Is variation in female aggressiveness across *Drosophila* species associated with reproductive potential?

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Data and code accessibility

Data and code for this paper are available in the Dryad repository: https://doi.org/10.5061/dryad.zpc866tfp.

Abstract

Aggression is a key determinant of fitness in many species, mediating access to mates, food, and breeding sites. Variation in intrasexual aggression across species is likely driven by variation in resource availability and distribution. To date, most studies have focused on male aggression, but female aggression is common and has fitness consequences. While males primarily compete over access to mates, females are likely to compete over resources to maximize offspring quantity and/or quality, such as food or breeding sites. To investigate why reproduction and aggression are often linked, we tested predictions about resource valuation, life-history, and physiological correlates of reproduction. We used machine learning classifiers developed for *D. melanogaster* to identify and quantify aggressive behaviors displayed in the presence of yeast for mated and unmated females of eight *Drosophila* species. We found that female aggression was linked to ovariole number across species, suggesting that females that lay more eggs are more aggressive. A need for resources for egg production or oviposition sites or similar physiological mechanisms regulating aggression and reproduction may therefore be drivers of female aggression.

Introduction

Aggression towards members of the same species is almost ubiquitous throughout the animal kingdom. In many species, aggression is a key determinant of fitness, establishing an individual's social dominance, access to resources (mates, food, shelter, and/or territory), and vulnerability to predation (Clutton-Brock and Huchard 2013b). Intrasexual aggression has been extensively studied in males, but, until recently, relatively neglected in females (Ah-King 2022). It is now commonly accepted that females compete aggressively in a wide range of taxa, with consequences for survival and reproduction (Clutton-Brock and Huchard 2013a; Rosvall 2013; Stockley and Campbell 2013).

Female-female aggression (hereafter 'female aggression') is diverse. Among species, female aggression varies in the frequency, intensity, and types of aggression, as well as the diversity of resources females compete over (e.g. food, nesting sites, territory,Clutton-Brock and Huchard 2013b; Rosvall 2011; Stockley and Campbell 2013). Within species, females commonly shift their levels of aggression in response to their current reproductive stage. For example, oestrous baboons fight over mates (Huchard and Cowlishaw 2011), mosquitofish increase fighting in the late stages of pregnancy (Seebacher et al. 2013), *Drosophila melanogaster* females increase fighting after mating (Bath et al. 2017; Nilsen et al. 2004). Although females fight predominantly over resources for reproduction, we have few testable predictions about the relationship between reproduction and patterns of female aggression across species because the majority of studies on female aggression focus on the benefits and costs within a species (Clutton-Brock and Huchard 2013a; Rosvall 2011; Stockley and Campbell 2013). The only systematic study (to our knowledge) investigating female aggression and resource valuation across taxa, found increased territorial aggression in cavity-nesting bird species relative to sister species that nested in open nests, suggesting selection for females to fight to protect limited nesting sites (Lipshutz and Rosvall 2021).

We propose that reproduction and female aggression may be related across species because of perceived resource value for maximizing reproduction, because life-history is closely tied to reproductive strategies, and because the physiology of reproduction is tightly linked to the physiology of aggression (all discussed below). We set out to test these ideas in a set of *Drosophila* species chosen for their differences in reproductive traits.

65 ***Drosophila* species**

66 The genus *Drosophila* encompasses over 2000 species (Markow and O'Grady 2008), which vary
67 in their ecology, physiology, mating behavior and life-history, all of which are likely to influence levels of
68 aggression (Markow and O'Grady 2005a; Markow and O'Grady 2005b; Markow and O'Grady 2008).
69 Aggression is understudied in non-melanogaster *Drosophila* species, with a few exceptions (Amino and
70 Takashi 2020; Belenioti and Chaniotakis 2020; Boake et al. 1998; Gupta et al. 2019; Ringo et al. 1983).
71 Most studies have focused on male aggression, though unmated females have been examined in a total
72 of 10 species other than *D. melanogaster* (Belenioti and Chaniotakis 2020; Shultzaberger et al. 2019).
73 Female aggression in *D. melanogaster* consists of a range of behaviors, from high-intensity headbutts to
74 low-intensity wing threats (Nilsen et al. 2004). Female aggression is linked to reproduction in *Drosophila*
75 *melanogaster*: mated females fight more than unmated females over food (Nilsen et al. 2004, Bath et al.
76 2017), while unmated females attack mating pairs but mated females do not (Gaspar et al. 2022). The
77 change in aggression with mating status over food is stimulated by the transfer of sperm and seminal fluid
78 proteins during mating (Bath et al. 2017) and may be driven by increased need for protein-rich food to fuel
79 egg production in mated females (Lee et al. 2013). The clear relationship between reproduction and
80 aggression in *D. melanogaster* sets the groundwork to test if this relationship holds when comparing
81 species with different reproductive strategies (e.g. how many eggs they lay, their mating system, and
82 lifespan).

83 In *Drosophila*, female fecundity is determined by the number of ovarioles (egg-producing
84 compartments in the ovaries), each of which has the capacity to produce two eggs per day (David 1970);
85 species with more ovarioles produce more offspring than females of species with few ovarioles (R'kha et
86 al. 1997; Sarikaya et al. 2019). Ovariole number varies across *Drosophila* species and is strongly
87 correlated with ecology (Sarikaya et al. 2019). Thus, the reproductive potential of females of different
88 species is limited by physiology. Because females need protein to produce eggs after mating (Walker et
89 al. 2015), competition for protein-rich food may lead to the possibility that variation in reproductive
90 potential (i.e. ovariole number) across species may drive aggressive responses to mating due to
91 increased need or valuation of the contested resource (see Predictions below).

Mating systems also vary across the genus: in some species, females mate multiply each day whereas in other species females have a refractory period or may never remate (Markow and O'Grady 2005b). Characteristics of the male ejaculate influence female remating rate. In *Drosophila melanogaster*, proteins transferred with sperm modify female physiology and behavior, inducing a refractory period (Avila et al. 2011). In addition, the quantity of sperm transferred affects the female's propensity to remate, with females receiving few sperm needing to remate often to maximize their reproductive potential (Markow 2002). Across species, the amount of sperm transferred is inversely related to the size of sperm (Markow 1996; Pitnick 1996; Syed et al. 2021) and sperm investment, either in size or number of sperm, varies across the genus (Lüpold et al. 2016; Markow 1996; Pitnick et al. 1995; Schärer et al. 2008; Syed et al. 2021). Variation in remating rate and sperm provisioning across species is likely to result in differences among species in the relationship between a female's mating status and her investment in aggression.

In this study, we tested if reproductive potential is related to female aggression in eight species of *Drosophila*. We used a comparative approach among *Drosophila* species to test the predicted drivers of female aggression across species: remating rate, sperm length, ovariole number, and lifespan. We used machine learning to automatically score common aggressive behaviors in eight *Drosophila* species, measuring aggression in unmated and mated females and investigating cross-species relationships with reproductive drivers of aggression. We tested the specific predictions below.

Resource valuation

Aggression theory predicts that an individual will be most motivated to fight (and continue fighting) when it perceives the resource it is competing over to be valuable (Arnott and Elwood 2008). The reproductive stage of a female modifies how valuable resources are at any given time, influencing her likelihood of engaging, persisting in, and escalating aggressive contests (Huchard and Cowlishaw 2011; Palanza et al. 1996; Seebacher et al. 2013). By framing resources in terms of their value to females, we can predict when females should compete over resources within and across species and when females should display the most aggression. For some species, resources required for offspring production and provisioning (e.g. food, nesting sites) may be the most limiting resource, causing aggression to peak at times when fecundity is at a maximum (Rosvall 2008; Seebacher et al. 2013; Viblanc et al. 2016). For other species, mates or the resources they provide (e.g. sperm, nuptial gifts, paternal care) may be a

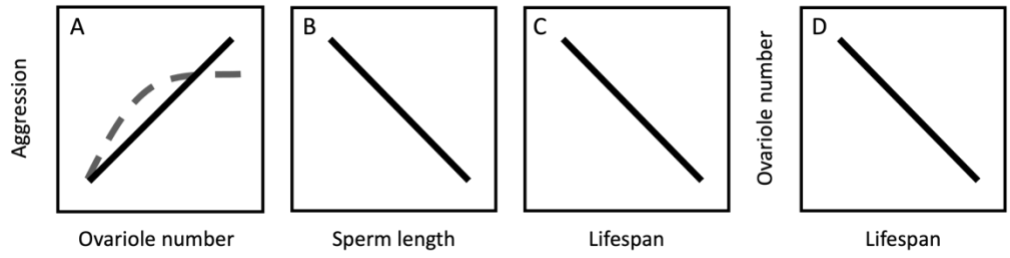
limited resource, resulting in increased female competition before mating for access to mates (e.g. Bro-Jørgensen 2002; Gwynne 1991; Papadopoulos et al. 2009). We, therefore, predict that that the mating system and reproductive potential of a species may explain patterns of female aggression.

