Social hierarchy is established and maintained with distinct acts of aggression in male *Drosophila*

Jasper C. Simon* & Ulrike Heberlein

Janelia Research Campus, HHMI, Ashburn, VA 20147, USA

*Correspondence: *simonj10@janelia.hhmi.org*

ABSTRACT

Social interactions pivot on an animal's experiences, internal states, and feedback from others. This complexity drives the need for precise descriptions of behavior to dissect the fine detail of its genetic and neural circuit bases. In laboratory contests, *Drosophila* males reliably exhibit aggression, conventionally scored using lunges as a proxy, allowing its study with the many available genetic tools. Here, we use an explicit approach to identify the onset and reversals in hierarchical relationships and observe that distinct aggressive acts precede, concur, and follow dominance. We find that lunges are inadequate in establishing social dominance. Lunges rather appear to reflect the dominant state of a male and help in maintaining his social status. Lastly, we characterize the recurring structure of aggression that emerges through subsequent reversals in dominance. Collectively, this work provides a framework for studying the complexity of agonistic interactions in flies enabling its neurogenetic basis to be understood with precision.

INTRODUCTION

Agonistic interactions important for establishing social hierarchies over food, territory, or mates often progress as a sequence of stereotyped acts. Like other goal-driven behaviors [1], the steps along this sequences are triggered by context and modified by past experience [2,3], through largely unknown mechanisms that are coupled to an animal's internal physiological state [4]. Determining these mechanisms remains difficult, however, because social exchanges proceed as a dynamic, reciprocal, and continual feedback loop of interactions between two (or more) decision-making individuals that progresses over both moment-by-moment and protracted times scales [5]. Intra-male adversarial contests in the fruit fly *Drosophila melanogaster* seem well suited for studying such complex social phenotypes, due to its established study in a laboratory settling [6,7] and the multitude of techniques available for manipulating and recording gene and neuron function [8]. Nonetheless, efforts to explain which acts lead to dominance hierarchies [9,10] have suffered from a lack of consensus on methods and results for studies of aggression (as discussed within [11,12])**.**

Here we use an explicit approach to characterize the temporal relationship among various aggressive acts and the establishment of social hierarchy between pairs of male flies in arenas previously developed for high-throughput phenotyping [13,14]. Using this approach, we observe that the acts of fencing, boxing, and tussling appear well positioned in time to be related to the establishment of social hierarchy, whereas lunging and chasing its maintenance. Unexpectedly, the commonly studied aggressive act of lunging, near-universally, follows the onset of social hierarchies and thus is

unlikely involved in its establishment. We carried out a simple social manipulation and observed that males in contests between mixed pairs of genotypically high versus low lunging lines became dominant at chance levels. This result suggests that the total number of lunges executed by an individual is inadequate in explaining his social outcome. We further observed that lunging persists as dominant males confront unfamiliar opponents in unexplored settings. Lastly, we report that a faction of the aggressive acts surveyed also intensify through subsequent reversals in social dominance adding to a fuller appreciation of this complex, recurrent social exchange. Together, this work provides a framework for untangling which aggressive acts causally relate to the establishment of social dominance and those that are a consequence

RESULTS

Identifying hierarchical relationships

Upon introduction to experimental arenas, pairs of males whom had never fought (henceforth, “naïve males”) display independent and haphazard movement that may include one or both males jumping, taking flight, and climbing the wall as they attempt to escape. With time males settle into a period of exploration during which they eventually meet, and commonly this initial contact leads to bouts of short, alternating pursuits that are characteristically restricted to the floor (which is entirely made up of food; see **Methods**). Predictably this engagement then progresses to a situation where one male consistently pursues the other with the retreating male leaving the floor (resource) presumably attempting to flee by climbing the wall. It is this explicit characteristic sequence – of one male pursuing and a second attempting to climb the arena wall –

that we use for identifying when a hierarchical relationship is established (see **Figure 1** and **Movie 1**).

Distinct aggressive acts precede, concur, and follow establishment

We observed adversarial contests in experimental arenas that have been widely used to study aggression [13,14]. These arenas increase the frequency of flies' interactions by restricting their movements near a food resource and yet retain an uncluttered background (no food cup, no decapitated female, nor region of the chamber that is blocked from camera view [15]). This arrangement permits methods based on computer vision to help automatically annotate various aggressive acts [13], followed by direct inspection for correcting erroneous annotations and identifying the onset of dominance. Together this complimentary workflow allows a robust, high-resolution portrayal of the aggressive acts displayed during the progression of adversarial contests (see **Figure S1** and **Table S1** for details of annotation and software used).

Naïve males were introduced simultaneously on food as pairs. This lessened the disparity in which individuals discovered the contested resource and allowed uninterrupted observation of their interactions leading up to and following the onset of social dominance. We considered "Wing flick" and the following acts reported to span the range of aggressive intensities (from low to high): "Fence," "Chase," "Lunge," "Box," and "Tussle" [6] (for descriptions of each act see **Table S1**). At times we found boxing and tussling difficult to separate, and thus for ease of scoring they were combined into the single category, "Box, Tussle" (as done by others [6]).

From both individual contests and aggregate data, we observed reproducible patterns of behavior (see **Figure 2**). Males consistently displayed the highest levels of the head-to-head interaction, “Fence,” from the beginning of contests until the onset of dominance. Thereafter, we observed only sporadic displays of fencing with no clear temporal structure. In contrast, the other head-to-head interactions, “Box, Tussle,” appeared abruptly, peaked, and then sharply decreased immediately prior to the onset of dominance (see **Figure 2D**), and on occasion also transiently reappeared preceding subsequent reversals in social status. The wing display, “Wing flick” was observed more broadly, frequent at a low level from the beginning of contests and making a salient uptick near the onset of dominance with males continuing this escalated flicking throughout the remainder of the contest. In contrast, displays of “Lunge” were rarely observed before the establishment of dominance, and began and continually increased in intensity thereafter. Lastly, occurrences of “Chase” were never recorded prior to dominance and emerged well after establishment (as reported previously [6]). Together, by aligning recordings from individual contests to the onset of social dominance, we expose which acts may causally relate to its establishment and those apparently more related to its maintenance.

Lunges are inadequate for explaining hierarchical outcomes

The act of lunging is commonly used for studying aggression (see reviews [11,16]), with several studies suggesting they play a pivotal role in establishing dominance [9,17,18]. We, however, observed that lunging emerged after the establishment of dominance (as

seen in **Figure 2**). To confirm this finding we ran a second, independent group of contests and carefully reviewed the incidences of lunging (both direct inspection with slow playback to correct for false negative displays and semi-automatically for false positives; see **Methods** for details). Again males lunged after the establishment of dominance, with the majority of the lunges executed by the dominant male (see **Figure S3**).

From the observation that lunging occurs after the onset of dominance we reasoned that they were unlikely to play a role in its establishment. If this were true executing more (or less) lunges should not influence whether an individual emerges dominant. To test this, we set up contests with mixed pairs of males from genotypically high and low lunging lines and measured the total number of lunges executed by those males that emerged dominant. We saw no influence of total number of lunges on dominance outcomes, even when males from mixed pairs executed dramatically lopsided amounts of lunging (see fourth columns in **Figure 3A, B**; and **Figure 3 – Supplement 1**, **Methods**, and **Table S2** for additional examples and details regarding the various fly lines). It was therefore unsurprising to observe that males from both the high and low lunging genotypes also executed lunges following the establishment of dominance (see **Figure S4**). Collectively, these results provide evidence that lunges do not establish dominance, and more likely play a role in its maintenance.

Males assert dominance by lunging at familiar and unfamiliar opponents

For the majority of contests, we observed that only a single, stable hierarchical relationship formed between pairs of naïve males (as shown in **Figure 2** and **Figure S3**). In approximately one in five contests, however, after the establishment of dominance there were subsequent reversals in social status, with lunges (sometimes by both males) clustering near each reversal. To better understand how these lunges relate to reversals in status, we determined the identities of males executing each lunge (see **Methods**). The majority of the lunges preceding a reversal in status were displayed by the currently dominant male; whereas, dominant males executed nearly all of the lunges following reversals (see **Figure S3**). This analysis provides evidence that lunging reflects current and likely past dominance status, and further support the interpretation that lunges play a role in maintaining a male's social status.

