and are not therefore not genetically distant from controls. To directly address this concern, we included new data from Bully $\times$ Bully pairs (Figure 1), using independently selected Bully lines. These experiments reproduce the key aggression and courtship phenotypes observed in Cs $\times$ Bully assays, indicating that the effects are not attributable to hybrid vigor.

(ii) Use of additional selected lines:

We now include data from two independently selected lines (Bully A and Bully B), both derived from Cs, which show consistent behavioral phenotypes. This supports the conclusion that the observed effects are associated with selection for aggression rather than line-specific artifacts. We note that generating such lines is time- and labor-intensive, and only a few laboratories have established aggression-selected lines in *Drosophila melanogaster* (e.g., Penn et al., 2010; Dierick et al., 2006; Edwards et al., 2006). Accordingly, we have revised the manuscript to explicitly acknowledge this limitation and to frame our conclusions in terms of association rather than causation.

Reviewer #1 (Recommendations for the authors):

I can't see any way to interpret the data using CsxCs vs CsxBully comparisons.

We thank the reviewer for this important point. This concern appears to arise from the assumption that Bully and Cs represent genetically distinct or non-coevolved populations. However, Bully lines were directly derived from Cs and therefore share a common genetic background. We have clarified this point in the Introduction (lines 104-106) and Results (lines 132-137).

Importantly, we now include additional data showing that key phenotypes, including reduced mating duration, are also observed in Bully $\times$ Bully pairings and across independently selected Bully lines (new Figure 1). These results demonstrate that the observed effects are driven by the male genotype and do not depend on the female background.

Because selection for aggression was applied specifically to males, we used Cs females as a standardized background to isolate male-specific effects while minimizing variability arising from female genotype or behavior. This rationale is now explicitly stated in the Results (lines 163-166) and at the beginning of the Discussion (lines 326-330). This experimental design allows interpretation of male-specific effects, and the observed differences cannot be attributed to cross design artifacts or hybrid vigor.

Reviewer #2 (Recommendations for the authors):

Major comments:

(1) Several passages currently imply causality, whereas the data support correlations between selection and trait differences rather than direct causation. This overstatement also appears in section subheadings within the Results, such as "Hyper-aggressive males display reduced mate-guarding efficiency, without compromising female fertility." Please consider toning down the wording by replacing active causal verbs with more neutral phrasing. Additionally, it would be important to include a clear, explicit sentence in the Discussion acknowledging this caveat, as the existing phrase "is associated with changes in reproductive traits" does not fully convey this nuance.

We thank the reviewer for this important comment. We have revised the manuscript throughout, including Results subheadings, to replace causal language with association-based phrasing. We also rephrase the first sentence of the Discussion to clarify that our conclusions are correlational (see line 320).

(2) Figure 1 would benefit from a simple schematic of the behavioral paradigm and the arena, since the authors' arena design minimizes manual handling; a cartoon would help readers quickly grasp the assay flow and the conditions under which interactions occur.

We thank the reviewer for this helpful suggestion. We have added a schematic to Figure 1 illustrating the behavioral paradigm and arena design. Additional details are provided in the Materials and Methods (Trannoy et al., 2015). This improves clarity and accessibility of the experimental design.

(3) In Figures 2A-B and A'-B', the higher post-mating UWE in Bully males is intriguing, but these panels do not actually measure the refractory period. It would be helpful to include 'latency' in the first UWE after mating in these swapped-female conditions. This could also be repeated with pheromone-standardized (cVA/CHC-equalized) decapitated females to disentangle effects of female pheromone load from male sensory perception. In addition, a baseline courtship control (naive males with decapitated virgins) is necessary to test whether Bully males simply have a lower threshold for initiating courtship.

We thank the reviewer for this suggestion. The referenced panels are now shown in Figure 3. We quantified post-mating courtship latency; however, latencies were very short across conditions, and no differences were observed between genotypes. We therefore revised the text to interpret these results in terms of post-mating courtship motivation rather than refractory period. The baseline courtship control with decapitated virgins is provided in Fig 2G. These changes clarify the interpretation of post-mating behavior and address the reviewer's concerns.

(4) Related to my above point, the results in Figure 2B-B' raise the possibility that Bully males have reduced perception or neural sensitivity to anti-aphrodisiac pheromones deposited by CS males, which could account for their elevated post-mating courtship; the authors might consider experiments that directly test male sensory responsiveness to these cues or mention this possibility in the Discussion.

We thank the reviewer for this point. We performed additional assays to test males' sensory responsiveness using binary choice assays and measured the time spent performing UWE towards decapitated females versus males. These results were added in Figure 3-Figure Supp 1, and indicate that both Cs and Bully males displayed courtship preferentially towards females, providing a control for sensory perception.