We focused on aggression over access to food as this is a form of competition common across taxa (Stockley and Campbell 2013). Reproductive costs include the energy demands for production of offspring, gestation, lactation, parental care, and locating appropriate breeding sites (Stockley and Campbell 2013). The more females invest in reproduction, the greater their energetic demands, leading to increased consumption of food in general, as well as increased intake of specific nutrients (e.g. protein, specific amino acids (Carvalho et al. 2006; Lorenz and Gäde 2009). Because females need food for investment in reproduction, females should be motivated to fight over access to food when it is limited, leading to a positive correlation between investment in reproduction and aggression over food. Thus investment in reproduction leads to higher resource need and/or valuation. When females compete for food, females should be most aggressive when energetic demands are highest, for example during gestation and lactation for mammals, during incubation for birds, and during egg production after mating for most invertebrates (Gittleman and Thompson 1988; Nord and Williams 2015; Shingleton 2015). We can also use this theory to predict patterns of aggression across species: species that have high reproductive energy demands (i.e. producing offspring at high rates) will be more aggressive over food than those with low demands.

With respect to *Drosophila*, resource valuation leads to several predictions. 1) Within species, mated females will show more aggression than unmated females as they have increased egg production and the need to find oviposition sites to lay eggs. Producing more eggs requires increased consumption of food, driving increased aggression after mating in all species. 2). Among species, species with more ovarioles will display more aggression than those with fewer (fig. 1A), as they produce more eggs so have higher energy and resource requirements. 3) Among species, we also predict that there will be a greater change in aggression after mating in species with more ovarioles as they should exhibit the largest change in resource demands. 4) Across species, we expect a negative correlation between mated female aggression and sperm length (fig. 1B). Species with longer sperm transfer fewer sperm in each mating, meaning that fewer eggs are fertilized than in species with shorter sperm. We would therefore expect

148 females receiving few, long sperm to have low energetic demands from egg laying and therefore show

149 lower rates of aggression than females of species who receive many, short sperm.



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151 **Figure 1: Predictions for the relationship between aggression and potential drivers of aggression**
152 **across species** A) All theories predict a positive relationship between reproduction (measured as
153 ovariole number) and female aggression. The exact nature of this relationship is unclear, so we have
154 plotted a solid line to indicate a linear relationship and a dashed line to indicate a situation where a
155 maximum level of aggression is reached and cannot increase any further. B) Predicted negative
156 correlation between sperm length and female aggression. C) Predicted negative correlation between
157 lifespan and female aggression. D) The underlying assumption for life-history theory is that there is a
158 negative relationship between reproduction and lifespan.

159 **Life-history**

160 Life-history theory provides additional insight into the link between reproduction and female

161 aggression across species. Species lie along a continuum of life-history strategies: on one end lie species

162 with 'fast' life-histories (short lifespans and fast rates of reproduction) while at the other end are species

163 with 'slow' life-histories (long lifespans and slow rates of reproduction, fig 1D, Stearns 1989). Behaviors

164 that increase access to resources or mates, such as aggression or boldness, come with costs in the form

165 of increased mortality, and are likely to coevolve negatively with lifespan (fig. 1C) for two different reasons

166 (Eckerström-Liedholm et al. 2016): 1) Species with different life-history strategies differ in their relative

167 energetic demands. Species with fast life-histories have high productivity and short lifespans so need

168 energetic resources to grow fast and start reproducing quickly (Dammhahn et al. 2018). Fast species

169 should therefore exhibit high risk-taking behaviors to acquire resources due to the high relative value of

170 the resources. 2) Species will vary in their valuation of current vs future reproduction. Traits that prioritize

171 current reproduction, even at the cost of future reproduction, are favored in short-lived species that

172 receive the benefits of such behaviors but tend to die before facing the costs (Eckerström-Liedholm et al.

173 2016). Aggression increases current reproduction through increasing access to vital resources but with an

174 elevated risk of injury or other costs (Smith and Blumstein 2008).

We can test the trade-off between reproduction and lifespan in our dataset with ovariole number and lifespan with the following predictions: 1) Within species, mated females will fight more than unmated females as there is no point taking risky behavior if an individual is not yet reproducing. 2) Among species, faster-reproducing species (those with more ovarioles) will display more aggression. We therefore expect to see a positive correlation between ovariole number and aggression (fig. 1A). 3) Among species, we also predict that there will be a greater change in aggression after mating in species with more ovarioles as they should need to increase their risk-taking more due to increased reproduction. 4) Across species, shorter-lived species will display more aggression, leading to a negative correlation between lifespan and aggression (fig. 1C).

Physiology

Changes in aggression associated with reproduction may be a by-product of changes in physiology involved in reproduction. Many of the hormones that regulate reproductive traits, including egg production, ovulation, mating, gestation and lactation, are also implicated in regulating aggression. For example, the monoamine octopamine, the invertebrate counterpart to vertebrate norepinephrine, functions as a neurotransmitter, neuromodulator, and neurohormone (White et al. 2021). Octopamine signaling regulates a range of reproductive behaviors, including ovulation, sperm storage, and physiological changes in mated females in a range of insect species (White et al. 2021). Octopamine also regulates aggression in both males and females, suggesting a possible link between reproductive and aggressive regulators (Cunningham et al. 2014; Jia et al. 2021; Zhou et al. 2008). In vertebrates, testosterone regulates aggression in both sexes and mediates ovulation or fertility (reviewed in Rosvall et al. 2020). Female testosterone levels fluctuate seasonally in bird species, peaking during territory establishment and diminishing during periods of parental care (reviewed in Rosvall et al. 2020), suggesting adaptive expression of hormones that are involved in both aggression and reproduction. Changes in aggression regulated by hormones may therefore track changes in reproductive state, and induce changes in aggression, but this may be a side-effect of reproduction rather than an adaptive trait (Sibbald and Plowright 2015). Conversely, the use of the same mechanisms in reproduction and aggression may be an adaptive way of linking the two to ensure aggression occurs at the optimal time.

202 If shared physiological mechanisms regulate reproduction and aggression, we predict: 1) Within
203 species, mated females will be more aggressive than unmated females due to the changes in physiology
204 stimulated by mating regulating both reproduction and aggression. 2) Across species, aggression and
205 ovariole number will be positively correlated. If the rate of aggression increases in response to the rate of
206 reproduction due to similar hormones regulating the behaviors, as reproductive rate increases, so should
207 aggression. 3) Because females that have more ovarioles should exhibit larger changes in physiology
208 due to reproduction, females in species with more ovarioles will have a greater increase in aggression
209 after mating than those with fewer. 4) Because females can only respond to sperm after mating, any
210 relationship between sperm production and female aggression should only be seen in mated, and not
211 unmated females. 5) If the number of sperm produced by a male is related to change in aggression, then
212 a larger response to mating will occur in species that produce many, small sperm.
213 To test these predictions and measure female aggression in a range of species, we used machine
214 learning to automatically score headbutting and fencing in mated and unmated females in eight
215 *Drosophila* species. The development of software to track animal locomotion and behavior has improved
216 our ability to study broad-scale and individual-level behaviors (Dell et al. 2014), but the use of such
217 analyses identifying and classifying behaviors is usually focused on one species at a time (though see
218 DeLellis et al. 2014; Hernández et al. 2021). Here we examined eight *Drosophila* species with a range of
219 phylogenetic relatedness, and differences in reproductive and life-history traits, to investigate cross-
220 species relationships with reproductive drivers of aggression.

221 ## Materials and Methods

222 **Strain maintenance and unmated fly collection**

223 All strains (table S1) were maintained on standard cornmeal-molasses food in multiple 75 ml
224 bottles at 25˚C on a 12:12 light:dark cycle. Unmated males and females were collected on ice within a
225 day of eclosion except for species that are sexually mature in a day or less (*D. willistoni* and *D. nebulosa*),
226 which were collected within 7 hours of eclosion. Males were housed in 12.5 ml vials in groups of 10 with
227 *ad libitum* access to food. Females were housed individually.