Both increased total time holding dominance and also the occurrence of reversals in social status contributed to a persistent high level of lunging by individuals against familiar opponents. Moreover, it has been reported that "winner" males lunged and became dominant earlier in contests against naïve opponents [19]; and also, that males appeared to change their fighting tactics as a consequence of winning or losing [20]. Therefore, to test if lunging functions as a general tactic for holding on to dominance irrespective of opponent or location, we paired the emergent dominant males (from a first contest between two naïve males; see **Figure 4A**) with naïve males in unexplored arenas (see **Figure 4B**). The most salient feature of these second contests was that unlike for pairs of naïve males, that rarely lunged before the establishment of

dominance, the majority of dominate males (although not their paired naïve opponents) lunged prior to establishing dominance (see **Figure 4C**). The temporal sequence for all other aggressive acts studied (including lunging after the initial establishment of dominance) appeared comparable to those observed in contests between two naïve males (data not shown). Finally, to examine if the precocious lunging helped dominant males reassert dominance against unfamiliar males in unexplored arenas, we quantified the number of contests in which dominant males that had lunged early were successful in reasserting their dominance status. For the majority of these contests, previously dominant males reasserted their dominance (see **Figure 4D**).

DISCUSSION

Social dominance forms when individuals yield to the agonistic advances of others, often in the context of conflict over resources such as access to food, territories, and reproductive opportunities [21]. However, the relationship between specific aggressive acts and the establishment of social hierarchies remains contentious largely for they appear so highly correlated, even with disagreements over which drives the other [22]. In this work, we use an explicit approach to characterize with high temporal resolution how a range of known acts of aggression relate to the establishment, maintenance, and subsequent reversals of social hierarchy.

The approach introduced within this work has several advantages. It is easy to describe and straightforward to implement. It accommodates that adversaries compete over resources and includes an operationally defined ‘escape,’ both which are important

ethological considerations [6,23]. Further, it provides the means to align and thus compare the onset of dominance, which varies in time across contests due to differences in various influences (e.g., inherited factors, past social histories, current physiological states, handing by the experimenter, environmental conditions, and feedback between adversaries). Lastly, it can be used to update the classification of dominance if and when reversals in status occur after an initial hierarchical relationship has been established.

A significant challenge to studying complex social behaviors such as those related to the establishment of social hierarchy is that the numerous interactions possibly regulating them are often highly correlated with outcomes. By using criteria to identify the establishment of dominance that are independent from the particular acts of aggression under study, we avoid a circular definition of social hierarchy, and thus allow for stronger claims of causality between specific acts and its emergence (for a graphical summary see **Figure 5**).

Unexpectedly, we show that near-universally only once males hold (or have held) dominance do they lunge, and moreover they continue lunging against familiar opponents even after a dominance hierarchy is settled (as has been described previously [9,24]). From this and our observation that dominant males precociously lunge and successfully reassert dominance over unfamiliar males in novel settings, we propose that lunging is likely uninvolved in establishing dominance, but rather

symptomatic of a changed internal state [25] and plays a role in maintaining an individual's social status.

The complexity of reversals in dominance requires more study. From a preliminary characterization of a larger number of contests with reversals, we observed that lunging appears as just one of several interacting aggressive acts likely involved in the process (see **Figure S5**). Taken together, it is clear that further consideration is needed for a more complete understanding of these dynamic, reversing, and escalating exchanges.

Collectively, this work advances our understanding for the causal relationships within this complex behavioral phenotype and should prove helpful for both refining the roles of known [6,26,27] and yet to be discovered acts of aggression. Finally, the approach described, when used in conjunction with high-throughput screens, provides a framework for helping to identify and clarify the genetic and neural mechanisms underlying the coordination of behaviors involved in establishing hierarchical social relationships in insects.

FIGURE LEDGENDS

Figure 1. Identifying social hierarchy within arenas used for studies of aggression. (A) Behavior independent of specific aggressive acts used to determine when social hierarchy forms between pairs of males. Pursuing males (red, solid arrow), that often proceed to guarding the floor (red, asterisk), are classified as dominant and those then attempting to escape by climbing the wall of the arena as subordinate (black, dashed arrow). (B) Twenty-minute contests ordered as rows from early to late establishment. Using the “Pursue-to-Climb” criteria clear hierarchies form in the majority of pairings. Periods of unestablished social standing (light yellow bars) precede clear social hierarchy (gray bars). Contests in which hierarchies formed prior to observation (full gray bar, top row, n=12) and those in which hierarchies never formed (full yellow bar, bottom row, n=6) were excluded from further analysis.

Movie 1. Established hierarchical relationship. Example movie from a 20-minute contest showing an established hierarchical relationship, wherein a subordinate male repeatedly attempts to climb the wall of the arena while a dominate male (clipped wing) guards the floor.

Figure 2. Fence, box, and tussle interactions precede the establishment of social hierarchy, whereas the majority of wing flicks, lunges, and chases follow. (A) Raster plot from a single contest illustrates the relationship between the observed reproducible sequence of aggressive acts and the establishment of social hierarchy. Each row and color corresponds to a different act. (B) Raster plots show the temporal

structure for the various acts (discrete act = black tick). Individual contests (shown as rows) between pairs of naïve males are ordered top-to-bottom by latency until establishment (red ticks). After establishment males exhibit clear hierarchical relationships (gray shading), with occasional reversals in dominance (blue ticks). (C) Peri-event plots with contests aligned by establishment (vertical, red line). Aggressive acts from each contest are binned into one-minute intervals and displayed above plots with corresponding collective medians (black line) and interquartile ranges (gray envelope). (D) Peri-event plots aligned to include the minute immediately preceding and following establishment (vertical, red line). Discrete acts are displayed above associated collective medians (black line) and interquartile ranges (gray envelope). (C, D) Statistical comparisons for the frequency of acts within five- (C) and one-minute (D) windows prior to and following establishment are noted above plots (horizontal, green lines). In all cases the Wilcoxon signed rank test was used to assess whether differences in the observed acts exist between paired, equal-length windows of time before and after the establishment of social hierarchy. See **Figure S2** for details regarding data exclusion. Common nomenclature for statistical significance used throughout study: not significant (n.s.), *p<.05* (*), *p<.01* (**), *p<.001* (***), *p<.0001* (****), *p<.00001* (*****).

Figure 3. Number of lunges executed by dominant individuals is inadequate for explaining hierarchical outcomes. (A) Focal males (F, above) and opponents (O, below) were paired and the percentage of contests in which males became dominant are shown within each stacked bar chart. Focal males from genotypically low (L, blue),

standard (S, green), and high (H, light red) aggressive lines became dominant at chance levels when paired with same-genotype opponents. The tested pairing between mixed high (H, light red) and low (L, blue) aggressive males also became dominant in comparable amounts (shaded in green). In contrast, for contests in which high-aggressive focal males (H, light red) were paired with socialized opponents (group housed) from the same genotype, focal males became dominant 100% of the time (compared to chance levels; Fisher exact, $p=.0005$; right-most bar). In all cases males were socially isolated unless noted. (B) Medians with interquartile ranges for the total number of lunges executed by dominant individuals from the contests reported in “A” above. The number of lunges executed by dominant focal males (F) from low (blue), standard (green), and high (light red) aggressive lines were no different than when those of paired opponents (O) from the same genotypes became dominant. Focal males from a high-aggressive line (F, light red) displayed a comparable, high level of lunging when paired with all opponents (from left to right): males from the same genotype (O’, light red), genetically low-aggressive males (O’’, blue), or socialized males (O’’’, light red) from the same genotype (Kruskal-Wallis, $H(3)=5.92$, $p=.1142$). However, in tested pairings the levels of lunges executed by the dominant, high-aggressive focal males (F, light red) were significantly greater than those of dominant, low-aggressive opponents (O’’, blue; Wilcoxon Rank-Sum, $Z=3.9244$, $p=.00009$; shaded in green) and socialized opponents from the same high-aggressive line (O’’’, light red; Wilcoxon Sign, $p<.00001$).

Figure 3 – Supplement 1. Independently isolated genotypically low, standard, and high aggressive lines identified from a P-element screen; additional genotypically high and low aggressive lines corroborate findings in Figure 3. (A) The distribution of P-element lines screened before outcrossing is skewed right (black, stair-step line) with the median (vertical, white line) and interquartile range (gray shading) centered around 67 total lunges, as calculated by summing the total lunges executed by pairs of same-genotype males from 20-minute contests. The median number of lunges executed by the genotypic standard (green) used throughout the study and also the low (blue, light blue) and high (light red, red) lines are shown after outcross. (B) Focal males (F, above) and opponents (O, below) were paired in various combinations and the percentage of contests in which males became dominant are shown within each stacked bar chart (adversaries are coded by letter and color as indicated in “A” above). Focal males became dominant at chance levels in all contests. (C) Lunging executed by dominant males in both same- and mixed- genotype pairings were consistent with those reported in **Figure 3**. Dominant focal or opponent males arising from same-genotype pairs displayed equivalent amounts of lunges (F vs. O, light blue and red). Focal males from an additional high-aggressive line (F, red) displayed a comparable, high level of lunging regardless of opponent (Kruskal-Wallis test; H (1)=0.0039, *p=0.9503*). However, in tested pairings the levels of lunges executed by both dominant, high-aggressive focal males (F, light red; F, red) were significantly greater than an additional dominant, low-aggressive opponent (vs. O’, light blue; Wilcoxon Rank-Sum, Z=3.8884, *p=.00010* and versus O’’, light blue; Wilcoxon Rank-Sum, Z=3.8111, *p=.00014*).