(5) In multiple figure panels, virgin and mated females are depicted with the same symbols, which makes interpretation confusing.

Thank you for pointing this out. We have updated the figure panels to use distinct symbols for virgin and mated females to improve clarity.

(6) For Figure 4, it would be helpful to provide standalone KM curves for Bully versus CS males, including a separate panel for isolated (never-mated) males, and present mating counts in a separate panel while reporting survival models that incorporate mating frequency (or use it as a time-dependent covariate). Although Figures 4C-D report median survivals, full KM plots and an isolated-male curve are important since mating itself elevates mortality and can otherwise confound intrinsic lifespan differences.

Thank you for this important point to improve clarity of this figure. We have reorganized this figure (now Figure 5) to now, present survival curves first (isolated and group-housed males), followed by lifetime mating counts. This reorganization separates survival from mating activity and addresses the potential confounding effect of mating on lifespan. For the lifetime mating data, we used bar plots rather than curves to better visualize individual mating events.

(7) The following sentences overstate the results and imply causality; consider toning down: "These findings suggest that 5-P and 5-T might contribute to promoting remating in females that have previously mated with Bully males (Figure 2F' and G'). Given that Bully males also showed higher levels of both 5-P and 5-T compared to naïve Cs males (Figure 3B), it is likely that the elevated levels of these compounds observed in females result from their transfer during mating."

We thank the reviewer for this important point. We have rephrased these sentences to remove causal language and instead describe associations between CHC profiles and behavioral outcomes. In particular, statements implying that 5-P and 5-T promote remating or are directly transferred during mating have been revised to reflect correlational evidence only (see lines 253-256).

(8) It is not entirely clear how aggression was quantified in each generation, what proportion of males were selected to breed, and whether the findings generalize beyond a single Bully line (Figure Supplement 1 shows data from a closely related Bully line). Without independent replicate lines or sham-selected controls, it remains difficult to rule out drift or line-specific artifacts, and this limitation should be explicitly acknowledged.

We thank the reviewer for this important point. We have clarified the aggression selection procedure by adding methodological details from Penn et al., including how aggression was quantified and how breeders were selected (lines 104-106 and 131-137). Briefly, independent selection replicates were initiated from the same Canton-S population, generating three lines (Bully A, B, and C), which were maintained separately.

To address generality, we now include data from multiple lines. In particular, a new Figure 1 presents aggression and courtship phenotypes across Bully A, B, and C, and key behavioral results are consistent across independent lines.

We acknowledge that additional independent lines would further strengthen generality; this limitation is now explicitly stated in the Discussion (lines 325-326).

These additions clarify the selection procedure and support that the observed phenotypes are associated with aggression selection rather than line-specific artifacts.

Minor Comments:

(1) Exact sample sizes for every experiment should be included in the main figure legends.

Thank you. We have added the number of replicates in each figure legends.

(2) In Figure 1-Supplement 1, the orientation for depicting mating success is reversed compared to Figure 1, which is a bit jarring; it would be clearer to keep the orientation consistent with the main figure.

Thank you. We have incorporated the results initially presented in Figure 1-Sup 1 into a new Figure 1 with additional results, and have taken into account reviewers' comment.

(3) For multivariate analyses, I suggest including important details such as group sample sizes, p-value, and the percent variance, etc., in the figure legend rather than keeping this only in Supplementary Table S1.

We have inserted these details directly into the figure legends for clarity.

(4) Why was the food cup used for arenas where decapitated virgins were used in mating assays?

Thank you for pointing this. We now have clarified the experimental procedure in the M&M of the revised manuscript (lines 465-469).

(5) For cartoons in Figure 2, the current yellow background makes it very difficult to distinguish flies drawn in yellow or green. Please adjust to a higher-contrast background or add darker outlines so that the cartoons are clearly legible.

Thank you. We have increased the contrast of the female bodies to ensure the cartoons are clearly distinguishable (now figure 3).

(6) Addition of line numbers in the manuscript would be helpful during the review process.

Line numbers have been added throughout the manuscript.

(7) I noticed a few typos in the manuscript. For example, in the Introduction, "seminal fluids" should be corrected to "seminal fluid." In the Discussion, the phrase "CHCs profiles compared those" requires a "to" before "those." Please carefully review the manuscript for similar errors.

Thank you for pointing this out. We carefully reviewed the manuscript for typos and corrected all identified errors.

<https://doi.org/10.7554/eLife.107559.2.sa0>