228 **Aggression assay**

229 The aggression assay followed the method of Bath et al. (2017). We collected flies before the age
230 at which they become sexually mature (table S1). When sexual maturity for one sex was unknown, we
231 collected flies at the same age as the sex for which we had age at maturity information on the basis that if
232 one sex was not mature, the other sex could not mate even if they were mature (e.g. *D. ananassae* males
233 are reported to be sexually mature at 4 days old, so we collected both males and females before they
234 were 4 days-old). Once both males and females were sexually mature, a subset of the unmated females
235 were paired with one or two males of their species at lights on for up to two hours or until the first mating
236 finished. Females that mated were separated from the male and transferred to fresh vials to be kept
237 overnight. Females that did not mate were discarded. Unmated females that were never exposed to
238 males were moved to fresh vials at the same time as mated females to be kept in identical conditions
239 overnight.

240 At 24-hrs after mating, single mated and unmated females of a single species were separately
241 placed in individual empty vials with a moist cotton ball. Females remained in these vials for two hours to
242 increase their motivation to compete for food (Edwards et al. 2006). Each female was then paired with a
243 female from the same treatment (mated or unmated) in a contest arena (20 mm in diameter), with apple
244 juice agar and a small patch of yeast paste (Supplementary Video). Up to 20 dyads were video recorded
245 simultaneously with a Basler ac A2440-75µm camera. Where possible, equal numbers of mated and
246 unmated dyads were included in each video to control for time-of-day effects. Pairs were given 5 minutes
247 to acclimatize to the arena. We recorded videos for 15 minutes using Kinovea software (v 0.9.5;
248 https://www.kinovea.org) at 30 frames-per-second. Flies were subsequently discarded.

249 **Automated tracking and machine learning methods**

250 Following recording, we used automated tracking and machine learning to score flies' aggressive
251 and movement behavior (Bath et al. 2020). Briefly, the Caltech fly tracker (Eyjolfsdottir et al. 2014)
252 determined the location and per-frame characteristics (e.g. velocity, distance to other flies, orientation) of
253 each fly within an arena. Each species was tracked separately, with species-specific tracking parameters
254 (e.g. background and foreground thresholds) to account for differences in body size. Videos were

inspected manually after tracking to check the accuracy of tracking. Videos that did not track correctly were retracked with video-specific parameters to ensure the highest quality video tracking.

The tracking data were transferred to JAABA (Kabra et al. 2013), where we used supervised machine learning to train classifiers for female aggression. The two most overt contact behaviors used to score female aggression are headbutts (attacks using the head) and fencing (striking the opponent with legs; see Supplementary Video). Headbutting is the most commonly used measure of female aggression in *D. melanogaster* studies (Bath et al. 2017; Chiu et al. 2021; Schretter et al. 2020), so we focus our results section on this behavior, with fencing results reported in the Supplementary Information. Both headbutting and fencing have been observed in females of multiple species (Belenioti and Chaniotakis 2020; Shultzaberger et al. 2019), suggesting consistent behavioral repertoires across species. The existence of a common behavioral repertoire allows us to use the same machine learning classifiers for all species in our experiments.

For all species, we used an existing lab classifier for headbutts trained on *Drosophila melanogaster* (Bath et al. 2020). We trained one new classifier for fencing and one new locomotion classifier, defined as any time a female moved around the arena. A new species-specific classifier was trained for the *D. nebulosa* wing lifting display, a behavior unique to this species (Supplementary Video).

All classifiers were ground-truthed by manually scoring a subset of videos with the scorer blind to the automated classifier score. Ground-truthing was conducted on each species to test for differences in accuracy among species. We used 30 randomly chosen intervals for each behavior in each species, using the ‘balanced random’ option in JAABA, which ensures that some of the intervals contain the behavior. We selected bout lengths (the number of frames an instance of that behavior was scored) the same length as the average bout length for each species, because bout lenghts differed among species (table S2). As is often the case when scoring behaviors, some instances of behaviors were clear (‘Certain’), while others were more difficult to classify (‘Difficult’). When manually scoring during ground-truthing, we therefore scored the frames as either containing the behavior (‘Behavior’) or not (‘None’), and within each of those two groups, whether the behavior was easy to distinguish (‘Certain’) or difficult to score (‘Difficult’). This resulted in four possible outcomes for a frame: ‘Behavior Certain’, ‘Behavior Difficult’, ‘None Certain’, ‘None Difficult’. We then separately analyzed the frames where the behavior was

clear from those where it was difficult, because we expected differences in the performance of the classifier between 'Certain' and 'Difficult' cases.

To quantify behaviors, we obtained the number of frames in which JAABA scored that a focal individual in a dyad displayed that behavior. We divided this number of frames by the total number of frames in which the individual was tracked to produce the proportion of frames an individual engaged in the behavior. Using the proportion of frames allowed us to account for slight differences in frame numbers among videos or individuals, due to recording or tracking discrepancies. We calculated values for each individual in a dyad, but for some analyses (see below), we calculated a combined score for the dyad based on both individual's tracking data.

For all behavioral classifiers, we used the proportion of frames scored for a behavior as our response variable (e.g. proportion of frames scored as headbutting). The proportion of frames in which one individual displayed aggression (headbutting and fencing) was positively correlated with the proportion of frames her opponent displayed aggression (fig. S1 & S2), thus counting all frames with aggression leads to pseudoreplication. The exception was *D. sturtevanti,* which did not have a positive correlation in the behavior of both females. Nevertheless, to avoid pseudoreplication in all species, we summed the number of frames for both individuals in a dyad to calculate the total number of frames in which the behavior was observed in the arena to produce one value per dyad. We then divided this number by the number of frames in the video to produce the total proportion of frames in which the behavior happened for the pair. We then used this composite value for each pair, resulting in only one value per dyad. For locomotion behavior, how much one individual moved was correlated with how much its opponent moved (fig. S3). However, both individuals could be moving at the same time so combining the number of frames in which each individual moved would have produced an overestimate of locomotion within a pair. We therefore randomly chose one individual from each dyad and ran the analysis on those individuals to avoid pseudoreplication.

Phylogenetic tree and species data

We built a phylogeny for our eight *Drosophila* species using the Open Tree of Life data store (McTavish et al. 2015) and custom synthesis tools (Redelings and Holder 2017), based on phylogenies from multiple sources (Finet et al. 2021; Oliveira et al. 2013; Sarikaya et al. 2019; Suvorov et al. 2022).

311 Branch lengths were estimated from date using Chronosynth (McTavish et al. 2023,
312 https://github.com/OpenTreeOfLife/chronosynth). Once a tree containing 200 species had been built for
313 another research project, we trimmed this tree to contain only our eight species (fig. S4). The eight
314 species were chosen as they represented a range of mating system characteristics (lifespan, remating
315 rate, sperm length, ovariole number; table S1). We attempted to account for phylogeny by pairing
316 species, when possible, in the same species group (i.e. *D. ananassae, D. bipectinata* in the melanogaster
317 group, *D. willistoni, D. nebulosa* in the willistoni group, *D. saltans, D. sturtevanti* in the saltans group).
318 In addition to the phylogeny, we collected values on ovariole number, sperm length, and remating
319 rate from the literature (table S1). For several of our species, we did not have data for ovariole number or
320 remating rate. We collected remating data for *D. saltans, D. sturtevanti,* and *D. willistoni*. We attempted to
321 collect these data for *D. nebulosa* but could not get a large enough sample size. To measure remating
322 rate, we mated females as described above. Once those pairs had finished mating, we removed the male
323 and left the female in the vial alone overnight. Twenty-four hours later, we placed two new unmated males
324 (which had not been used the previous day) in the vial with the female. Flies were observed for two hours
325 to determine if remating occurred (following Denis et al. 2017; Holman et al. 2008). Our sample sizes
326 were 29 *D. saltans* vials (4 remated), 37 *D. sturtevanti* (37 remated), and 48 *D. willistoni* vials (1 remated).
327 We then calculated the percentage of females that remated for use in our analysis of remating rate.
328 We counted the number of ovarioles in species for which we could not find ovariole number in the
329 literature. We followed the procedures outlined in Lobell et al. (2017). Briefly, unmated females were
330 collected and housed individually. When sexually mature, a female was placed on food with yeast paste
331 overnight before freezing at -20˚C. Within a month, females were dissected using fine needles in a drop
332 of PBS and stained in a solution of PBS saturated with crystal violet (Thermo Scientific). Ovarioles were
333 counted from both ovaries. Strains measured are given in table S1. At least 20 females were dissected
334 from each species.