Figure 4. Lunging persists as dominant males confront unfamiliar opponents in unexplored arenas. (A, B) Raster plots showing the temporal structure of lunges executed by naïve (black ticks) and previously-dominate (red ticks) males in relation to establishing social hierarchy. Individual contests (shown as rows) between pairs of males are ordered top-to-bottom by latency until establishment. Periods preceding establishment (yellow shading), and the prevailing dominance status for naïve (gray shading) and previously-dominant (light red shading) males are noted as observed within 20-minute contests. (A) Pairs of naïve males. (B) Dominate males paired with naïve opponents in a second, unexplored arena. (C) The number of contests in which the previously-dominant males (red bar; "Dm") lunged prior to reasserting dominance over naïve males was significantly greater than those in which randomly selected individuals from pairs of naïve males (black bar; "N") lunged prior to establishing dominance (Chi-Square: X^2 (1, N=56) = 16.07, *p=.00006*). (D) The number of contests where previously-dominant males (light red bar; "Dm") lunged prior to reasserting dominant was also significantly greater than those in which they lunged and failed (gray bar; "N") and became subdominant (Chi-Square: X^2 (1, N=25) = 4.89, *p=.02701*).

Figure 5. Sequence among aggressive acts in relation to establishing social hierarchy. Depiction of the reproducible sequence among distinct aggressive acts as observed in relationship to the emergence of social hierarchy. After adjusting to the experimental arena, pairs of males typically recognize each other as opponents. Successively they then exhibit fencing (red line) and then display low levels of wing flicks (purple line). As contests escalate (graded yellow box) the frequency of fencing

bouts lessens and is replaced by boxing and tussling (orange line) concomitantly with an increasing number of wing flicks. Transiently the amount of boxing and tussling peaks and then sharply drops immediately preceding the establishment of hierarchical relationships (time = 0). Lunging (blue line) follows establishment, is the principal aggressive act during the maintenance of dominance (gray box), and is accompanied by a steady display of wing flicks. Finally, increased displays of chase (green line) observed after some time appear to connote stabilized hierarchical outcomes.

Figure S1. Details for including and classifying fence versus box, tussle interactions. (A) Illustration drawn from a top viewing angle to clarify the criteria for inclusion. For an interaction to be included the focal male must touch the front legs or head of the opponent (blue dashed box). (B) Side views showing the postural difference between “Fence” and “Box, Tussle.” Interactions that included touch from a prone stance were classified as “Fence.” Any touching when one or both males exhibited an erect posture with both front legs lifted off the floor were classified as “Box, Tussle.” Interactions in which males disengaged, directly executed a 360-degree rotational turn, and then reengaged were annotated as a single continuous act.

Figure S2. Example data to show the requisite exclusions for balanced statistical analyses. An example dataset shows the data used for statistical analyses. In order to apply paired statistical tests, only contests allowing five minutes or more (always equal) windows of time before and after the establishment of social hierarchy were used unless otherwise noted. In the example shown only contests with five minutes of recorded data

before and after establishment were used (blue shaded box, above; median frequency of behavior for corresponding period denoted as bold, blue line, below). In this example, data recorded earlier than five minutes preceding and following establishment and also the data from the entire first five and last two contests were excluded. The exclusions occasionally decreased the sample size, and thereby the statistical significance, yet never qualitatively changed results.

Figure S3. Males assert dominance by lunging. (A) Raster plot displaying the temporal structure of lunges executed by transiently dominant males (red ticks) and those observed dominant at end (black ticks) in relation to the establishment and reversals in social hierarchy. Individual contests (shown as rows) between pairs of males are ordered top-to-bottom by latency until the establishment. Periods preceding establishment (yellow shading), ultimately transient (light red shading), and of final dominance status (gray shading) are noted as observed within 20-minute contests. Terminal reversals in social status for each contest are indicated with blue ticks (ticks for intermediate reversals were excluded for clarity). (B) Lunging reflects dominance. Uncommonly lunges were observed before establishment (bar plots highlighted by yellow shading). Prior to terminal reversals (vertical, blue line), the currently dominant male executed the majority of the lunges executed. Thereafter, near-universally, all lunges came from dominant males (D, black bar), and subordinate males (t, red bar) displayed almost none (Wilcoxon rank sum, Z=7.1057, *p<.00001*). This figure includes the identities for which male lunged and also denotes any reversals in social status

between the pairs of naïve males as observed within the contests reported in **Figure 4A.**

Figure S4. Lunges are executed after establishing dominance irrespective of genotype. (A) Raster plots show the temporal structure for lunges executed by males from genotypically high (top) and low (bottom) aggressive lines (lunge = black tick). Individual contests (shown as rows) between pairs of naïve males from the same genotype (high vs. high and low vs. low) are ordered top-to-bottom by latency until establishment (red ticks). After establishment males exhibit clear hierarchical relationships (gray shading). (B) Peri-event plots with contests aligned by establishment (vertical, red line). Lunges from each contest are binned into one-minute intervals and displayed above plots with corresponding collective medians (black line) and interquartile ranges (gray envelope). (C) Peri-event plots aligned to include the minute immediately preceding and following establishment (vertical, red line). Lunges are displayed above associated collective medians (black line) and interquartile ranges (gray envelope). (B, C) Statistical comparisons for the frequency of lunges within five- (B) and one-minute (C) windows prior to and following establishment are noted above plots (horizontal, green lines). In all cases the Wilcoxon signed rank test was used as described in **Figure S2**.

Figure S5. Recurring aggressive acts intensify through subsequent reversals in dominance. (A) Schematic illustrating the establishment and subsequent reversals in social dominance in contests between pairs of naïve males. The gray, switching line

indicates the state of dominance with colored arrows indicating the onset (white dot) for the establishment (Initial, black), subsequent reversals (Intermediate, blue), and the final hierarchical outcome (Terminal, red). (B) Idealized results for the observed changes of aggressive acts among establishment (black line) and intermediate (blue line) and terminal (red line) reversals of social dominance (gray shaded box). A value of "1" indicates the highest level for any particular act to help clarify its relative increase or decrease; drawings are based on the data in **Figure 2**, those reported here in: "C-E," and observations of contests using males from the genotypically high and low aggressive lines (data not shown). Depicted in the top panel of "B," early on males exhibit the highest levels of "Fence," which then drops during the minute immediately preceding the initial establishment (black line). From then on only intermittent and low levels of fencing were observed through subsequent reversals (Intermediate, blue and Terminal, red lines). In contrast, as shown in the next panel down, the amount of "Box, Tussle" peaks during the minute immediately preceding the initial establishment (black line), and then peaks again and again, increasing and broadening through subsequent reversals (Intermediate, blue and Terminal, red lines). (C-E) Peri-event plots centered on the minutes immediately preceding and following the initial establishment (C; vertical, black line), the intermediate subsequent reversals (D; vertical, blue line), and final outcome (E; vertical, red line) of social hierarchy. Discrete acts are displayed above associated collective medians (black line) and interquartile ranges (gray envelope). (C-E) Statistical comparisons for the frequency of aggressive acts within one-minute windows prior to and following establishment are noted above plots (horizontal, green lines; with ±10-second window comparisons denoted within parentheses). In all cases

the Wilcoxon signed rank test was used as described in **Figure S2**. Lunges increased, formed clusters, and the peaks of clusters shifted forward in time relative to each subsequent reversal (C-E; shaded, green box). Lunging increased in a scalable manner (One-way ANOVA: df(2), F(22.5470), *p<.00001* and followed by post hoc comparisons: Initial versus Intermediate, *p=.0135*, Intermediate versus Terminal, p=*.0475*, and Initial versus Terminal, *p<.00001*; all with Bonferroni correction). The shift in peaks was quantified as a procession index; PI = (Lunges *minute preceding* – Lunges *minute following*) / (Lunges *minute preceding* + Lunges *minute following*). One-way ANOVA: df(2), F(6.1421), *p=.0049* and followed by post hoc comparisons: initial versus Intermediate, *p=n.s.*, Intermediate versus Terminal, *p=n.s.*, and Initial versus Terminal, *p=.0046*; all with Bonferroni correction). The intensity for several of the aggressive acts increased alongside that of lunges. Collectively, the characterization exposes a recurring structure that emerges during protracted engagements – one in which both males reciprocally escalate fights just prior to reversals in status – seemingly in attempt to maintain or reclaim social dominance. Data within this figure come from contests containing reversals in dominance (n=25) identified from 146 adversarial contests.

TABLES

Table S1. Details for studying the aggressive acts reported within manuscript.

Descriptions and process for generating the data used to characterize the temporal structure of the various aggressive acts reported within manuscript.

Act	Description	Method	Annotation	Software
Fence	Extending one or both forelegs and pushing against the forelegs or head of opponent from a prone position.	Manual	See **Figure S1**.	VCode [28]
Box, Tussle	Rearing up on hindlegs while striking, pushing, or pulling opponent with forelegs.	Manual	See **Figure S1**.	VCode [28]
Wing flick	Brief spreading followed by furling of one or both wings.	Automatic	Left and right wings flicks from both focal and opponent males outputted by CADABRA were combined (using Matlab) as follows: wingFlick = sort([wing.fli.r.obj1.t wing.fli.l.obj1.t wing.fli.r.obj2.t wing.fli.l.obj2.t])	CADABRA [13]
Lunge	Forward thrust of body with outstretched forelegs while snapping down on opponent.	Semi-Automatic	Assigning identification to time-stamped lunges classified by CADABRA (see Supplementary Tables 2,3 therein for details) provided information for which male executed a particular lunge.	CADABRA [13] followed by scoreMoviesLunges [29]
Chase	Running after opponent.	Automatic	First male following another at a speed greater than 5mm/s for ≥1s (see Supplementary Table 9 within [13] for details).	CADABRA [13]

Table S2. Behavioral measures for the out-crossed P-element lines used within manuscript. P-element lines ordered by row from fewest to greatest number of lunges (mean ±quartile) per 20-minute contest as identified within screen after outcrossing. Line names indicate batch followed by trial number and come from the labelling scheme of our laboratory. Color and intensity may be used to associate lines with results from **Figure 3** and **Figure 3 – Supplement 1**. In addition to its high levels of lunge displays, the line 9.3 was chosen for its high amounts of chase (see below). Consequently, to support the notion that chase events follow the establishment of social hierarchy as observed in **Figure 2**, we analyzed chase events in contests between pairs of males from 9.3 and also in mixed contests between males from 9.3 and the those from the low aggressive line 11.261. In both cases, we observed that all chase events occurred following the establishment of hierarchy (compared to equal numbers of chase events before and after establishment; 9.3 versus 9.3, Fisher exact, n=18; *p=.0010*; 9.3 versus 11.261, Fisher exact, n=11; *p=.0351*). Together, combining the above results with those from **Figure 2**, we report that we have only observed chases following the establishment social hierarchies, Fisher exact, n=34, *p<.00001*.

Line	Color	Intensity	Sample size (n=contests)	Tussle	Wing flick	Lunge	Chase
11.261	Blue	Low	62	1 (0/4.3)	66.5 (12/128)	12.5 (1/26)	0 (0/1)
15.46	Light blue	Low	64	1 (0/4)	66 (24.5/213.8)	16 (6.5/37.5)	0 (0/1)
5.116	Green	Standard	301	3 (0/8)	95 (57/149)	48 (23.5/104)	0 (0/3)
9.3	Light red	High	24	2 (0.9/7.6)	138 (73/212.6)	184 (98.5/236)	4 (0/16)
11.27	Red	High	52	3 (1/8.3)	138 (85.9/214.9)	277 (186/391)	0 (0/2)

MATERIALS AND METHODS

Fly lines

All *Drosophila* lines originate from a P-element collection generated by the Heberlein laboratory that were then outcrossed for at least six generations to w^{1118} Berlin.

Animal rearing

To lessen developmental heterogeneity, males used in experiments were collected from lines maintained with controlled densities (by seeding vials with 5 males crossed to 10 females and removing these parental animals after 3 days), reared in customary 8-dram plastic vials on standard media (cornmeal/yeast/molasses/agar), maintained at 25°C and 65% relative humidity, and entrained on a 12:12-hour light-dark cycle. The lights-on phase started at 1pm EST. Transitions between dark and light were immediate.

Animal handling

To model a uniform, probable ‘ecological baseline’ amount of social experience including to have had mated, males for experiments were collected after 7 hours, yet within the first 24 hours following eclosion using CO_2. For experiments in which the identity of individuals were required, during collection, a small section of wing from one of an eventual pair was removed, a procedure shown inconsequential to the outcomes of adversarial contests [29].

Animal housing

In order to increase or suppress the level of aggression, males were housed individually in 10-mm diameter x 75-mm tall glass tubes (Fisher, Waltham, MA) or as groups of 15 males in 8-dram vials [30,31]. For both housing conditions flies had *ad libitum* access to standard media and were incubated in the same conditions as they were reared.

Animal observation

Experiments were performed on 4~6-day old males. Trials began after a 30-minute adjustment period to “lights on” and the environmental conditions of the observation room. All runs were completed within the first 4 hours of “day.” Replicate experimental and control trials were intercalated and duplicate batches of trials were run over several days at least twice, weeks to months apart.

Aggression assay

Unless noted, aggression assays were performed similar to those previously described [25] modified from [14]. Briefly, pairs of males were aspirated simultaneously into a 16-mm diameter x 10-mm tall enclosed arena staged in an environmentally-control room held at 25°C and 45% humidity and their behavior was recorded for 20 minutes. To encourage consistent aggressive interactions, the entire floor of the arena was composed of a thin layer of apple juice-sugar agar made as 10g sucrose and 9g agar boiled in 400mL 100% apple juice (Mott’s, Plano, TX). To keep the quality of the agar floor consistent, it was used either immediately after a 2-hour setting period or air-dried for 1 hour, wrapped inside of plastic (Saran, Racine, WI), and held at room temperature

until the following day. To impede flies from climbing, the wall of the arena was made slippery by coating it with Fluon (BioQuip, Rancho Dominguez, CA). Similarly, to limit flies from hanging from the ceiling, the lid of the arena was brushed with Sigmacote (Sigma-Aldrich, St. Louis, MO), a transparent silicone paint allowing an unobstructed view for recording from above. Both coatings were left to dry for at least 24 hours before running experiments.

Aggression screen

The genetically high- and low- aggressive males used came from a P-element screen (Mark Eddison, J.C.S, U.H.; unpublished data; see **Figure 3 – Supplement 1A** for the distribution of median total lunges for all lines observed in screen). From this screen only lines that maintained a stable phenotype after outcrossing, had normative levels of activity as estimated by measuring their total distance travelled and total number of jumps, and appeared otherwise healthy were further used. A line, 5.116, exhibiting normative levels of aggression and activity was also identified in the screen. This "standard" was used for all principal experiments reported within this work. **Table S2** includes measures of the aggressive acts from the various lines used within the manuscript.

Data acquisition

Intra-male interactions were recorded at 30 frames per second using a Basler A622f camera (Edmund Optics, Barrington, NJ), with movies from an array of arenas saved simultaneously with gVision video acquisition software [32] for further analysis.

Experimental arenas were lighted from behind with a flicker-free, uniform, white backlight (Coherent, Santa Clara, CA), making recordings suitable for machine vision tracking and behavioral classification methods. For the principal experiments reported in this work, recordings started within 2 minutes after introduction. For experiments analyzed from the P-element screen, recordings started 5-7 minutes after introductions, allowing sufficient time for loading and 5 minutes for flies to settle.

Behavior classification

To catalog the changes in social hierarchy and also the aggressive acts, we used a combination of manual and automatic classification methods (see **Figure S1** and **Table S1** for methods, descriptions, and software used). In summary, the establishment and reversals of social dominance and also the aggressive acts, fence, box, and tussle, were manually annotated with VCode [28] and we used CADABRA [13] to automatically classify wing flick, lunge, and chase. To assign specific lunges to identified males we used a semi-automated method previously reported [29]. This software application uses as inputs both original full-length movies and corresponding time-stamped behavioral events (generated with CADABRA [13] in our case) and iteratively makes short movies inclusive of each event, allowing users to accurately assess if or which male executed a particular lunge. For the P-element screen, pairs of males were tracked and automatically scored for total number of wing flicks, tussles, lunges, chases, and jumps using CADABRA [13].