335 **Data analysis**

336 All analyses were run in R v4.2.1 (R Core Team 2017). To analyze our behavioral data, we used
337 generalized linear models (GLMs) from the *lme4* package (Bates et al. 2015). Model fit and assumptions
338 were checked by visual examination of diagnostic plots. We acquired p-values using the 'Anova' function

339 from the *car* package (Fox and Weisberg 2018) To test if classifier accuracy was correlated with the
340 frequency of the behavior, we conducted a linear model with classifier accuracy (% of instances correct)
341 as the response variable and the proportion of frames in which the behavior was observed as the
342 explanatory variable. To analyze the effects of species and mating status, we used the total proportion of
343 frames in which flies performed the behavior as the response variable, with species, mating status, and
344 their interaction as explanatory variables. All variables and their interaction were included in all models as
345 these were key to the experimental design and answering the question at hand. We fitted these models
346 with a quasibinomial error distribution to account for underdispersion in our proportion data. We
347 performed this analysis for all species for headbutting, fencing, and locomotion (tables S3, S4).
348 To assess whether locomotion and aggression were correlated, we ran the following GLM with a
349 quasibinomial distribution:
350 *Proportion of frames aggressive ~ Proportion of frames moving * Species * Mating status*
351 As differences in aggression may be related to the proximity of flies within the arena, we also tested if the
352 average distance between females varied across species, as has been found in previous studies
353 (Shultzaberger et al. 2019). We used the minimum distance from the focal fly's head to the closest side of
354 the other fly that lies in front of the fly (-30°- 30° in front of the fly). We chose this measure as the distance
355 to an opponent in front of the fly is more relevant to aggression than absolute difference between flies as
356 flies can only headbutt flies in front of them (Nilsen et al. 2004); the measure is generated automatically
357 by the JAABA per-frame feature: 'dnose2ell_angle_min30to30'.
358 To test relationships between aggression and the reproductive characteristics, we performed
359 phylogenetic analyses using the mean values for each behavioral trait (i.e. headbutting and fencing) for
360 each species and mating treatment as the response variable and the mean value for the reproductive trait
361 (i.e. ovariole number, sperm length, remating rate) as the explanatory variable. We ran a phylogenetic
362 generalized least squares models with female median lifespan, average ovariole number, sperm length
363 and remating rate per species as explanatory variables in separate models (ape package: Paradis and
364 Schliep 2019; nlme package: Pinheiro et al. 2022). For the analyses with ovariole number and lifespan,
365 we included average body size as a covariate because ovariole number and lifespan scale with body size
366 across *Drosophila* species (Sarikaya et al. 2019; Syed et al. 2021). We log-transformed ovariole number,

367 body size, and sperm length before including them as explanatory variables in phylogenetic generalized
368 least squares models. As we ran four models for each of our response variables, we corrected for multiple
369 testing by applying a Bonferroni correction and reducing the threshold for significance (dividing our
370 significance threshold by the number of models: 0.05/4 = 0.0125). We then judged whether terms were
371 significant based on this new threshold.

372 ***Drosophila nebulosa* specific analysis**

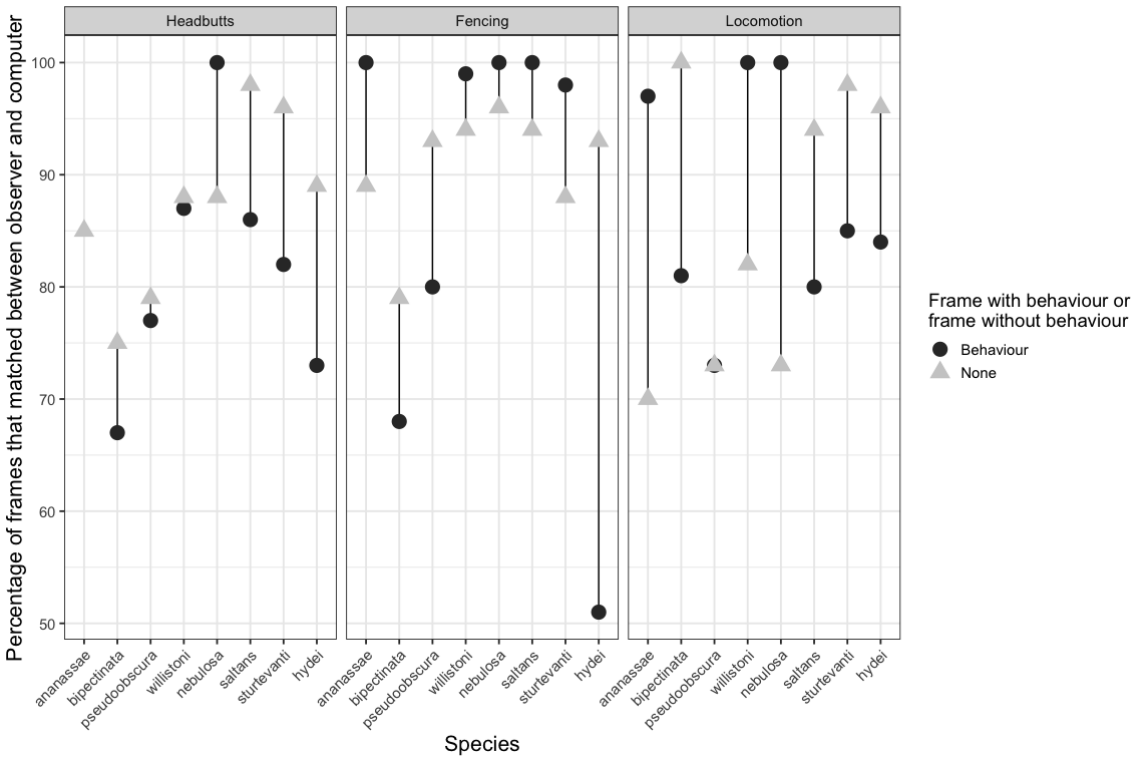
373 We observed female *D. nebulosa* performing a wing lifting movement that was first described with
374 courtship (Spieth 1947). In this movement, wings are lifted, spread, and dropped. Both *D. saltans* and *D.*
375 *sturtevanti* also performed a wing spreading behavior, but the movement was subtle and difficult to score.
376 The *D. nebulosa* movement is speculated to be a signaling behavior (Spieth 1947), thus we tested if its
377 performance was consistent with being an aggressive signal by describing when the behavior is
378 performed. We tested if the proportion of frames an individual spent performing the wing movement
379 varied with mating status. Because the proportion of frames one individual spent doing the wing behavior
380 was correlated with how much time their opponent spent performing the behavior, we randomly selected
381 one individual from each pair for the analysis to test if the wing movement varied with mating status. To
382 investigate if the wing behavior was a social behavior, we also tested if the distance between individuals,
383 and the orientation of the individuals, differed between frames in which flies were performing the wing
384 behavior and the frames in which they did not.

385 **Results**

386 **Accuracy of machine learning across species**

387 We used machine learning classifiers (Bath et al. 2020) to automatically score headbutts, fencing,
388 and locomotion in eight species. We scored classifier accuracy by ground-truthing a subset of frames
389 within each species (fig. 2 and table S2). We evaluated frames in which the behavior was unequivocally
390 happening or clearly not happening as 'Certain' instances and frames where it was less clear if the
391 behavior was happening or not as 'Difficult' frames. We used these two categories to allow us to separate
392 clear instances from less clear instances where manual observers often struggle to define behaviors. For
393 the headbutting classifier, accuracy ranged from 67-100% in the 'Certain' frames and from 63-100% in the

394 ‘Difficult’ frames. Fencing was more variable with 51-100% for ‘Certain’ frames and 44-100% accuracy in
395 the ‘Difficult’ frames. We included locomotion to investigate differences induced by mating, as increased
396 movement is a post-mating response in *D. melanogaster* (Isaac et al. 2010). Locomotion is a less
397 complicated behavior for the classifier to score because it focuses only on the behavior of one fly,
398 providing us with a non-aggressive behavior for assessing classifier accuracy. Locomotion was scored
399 with similar accuracy to headbutts and fencing with a range of 73-100% for the ‘Certain’ frames and 60-
400 100% for the ‘Difficult’ frames (Table S2). Overall, headbutting and locomotion were more accurately
401 classified than fencing, though this did vary between species (e.g. for *D. nebulosa*, all behaviors had
402 similarly high levels of classifier accuracy, but for *D. hydei* headbutting was more accurately classified
403 than fencing).