Statistical analysis

Data processing, plotting, and statistical analyses were all conducted in Matlab (MathWorks, Natick, MA). Details for analyses are explained and reported within each figure legend. See **Figure S2** for specifics regarding data exclusion.

Code availability

Documentation and code used for validating the identification of which male lunged is available at: https://github.com/JasperCSimon/scoresMovie.git.

Acknowledgments

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Authors Contributions

J.C.S. proposed project, designed, conducted, analyzed experiments, and wrote the manuscript; U.H. supported, advised project, and helped to write manuscript.

Declaration of interests

The authors declare no competing interests.

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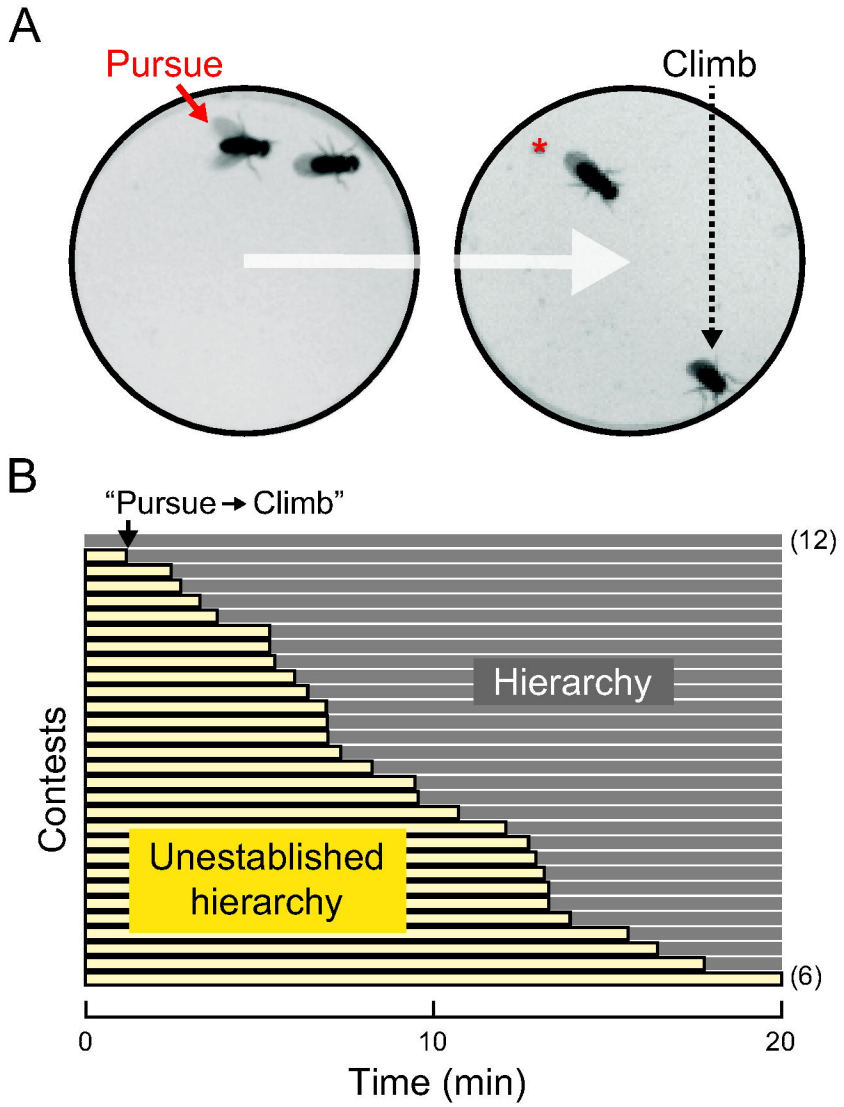
Figure 1. Identifying social hierarchy within arenas used for studies of aggression.



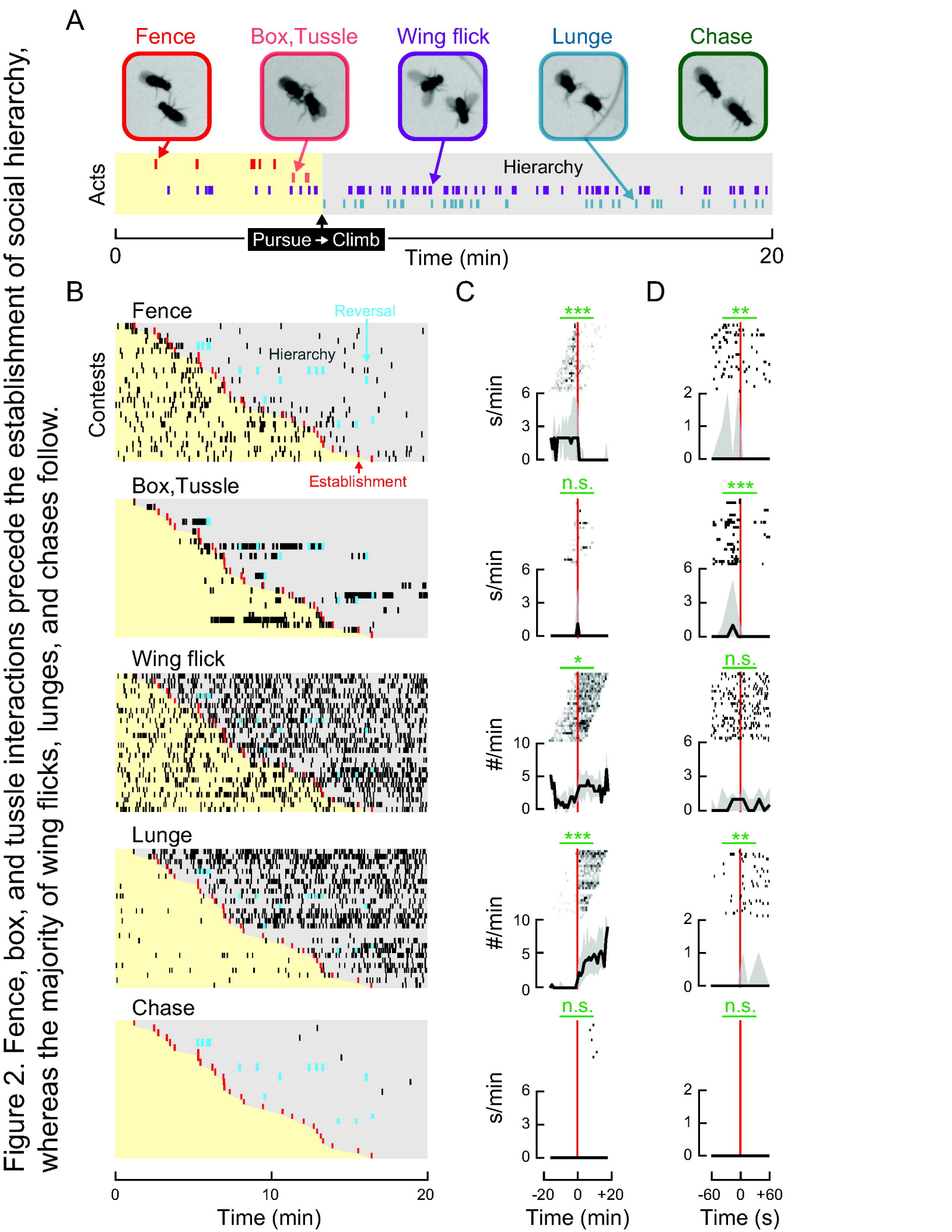


Figure 2. Fence, box, and tussle interactions precede the establishment of social hierarchy, whereas the majority of wing flicks, lunges, and chases follow.

Figure 3. Number of lunges executed by dominant individuals is inadequate for explaining hierarchical outcomes.

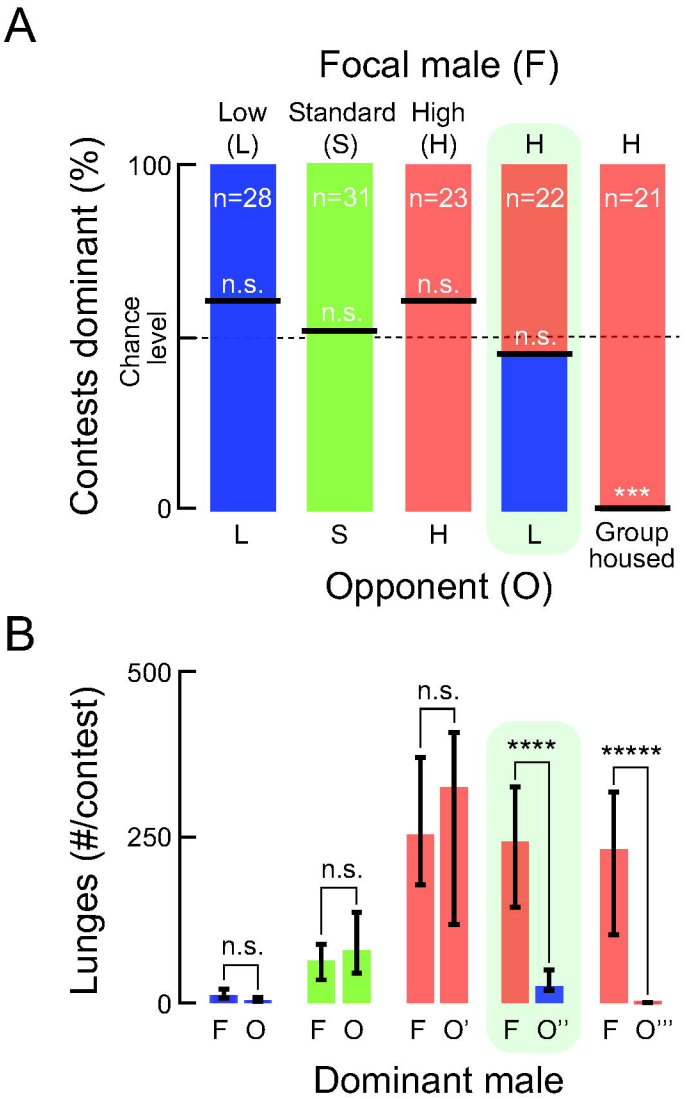


Figure 3 – Supplement 1. Independently isolated genotypically low, standard, and high aggressive lines identified from a P-element screen; additional genotypically high and low aggressive lines corroborate findings in Figure 3.

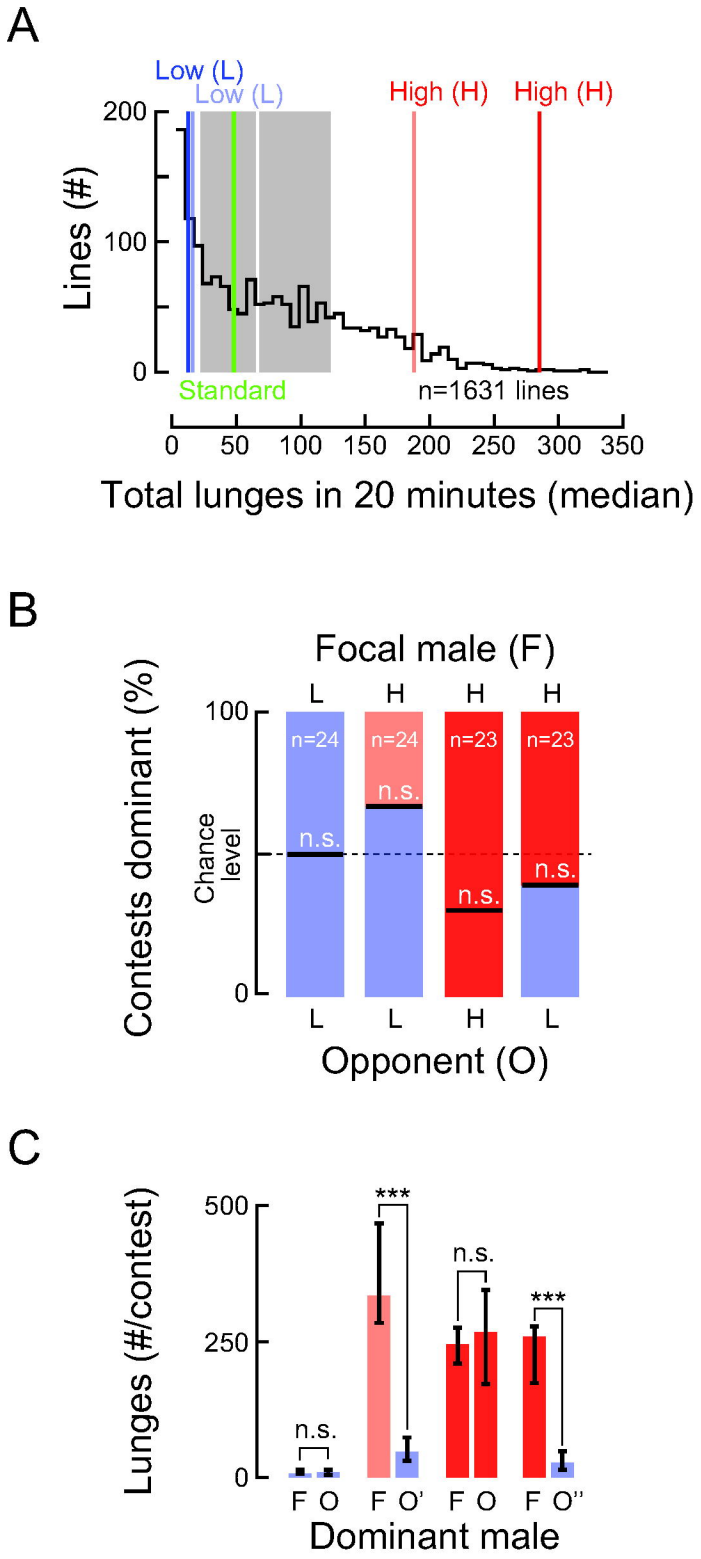


Figure 4. Lunging persists as dominant males confront unfamiliar opponents in unexplored arenas.

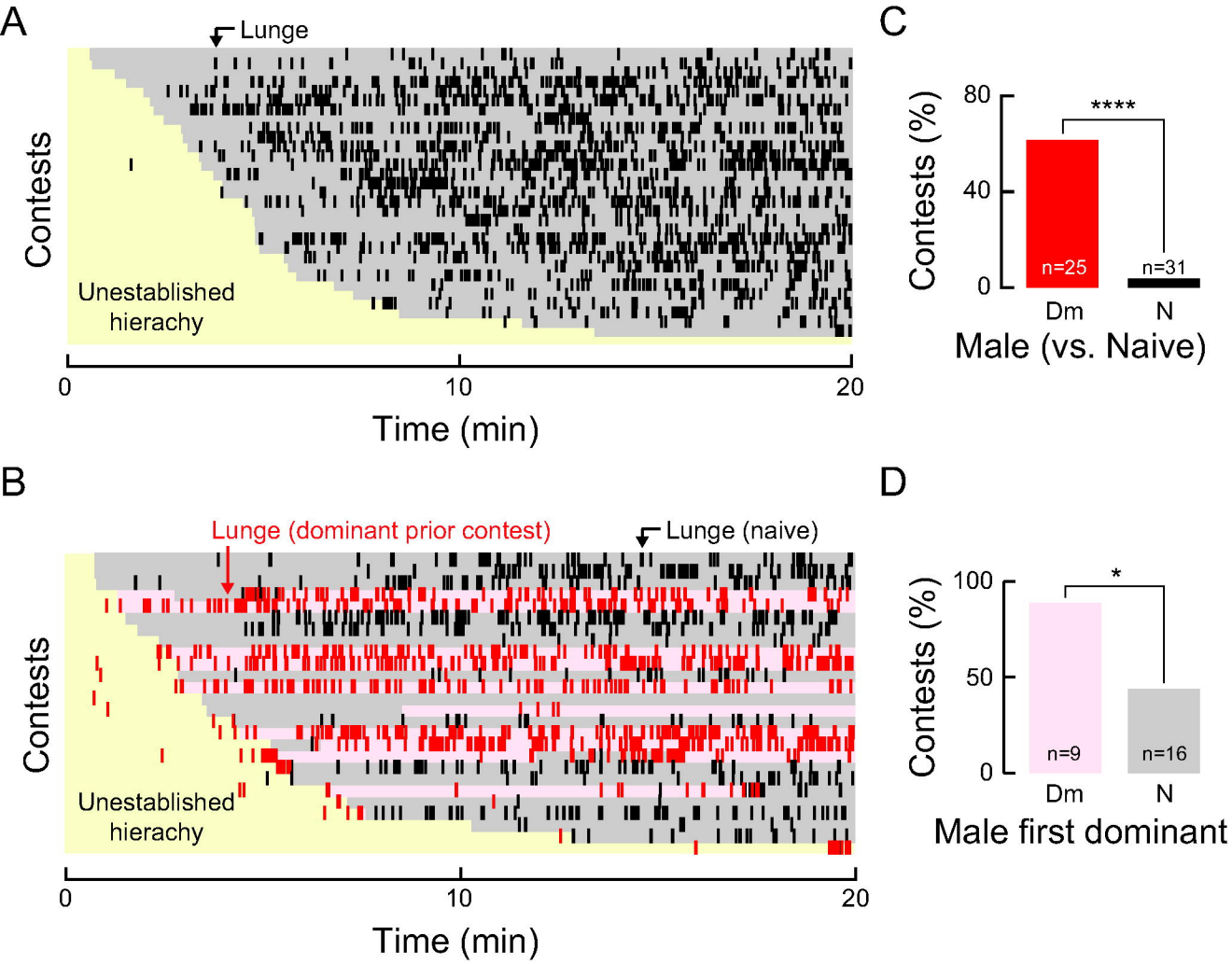


Figure 5. Sequence among aggressive acts in relation to establishing social hierarchy.