404

405 **Figure 2: Machine learning classifier accuracy across eight species and three behaviors.** Black
406 circles represent the percentage of frames in the ground-truthing dataset where both the machine
407 learning classifier and the manual observer scored the frame as containing that behavior. Grey triangles
408 represent frames where both the machine learning classifier and the manual observer scored the frame
409 as not containing that behavior. The lines connect points within a species for ease of visualization across
410 species. These represent the ‘Certain’ instances of the classifier—those that were judged to be
411 unequivocally containing the behavior by the manual observer. Results for frames that were less clear are
412 included in table S2. For Drosophila ananassae, no frames were scored as containing the ‘Certain’
413 behavior by either the classifier or the manual observer, so no circle is included. Points higher on the y-
414 axis mean the classifier was more accurate than those lower. When the black circle is higher than the

gray triangle, false positives exceeded false negatives in that species. If the points are the other way around, false negatives exceeded false positives.

We tested if the frequency of behaviors influenced classifier accuracy, on the assumption that species that had more instances of behaviors may be more likely to have higher accuracy. We found that classifier accuracy was not related to how frequently the behavior was observed for headbutting ($F_{1,5}$ = 0.3, P = 0.61) or locomotion ($F_{1,5}$ = 1.03, P = 0.35). For fencing, accuracy and frequency were slightly negatively associated ($F_{1,5}$ = 6.76, P = 0.041): species that had more instances of fencing were less accurate, driven primarily by low accuracy in *D. hydei*. Therefore, the differences between species and behaviors in accuracy were not due to how often the behaviors were displayed.

Effects of species and mating status on aggression

Headbutting is a commonly measured in the analysis of female aggression (e.g. Bath et al. 2017; Nilsen et al. 2004; Schretter et al. 2020). Results for fencing were broadly the same as for headbutting, thus we present the higher intensity headbutting (Nilsen et al. 2004). Results for fencing are presented in the online supporting information. We found a significant effect of species on the proportion of frames in which pairs were headbutting ($\chi^2_{7, 538}$ = 49.76, P < 0.001; fig. 3). Mating status did not have a significant effect ($\chi^2_{1, 538}$ = 0.35, P = 0.55), nor was there an interaction between species and mating status ($\chi^2_{7, 538}$ = 6.82, P = 0.45). *Drosophila pseudoobscura* was the most aggressive species with respect to headbutting (proportion of frames: 0.018 ± 0.0017) and was significantly more aggressive than all species except *D. saltans* (post-hoc Tukey tests, table S3).

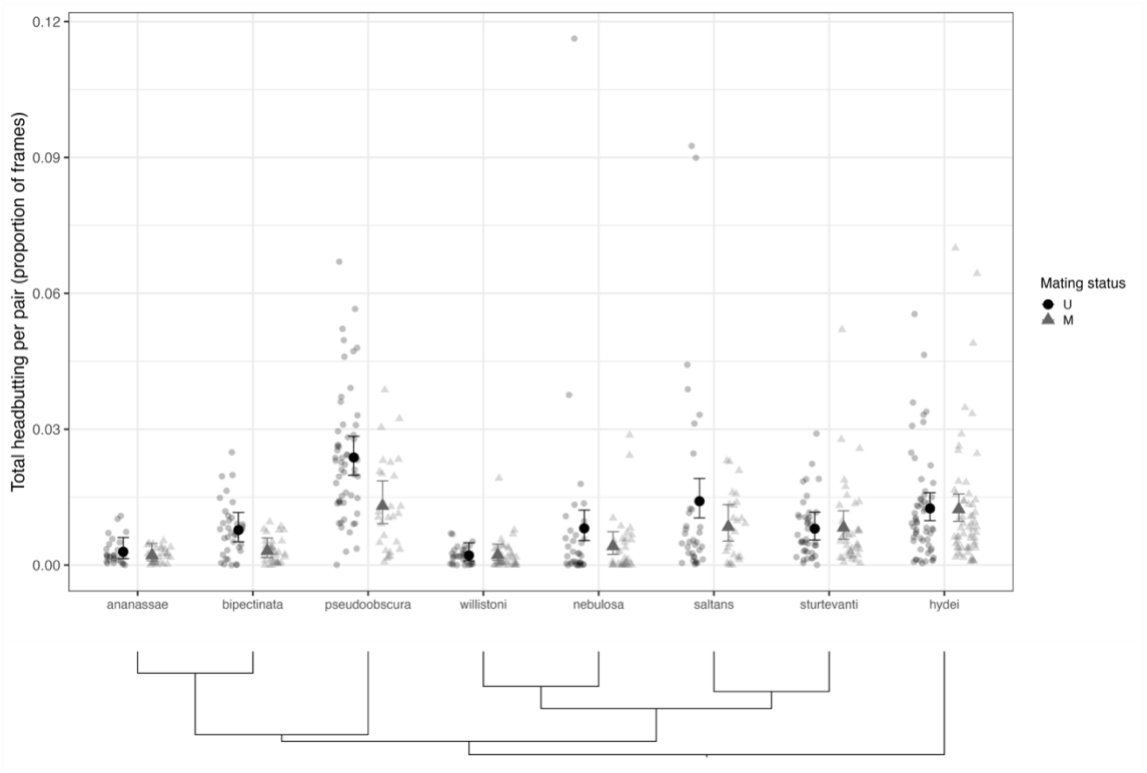


Figure 3: Total proportion of frames labeled with headbutting by species and mating status. Each small point represents one dyad of females, with black circles representing unmated pairs (U) and grey triangles representing mated pairs (M). Large points represent model means with error bars representing 95% confidence interval (asymmetric due to proportion data). The phylogenetic relationships among the species are shown underneath the figure (see fig. S4 for branch lengths).

Although we found no evidence for an interaction between species and mating status, visual inspection o the data revealed trends in how species responded to mating. We performed pairwise comparisons within each species between mated and unmated females. Unmated females were significantly more aggressive than mated females in *D. bipectinata* (Odds Ratio Unmated/Mated = 2.46, z = 2.409, P = 0.016; fig. 3) and *D. pseudoobscura* (OR = 1.84, z = 3.065, P = 0.002). Trending in the same direction were *D. hydei* (OR = 1.55, z = 1.672, P = 0.0945), *D. nebulosa* (OR = 1.96, z = 1.943, p = 0.052), and *D. saltans* (OR = 1.68, z = 1.898, P = 0.058).

To investigate the effect of phylogeny on headbutting, we ran a phylogenetic generalized least squares model with a phylogeny containing our eight species for mated and unmated headbutts separately. We found that both mated and unmated headbutting had a significant phylogenetic signal with λ = 1.23 and λ = 1.07 respectively. Closely related species were similar in their levels of headbutting for

both mated and unmated females. However, the relative change in aggression after mating did not have a significant phylogenetic signal (λ = - 0.24).

Locomotion and distance between individuals

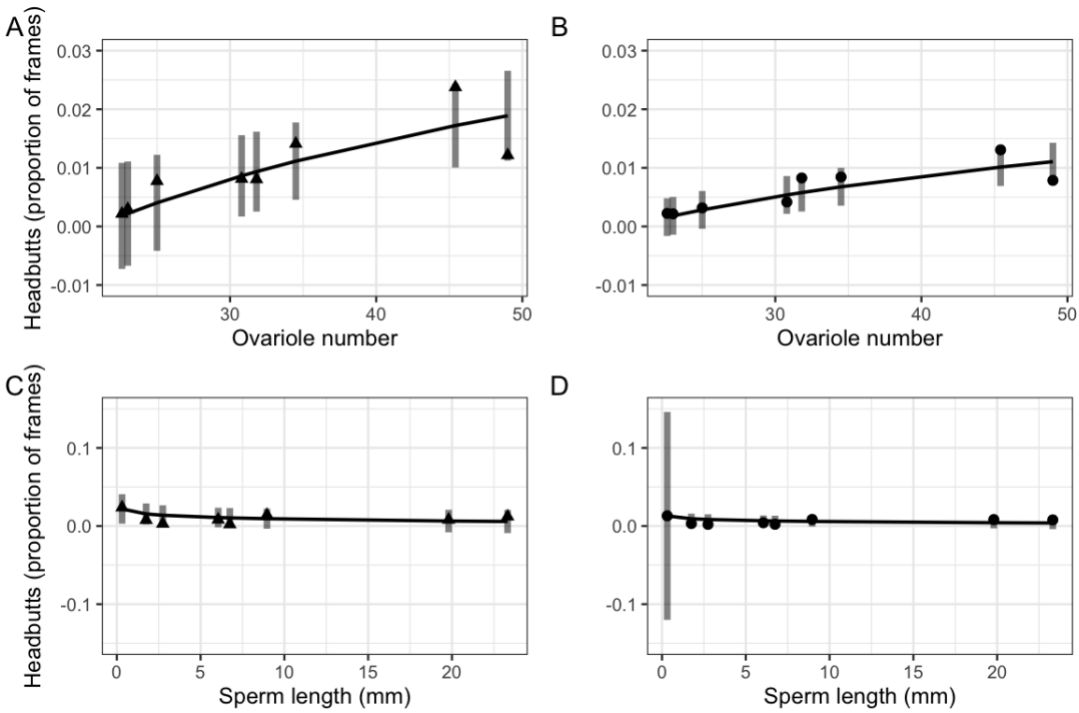
Species and mating status had a significant interaction with respect to the proportion of frames in which individual flies were moving ($\chi^2_{7, 538}$ = 34.569, P < 0.0001; fig. S5). Unmated females moved significantly more than mated females in *D. bipectinata* (OR V/M = 5.64, z = 3.804, P = 0.0001), but mated females moved more than unmated female *D. sturtevanti* (OR = 0.42, z = -3.071, P = 0.0021). No other species exhibited a significant difference in locomotion with respect to mating status (fig. S5). We found no significant effect of locomotion on headbutts, nor were there significant interactions between locomotion, mating status or species (fig. S6). Observed differences in aggression, therefore, are not attributable to differences in how much flies move.