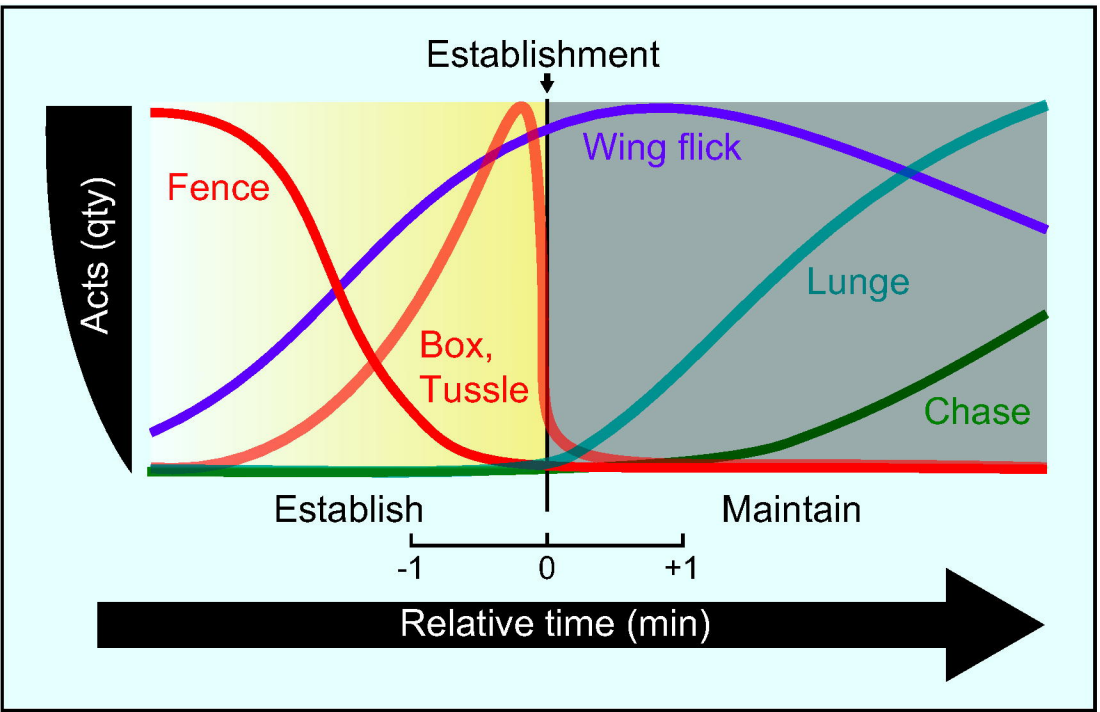


Figure S1. Details for including and classifying fence versus box, tussle interactions.

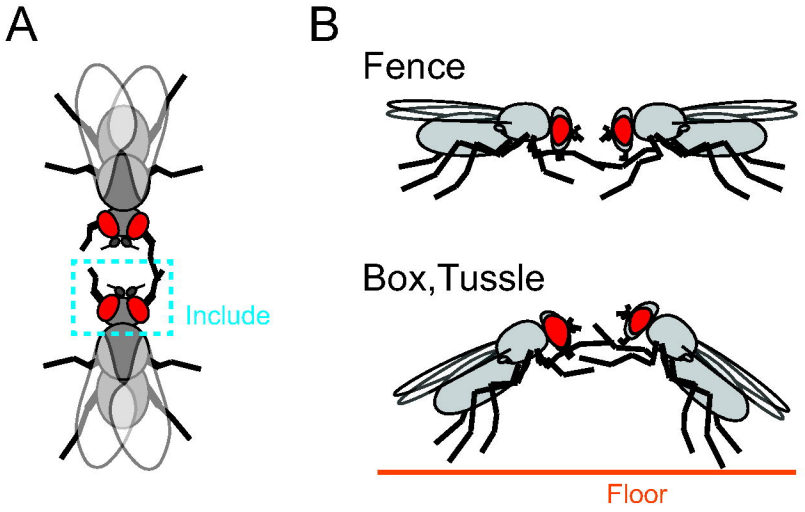


Figure S2. Example data to show the requisite exclusions for balanced statistical analyses.

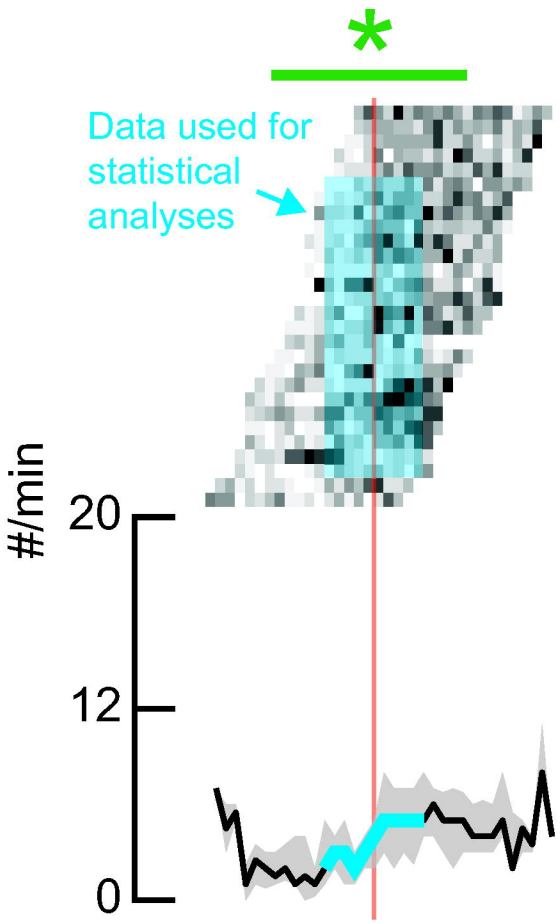


Figure S3. Males assert dominance by lunging.

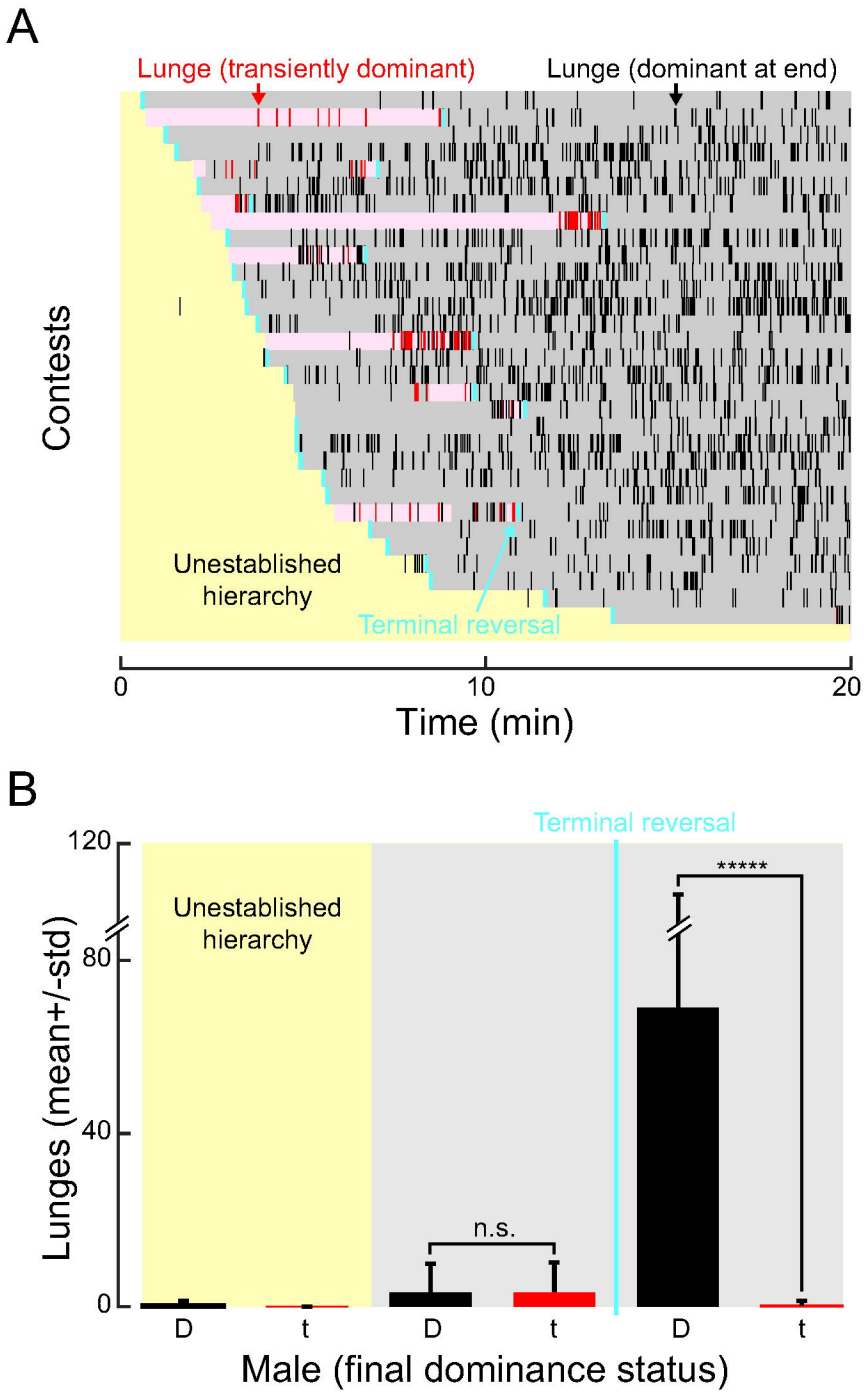


Figure S4. Lunges are executed after establishing dominance irrespective of genotype.

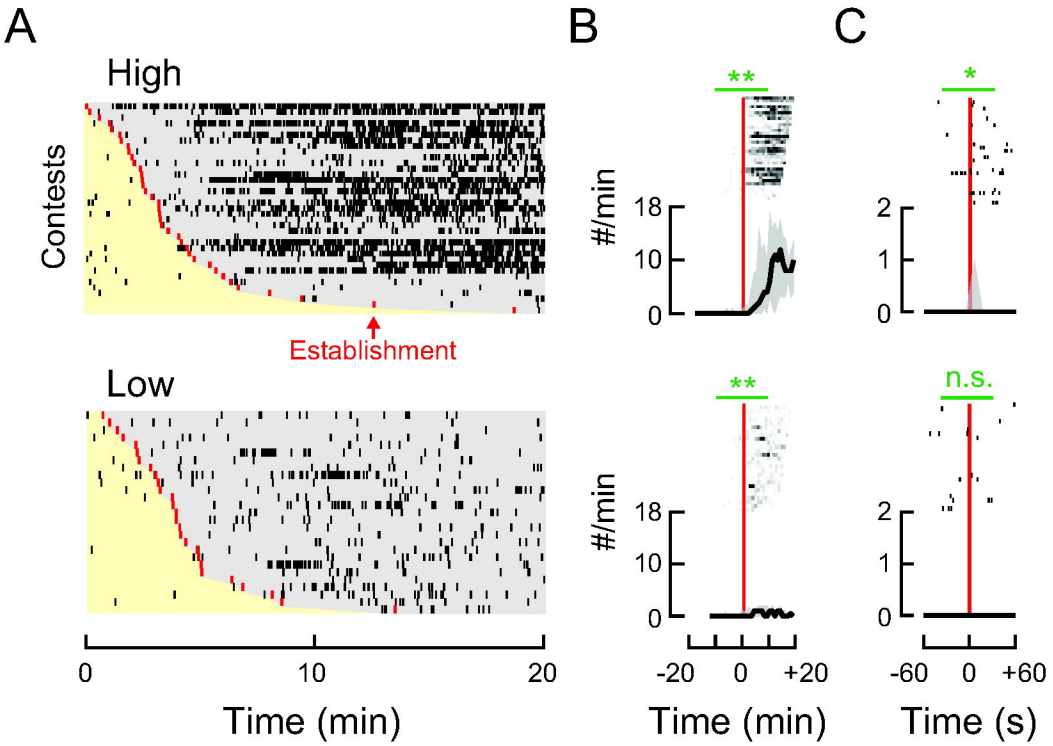
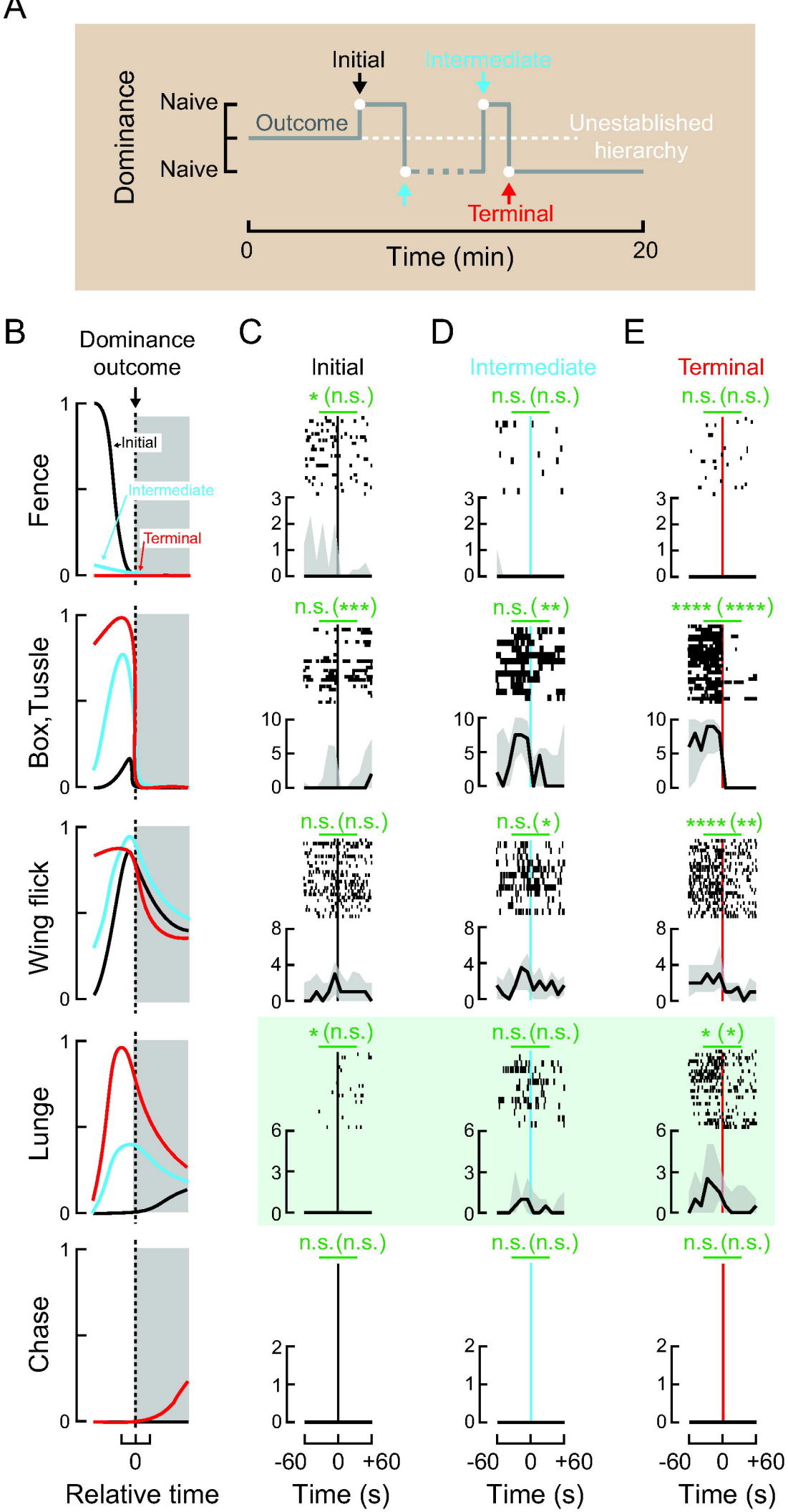



Figure S5. Recurring aggressive acts intensifies through subsequent reversals in dominance