We tested if the proximity of flies within the arena differed across species. We found a significant effect of species in how close an opponent was in the area in front of the focal fly ($\chi^2_{1, 22}$ = 10.83, P = 0.0005, fig. S7). Female *D. hydei* were significantly further apart than *D. saltans* and *D. sturtevanti* when not headbutting (Tukey post-hoc comparisons: *D. hydei vs. D. saltans*: z = 8.44, P < 0.001; *D. hydei vs. D. sturtevanti*: z = 7.58, P < 0.001), but no other pairs of species were significantly different.

Ovariole number and aggression

Ovariole number and body size were not correlated with each other in our sample of species ($F_{1,6}$ = 3.24, p = 0.12). The relationship between ovariole number and the proportion of frames with headbutting was positive– i.e. the more ovarioles a species had, the more time they spent headbutting (Unmated females: $\chi^2_{1, 5}$ = 40.2, P < 0.0001, fig. 4A; Mated females: $\chi^2_{1, 5}$ = 14.43, P = 0.0001; λ= 1.26, fig. 4B). Unmated females had a negative relationship between body size and headbutting—larger species spent less time headbutting ($\chi^2_{1, 5}$ = 8.51, P = 0.004; λ= 0.82). This relationship was not seen in mated females ($\chi^2_{1, 5}$ = 0.37, P = 0.54). Ovariole number did not predict the difference in aggression between unmated and mated flies ($\chi^2_{1, 5}$ = 2.6, P = 0.11; λ= -0.68), whereas mating-induced changes in aggression were linked to body size: species with small body sizes experienced a larger difference in

477 headbutting between mated and unmated females ($\chi^2_{1,5}$ = 5.88, P = 0.015).



478

479 **Figure 4: Relationship between headbutts and fecundity traits.** Relationship between headbutts and
480 ovariole number in A) unmated females B) mated females and relationship between headbutts and sperm
481 length in C) unmated females D) mated females. Dots (triangles in A and C and circles in B and D)
482 represent the species mean proportion of frames in which headbutting was recorded. Confidence
483 intervals represent 95% confidence intervals calculated in the phylogenetic analysis including body size
484 as a covariate. The black line represents the model predictions. Species in the order from least ovariole
485 number to highest are *Drosophila willistoni*, *D. ananassae*, *D. bipectinata*, *D. nebulosa*, *D. sturtevanti*, *D.*
486 *saltans, D. pseudoobscura, D. hydei*. For sperm length, the order is: *Drosophila pseudoobscura, D.*
487 *bipectinata, D. ananassae, D. nebulosa, D. willistoni, D. saltans, D. sturtevanti, D. hydei.*

488 **Sperm length and aggression**

489 Sperm length was not correlated with unmated female headbutting ($\chi^2_{1,6}$ = 3.07, P = 0.08; λ=
490 1.17; Fig. 4C), whereas species with larger sperm tended to have less headbutting in mated females ($\chi^2_{1,6}$
491 = 20.86, P < 0.0001; λ= 1.26; fig. 4D). The relative change in headbutting behavior between mated and
492 unmated females was not associated with sperm length across species (sperm length: $\chi^2_{1,6}$ = 0.47, P =
493 0.49; λ= -0.69).

494 **Lifespan**

495 Across species, body size and lifespan were not correlated ($\chi^2_{1,5}$ = 0.12, P = 0.73; λ= -0.57), but
496 ovariole number and lifespan had a significant positive correlation ($\chi^2_{1,5}$ = 6.88, P = 0.009; λ= -0.57),

497 which was the opposite to the life-history prediction that there should be a negative relationship between
498 lifespan and ovariole number (fig. 1D). There was no association between lifespan and mated or unmated
499 headbutting (mated: $\chi^2_{1,5} = 0.12$, $P = 0.73$; $\lambda = 1.26$; unmated: $\chi^2_{1,5} = 3.46$, $P = 0.06$; $\lambda = 1.26$). Lifespan
500 and change in headbutting with mating were not associated ($\chi^2_{1,5} = 1.66$, $P = 0.2$; $\lambda = -0.33$).

501 **Species-specific aggressive behavior: *Drosophila nebulosa***

502 The only species in which we observed a potential alternative aggressive behavior was *D.*
503 *nebulosa*. Females periodically performed wing flicks in which they lifted their wings (Supplementary
504 Video), which might be a signal of size or aggressive intent. To test if wing movements happened in a
505 social context, we identified which per-frame features were significantly different between frames in which
506 females were performing the movement and those where they were not. We found no significant
507 difference in distance to the opponent between frames in which a female was performing and not
508 performing the wing movement ($t_7 = 2.08$, $P = 0.076$). However, females were more likely to be facing
509 their opponent when performing the movement than when they were not ($t_7 = 3.2$, $P = 0.015$).

510 Mating status and the number of wing flicks that the opponent performed had an interaction effect
511 on the number of wing flicks the focal individual performed ($\chi^2_{1,62} = 4.4$, $P = 0.036$). For unmated females,
512 the number of wing flicks was positively correlated within a pair ($t_{1,62} = 5.72$, $P < 0.0001$), but not in mated
513 females ($t_{1,62} = 0.84$, $P = 0.41$; fig. S8). As a result of this correlation, we chose one individual randomly
514 from each pair to test if mating influenced how often females performed the wing behavior. Unmated
515 females performed the wing behavior more than mated females ($\chi^2_{1,62} = 4.76$, $P = 0.03$; fig. S8).

516 **Discussion**

517 When evaluating our results with respect to the predictions from resource valuation, life-history,
518 and hormonal correlation (Table 1), life-history is the least supported hypothesis. Aggression in the
519 species studied is not correlated with lifespan, though theory predicts a negative relationship. In addition,
520 with the life-history hypothesis, a negative relationship is predicted for the relationship between ovariole
521 number and lifespan, but we found a positive relationship in our species. Distinguishing between the
522 resource evaluation hypothesis and the hormonal correlation hypothesis is not possible because they
523 have the same predictions (table 1) but, for the most part, our results support these hypotheses.

524 **Table 1: Predictions from each hypothesis and the results from this study**

Relationship	Hypothesis			Results from this study	
	Resource valuation	Life-history	Hormonal correlation		
Within species predictions					
Aggression and mating	Mated > Unmated	Mated > Unmated	Mated > Unmated	No overall effect; in no species were Mated > Unmated *D. bipectinata* and *D. pseudoobscura:* Unmated > Mated	
Across species predictions				Unmated	Mated
Aggression and ovariole number	**Positive**	**Positive**	**Positive**	**Positive**	**Positive**
Change in aggression after mating and ovariole number	Positive (species with many ovarioles show large response to mating)	Positive	Positive	-	None
Aggression and lifespan	NA	Negative	NA	None	None
Aggression and sperm length	**Negative (mated females)**	NA	**Negative (mated females)**	None	**Negative**
Change in aggression after mating and sperm length	Negative (species with long sperm show small responses to mating)	NA	Negative	-	None
Ovariole number and lifespan	NA	Negative	NA	Positive	Positive

525 Notes: For the results, only statistically significant relationships are given, or "none" is indicated. Bold text
526 indicates that the hypothesis is supported by the results. NA= not applicable (no prediction).

527 **Within species predictions**

528 All three hypotheses predicted that mated females are more aggressive than unmated females
529 within species. Unlike *D. melanogaster*, in many of these species unmated and mated females had
530 similar levels of headbutting. The lack of change after mating was surprising because females of all
531 species are expected to experience an increased demand for protein and other nutrients essential for egg
532 production after mating. However, we do not know if females of other species experience the same post-
533 mating shifts in food preference and feeding behavior as *Drosophila melanogaster* (Barnes et al. 2008;
534 Ribeiro and Dickson 2010). Aggression may not change if nutrient requirements do not change
535 substantially. We know little about nutritional needs across species, or if investment in eggs changes after
536 mating. For instance, *D. melanogaster* females can produce mature eggs on a sucrose-only diet, while *D.*
537 *hydei* females require protein to synthesize essential proteins and develop eggs to maturity (Lamnissou
538 2000). Some species may therefore require yeast as a high value resource, increasing motivation to
539 compete over yeast, whereas other species may not. Further research investigating the nutritional
540 requirements and patterns of egg production across species are needed to understand changes in female
541 aggression, as well as the patterns of female post-mating responses.

542 The higher level of unmated females aggression relative to that of mated females in two species
543 (*D. bipectinata* and *D. pseudoobscura*) is contrary to previous work on *D. melanogaster* (Bath et al.
544 2017). Females in these species possibly acquire food and/or oviposition sites before mating, resulting in
545 a peak in aggression before they mate. Unmated females in these species may therefore value food
546 before mating to supply future egg production, rather than having a shift stimulated by mating.
547 Alternatively, aggression may be suppressed by mating through seminal fluid proteins or changes in
548 hormones regulating behaviors, such as juvenile hormone (Scott 2006; Tibbetts et al. 2013). Regardless,
549 these results illustrate that the behavior and physiology of *Drosophila* species is not always the same as
550 *D. melanogaster*. These results raise questions about why *D. melanogaster* shows an increase in
551 aggression after mating when none of the other species studied do.

552 **Across species predictions – ovariole number**

553 Ovariole number determines the number of eggs that can be produced in a day and,
554 consequently, lifetime fecundity (Sarikaya et al. 2019). All three of our hypotheses predicted a positive
555 correlation between ovariole number and aggression (table 1), but with different underlying reasons. The
556 resource valuation hypothesis is based on egg production requiring a large quantity of protein (Wheeler
557 1996) suggesting that females who have more ovarioles (and therefore produce more eggs) are likely to
558 require more protein than females producing fewer eggs over the same time. The increased need for
559 protein may drive increased aggression, helping to explain the pattern we see across species (the
560 resource valuation hypothesis). However, this pattern may also be explained by differential changes in
561 hormones and physiology across species that are associated with differences in ovariole number
562 (hormonal correlation). Alternatively, life-history theory suggests that faster-reproducing species (those
563 with more ovarioles) prioritize current reproduction over future reproduction, thus they are aggressive to
564 increase their current brood's chance of success. We found a positive correlation between ovariole
565 number and aggression, but are currently not able to distinguish the underlying cause of this relationship
566 in this experiment.

567 We also predicted that ovariole number is positively correlated with changes in aggression after
568 mating—i.e. that species with more ovarioles show a greater change in aggression after mating than
569 those with fewer ovarioles. However, we found no evidence of this in these species, which is likely driven

570 by the few species in which aggression changed in response to mating. Because these species differ in
571 ovariole number but do not change aggression in relation to mating may indicate that aggression is not
572 driven by energetic requirements of egg production. Alternatively, females across species may not differ
573 substantially in their valuation of this particular food resource despite laying very different numbers of
574 eggs.

575 *Drosophila s*pecies vary in their ecology, which is likely to generate different selection pressures
576 on female aggression with respect to different resources (Clutton-Brock and Huchard 2013b). However,
577 we know little about the behavior and selective pressures in nature for any of the species we studied. All
578 the species in this study, with the possible exception of *D. saltans* (Valadão et al. 2019), are generalists
579 that eat and lay eggs on rotting fruit (Nunney 1996; Valadão et al. 2019). Generalist species may
580 experience less competition for food or oviposition sites than specialists because of their ability to use
581 multiple hosts. The Drosophilidae family consists of cosmopolitan generalists (e.g. *D. melanogaster*), as
582 well as specialized species (e.g. *Scaptomyza chauliodon* and *Scaptomyza magnipalpa*; Sarikaya et al.
583 2019), making the group an excellent study system to explore how ecology affects female aggression
584 over food and oviposition sites.

585 **Across species predictions – sperm length**

586 Mated female aggression and sperm length were negatively correlated, which was predicted by
587 both the resource valuation and hormonal correlation hypotheses. The resource valuation hypothesis
588 argues that some species may compete over access to mates, particularly if mates are potentially limiting,
589 while the hormonal correlation hypothesis suggests that the strength of female post-mating responses will
590 be related to the amount of sperm female receive in each mating. Female *D. pseudoobscura* receive up
591 to 25,000 sperm per mating and can continue to lay fertilized eggs up to 2 weeks after a single mating
592 (Markow et al. 2012; Snook et al. 1994). In contrast, female *D. hydei* receive around 120 sperm per
593 mating, remate frequently, and appear to become sperm depleted after 1-2 days in the absence of males
594 (Pitnick and Markow 1994). Wild caught female *D. hydei* are less likely to be fertilized than are *D.*
595 *melanogaster* and *D. pseudoobscura* (Markow et al. 2012). Thus, for female *D. hydei*, sperm is potentially
596 a limiting resource. All the species in this study, except *D. pseudoobscura,* transfer few sperm during
597 mating, suggesting sperm may be a limiting factor for these species. Nonetheless, females of some

species fought over food, so females may compete over multiple resources. Future research using a range of contested resources in experiments is likely to yield different results. For example, unmated *D. melanogaster* females are more aggressive than mated females towards mating pairs (Gaspar et al. 2022) implying access to mates is driving aggression in that context.

Species with long sperm generally transfer few sperm to females in each mating (Markow 1996; Syed et al. 2021). Receiving few sperm may explain the negative relationship between sperm length and mated female headbutting. In *D. melanogaster*, the transfer of sperm induces more fighting than does mating alone, and the strength of this response may depend on the quantity and/or quality of sperm received (Bath et al. 2021a; Bath et al. 2021b). If sperm induction of a female's response is dose dependent, then females receiving few sperm will have less altered aggressive behavior (either up- or down-regulated) than those who receive more sperm. In contrast to our headbutting results, we found a significant effect of sperm length on changes in fencing after mating (Supplementary Information): females of species with longer sperm fenced more after mating than before. Headbutting and fencing may be regulated separately and serve different functions in aggressive interactions. If fencing is less energetically costly than headbutting, then fencing may be a low-cost form of aggression favored by mated females in species that need to expend energy in finding new mates to maximize reproductive output (i.e. females receiving large sperm).

Male ejaculate components transferred during mating can trigger changes in female behavior and physiology. Seminal fluid proteins (Sfps) induce strong post-mating responses in *D. melanogaster* females, altering their receptivity to mating, egg production, sleeping and locomotion patterns, feeding preferences and behavior, and lifespan (reviewed in Avila et al. 2011; Kubli and Bopp 2012). One Sfp, sex peptide, is involved in the mating-induced increase in aggression in *D. melanogaster* (Bath et al. 2017). Differences in aggressive responses to mating among species may indicate different effects of Sfps on female aggression, and potentially female behavior more broadly. We found contrasting effects of mating on locomotion in our species: *D. bipectinata* increased movement after mating, while *D. sturtevanti* reduced movement after mating, and other species showed no change in movement due to mating. While the functional categories of Sfps are well-conserved across *Drosophila* taxa, the genes encoding Sfps turnover and diverge rapidly (Hurtado et al. 2022; Sirot 2019; Wigby et al. 2020). Putative Sfps have been

identified in several species in our dataset (e.g. *D. ananassae*, Findlay et al. 2014; *D. pseudoobscura*, Karr et al. 2019 ; *D. bipectinata*, Santhosh and Krishna 2013), but few have been functionally validated or empirically tested for variation in female post-mating responses due to a lack of tools in non-model organisms. However, recent developments in gene editing technologies may allow the targeting of specific seminal fluid proteins to determine function and effects on female post-mating responses (Denis et al. 2017).

Across species predictions - lifespan

Species appear along a continuum of fast to slow life-history traits, where 'fast' species have high growth and reproductive rates, accompanied by shorter lifespans than 'slow' species (Stearns 1989). In our species, we predicted a negative correlation between lifespan and ovariole number such that species with high ovariole numbers invest more in reproduction and have short lives. However, we found a positive correlation between ovariole number and lifespan, suggesting species with more ovarioles live longer. Why this is the case is unclear. In *Drosophila* species, sperm length is positively correlated with the number of days post-eclosion for males to reach sexual maturity, which is likely due to the amount of time it takes for larger sperm to develop (Pitnick et al. 1995; Syed et al. 2021). However, for the majority of species, sperm length was not related to male lifespan (for an exception, see Syed et al. 2021). Adult lifespan and maturation time are unlikely to be determined by the development of ovarioles, as ovaries develop during the larval and pupal stages and ovariole number is set upon eclosion (Büning 1994). Within species, females with larger bodies tend to have more ovarioles (Honek 1993), but the pattern does not hold across insect species (Church et al. 2021; Sarikaya et al. 2019). Large species might have both long lifespans and many ovarioles, but we found no evidence for this in our dataset. Why species with more ovarioles live longer than species with fewer ovarioles is not clear, but testing more species will enable us to see if this is a broad pattern among *Drosophila*.

Based on the assumption of a trade-off between reproduction and lifespan, theory predicts a negative relationship between lifespan and aggression, as short-lived species can receive the benefits of aggression but die before facing the costs (Eckerström-Liedholm et al. 2016). However, we did not find a relationship between aggression and lifespan. Either the relationship may not exist without a pre-existing trade-off between reproduction and lifespan or all our species are reasonably short-lived, though there

654 was a significant amount of variation within our species (female median lifespan varies from 28-59 day;
655 table S1). The lack of a trade-off between reproduction and lifespan in our species, as well as the lack of
656 relationship between lifespan and aggression, suggests that differences in life-histories do not explain the
657 observed relationship between reproduction and aggression.

658 **Evolutionary History**

659 Both headbutting and fencing (Supporting Information) had a high phylogenetic signal, suggesting
660 aggression is conserved among closely related species. Behavioral traits are more evolutionarily labile
661 than morphological or physiological traits (Blomberg et al. 2003), although numerous behavioral traits
662 have significant phylogenetic signal (reviewed in Cicero et al. 2020). Our study consisted of only eight
663 species, which reduces our ability to detect phylogenetic signal, but some of our species were paired
664 within species groups (i.e. *D. ananassae* and *D. bipectinata*, *D. willistoni* and *D. nebulosa*, *D. saltans* and
665 *D. sturtevanti*), which may artificially inflate our measure of phylogenetic signal.

666 Aggression is a very plastic trait, responding to the social and asocial environment, internal
667 condition, and resource availability and distribution (Clutton-Brock and Huchard 2013b; Stockley and
668 Campbell 2013). In *D. melanogaster*, for instance, females double the amount of time they spend fighting
669 after mating (Bath et al. 2017), while unmated *D. bipectinata* and *D. pseudoobscura* in the current
670 experiment were around twice as aggressive as mated females (fig. 3). Given how plastic aggression is
671 within species in response to mating and environmental factors, phylogenetic conservation of aggression
672 in both mated and unmated females seems surprising. Closely related species may inhabit similar niches
673 or have similar mating systems and morphologies, leading to similar selection pressures shaping
674 aggression. For example, *D. ananassae* and *D. bipectinata,* which belong to the same subgroup, have
675 relatively small sperm and low remating rates, which may shape their response to mating in a similar way
676 (Syed et al. 2021). Further studies expanding the range of species in which we study both male and
677 female aggression will give us more insight into the selection pressures and constraints shaping
678 aggression.

679 **Machine learning for comparative behavior**

680 We used machine learning classifiers originally developed for *D. melanogaster* across eight
681 different species. To our knowledge, this is the first time machine learning has been used for a
682 comparative analysis of social behavior in arthropods (for an example of non-social behavior, see
683 Hernández et al. 2021). The classifier trained to identify headbutts in female *D. melanogaster* scored
684 headbutts for all eight species, with varying levels of accuracy. Our range of accuracies was similar to
685 other classifiers used only for *Drosophila melanogaster* (Bath et al. 2020; Chiu et al. 2023; Robie et al.
686 2017), with some of our accuracies exceeding that of same species (*D. melanogaster*) classifiers. For our
687 species, classifiers were less accurate in some species, suggesting potential caution and ground-truthing
688 must be used in interpreting data from the same classifier on multiple species.

689 For most species, in clear instances of headbutting, the classifier was more likely to miss
690 instances of aggression than falsely categorize frames with no headbutting (table S2). Our scores of
691 aggression are, therefore, likely underestimates rather than overestimates of female aggression. For
692 frames in which the behavior was difficult to categorize, the classifier had an even distribution of false
693 negatives and false positives. Classifier accuracy was dependent on tracking quality; however, we
694 ensured high-quality tracking for all species (see Materials and Methods), thus differences in tracking
695 accuracy among species are unlikely to be responsible for differences in classifier accuracy. Differences
696 in classifier accuracy may be due to species-specific differences in headbutting and fencing behaviors.
697 Slight differences in speed, direction, and duration of flies' movements during aggression may alter the
698 classifier's ability to score behaviors (Bath et al. 2020). Detailed studies are needed for an in-depth
699 analysis of qualitative differences among species in these shared behaviors.

700 Behaviors differed in the accuracy of their relevant classifier, which is consistent with other uses
701 of other classifiers (Robie et al. 2017). The headbutting classifier was the most accurate, while the
702 classifiers for fencing and locomotion were similar to each other in accuracy. We expected the
703 headbutting classifier to have higher accuracy than the fencing classifier because headbutting is easier to
704 score manually. We expected the locomotion classifier to be more accurate than either of the aggressive
705 behaviors because locomotion is easiest behavior to score manually (i.e. is the fly moving or not). The

706 similar level of accuracy between the locomotion and fencing classifiers implies that species may have
707 qualitative (different movement styles) and quantitative (duration of movement) differences in locomotion.
708 Using the same classifiers on all species allowed us to quickly calculate aggression scores and
709 directly compare species in shared experimental conditions. The ability to use one algorithm for multiple
710 species (with caveats) greatly increases the number of individuals and species that can be scored for
711 complex and subtle behaviors. We therefore demonstrate the potential use of machine learning for
712 comparative behavioral analysis, though caution that a human still needs to do ground truthing as well as
713 determine if additional behaviors exist that are not shared across species. Comparative behavioral
714 analysis using machine learning can help us to understand the evolution of complex social behaviors,
715 including elucidating ecological and genetic factors that shape behaviors across species (Hernández et
716 al. 2021; Moulin et al. 2021).

717 **Wing-flicking in *D. nebulosa***

718 Although our study focused on aggressive behaviors shared by species, we observed a
719 distinctive species-specific behavior that may function in aggressive encounters. A female *D. nebulosa*
720 was more likely to be facing her opponent when performing the wing flicking behavior than when she was
721 not performing the behavior, suggesting communication. A female often wing-flicked before approaching
722 the other fly and engaging in headbutting or fencing. The wing behavior may provide a signaling function,
723 perhaps indicating size or motivation to engage in aggression. If large females are more likely to win
724 encounters, wing size may be a signal of resource holding potential and a female's likelihood of winning a
725 subsequent aggressive encounter. Selection favors processes that allow individuals to evaluate the costs
726 and benefits of aggression, leading to the evolution of signals that demonstrate resource holding potential
727 to reduce the costs of engaging in aggression (Parker 1974). Large flies are more likely to win aggressive
728 encounters for both male and female *D. melanogaster* (Bath et al. 2018; Hoffmann 1987), but how
729 aggressive encounters are decided is unstudied in *D. nebulosa* and most other Drosophilids. However,
730 both male and female *D. nebulosa* perform this behavior in mixed-sex groups, as well as in male-female
731 pairs (Gleason et al. 2012; Spieth 1947) so it is possible it serves an alternative signaling function, or no
732 function at all. Further work on the sequence of aggressive behaviors, assessment rules, and what

determines winners in a variety of species, will help to elucidate the behaviors involved in aggression across species.

Conclusion

Species vary in female aggression level in the presence of yeast. The effect of mating in changing female behavior is not universal among *Drosophila* species and therefore additional study is needed in species other than *D. melanogaster*. The results indicate that resource valuation and hormonal correlation are the most likely drivers of female aggression, but more work needs to be done to determine which has a stronger effect. Studies are needed to understand how female behavior varies and if variation is dependent on ecology, mating system, phylogenetic history, or other aspects of female biology.

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