Indirect Genetic Effects on Male Territoriality in *Drosophila melanogaster*

TRISTAN DUCHARME

Thesis submitted to the University of Ottawa
partial fulfillment of the requirements of the
MSc degree at the Ottawa-Carleton Institute of Biology

Department of Biology
Faculty of Science
University of Ottawa

Abstract

When an individual interacts socially with a conspecific, their behavioural phenotype is affected directly by their genotype ('direct genetic effect', DGE), but may also be affected indirectly by the genotype of the opposing individual ('indirect genetic effect', IGE). While there is no doubt that IGEs occur in various organisms and contexts, it is unknown how properties of the environment may influence the relative magnitude of DGEs, IGEs, and their covariance. To gain insight into this, I examined territorial interactions in *Drosophila melanogaster*. Due to their short generation time and relatively simple care requirements, *D. melanogaster* has been used extensively in quantitative genetic research. Using offspring from a half-sib breeding design, I constructed an arena for documenting multiple dyadic territoriality assays with two sizes of a food resource. With this apparatus, 618 territoriality contests between 1,236 individuals were recorded and scored for four key aggressive behaviours. The results revealed significant genetic variation in how opponent effects on focal individuals changed between environments (i.e., genetic variation in the plasticity of IGEs). In addition, changes in DGEs and IGEs between environments were strongly and positively correlated (i.e., there was a DGE × IGE × environment interaction), although confirmation of this result in further studies is warranted because it was non-significant ($P = 0.10$), likely due to large uncertainties arising in part from some small variance component estimates. As a high throughput system for quantify IGEs in territoriality in *Drosophila*, my approach holds promise but there are issues to resolve, including automating phenotyping behaviors in place of manual scoring to enable many more trials. Additionally, modifications to increase humidity during trials might result in increased expression of certain territorial behaviours.

Résumé

Lorsqu'un individu interagit avec un congénère, son comportement est affecté directement par son génotype (« effet génétique direct », EGD), mais peut également être affecté indirectement par le génotype de l'individu adverse (« effet génétique indirect », EGI). Bien qu'il ne fasse aucun doute que les EGI se produisent dans divers organismes et contextes, on ne sait pas comment les propriétés de l'environnement peuvent influencer l'ampleur relative des EGI ainsi que leur covariance avec EGD. Pour mieux comprendre cela, j'ai examiné les interactions territoriales chez *Drosophila melanogaster*, une espèce largement utilisée dans la recherche en génétique quantitative en raison de sa courte durée de génération et facilité à maintenir en laboratoire. En utilisant un système d'élevage produisant des demi-frères, j'ai construit une arène pour documenter plusieurs essais de territorialité dyadique avec deux tailles de ressource alimentaire. Avec cet appareil, 618 tests de territorialité entre 1236 individus ont été enregistrés et notés pour quatre comportements agressifs. Les résultats ont révélé une variation génétique significative dans la façon dont les effets de l'adversaire sur les individus focaux changent entre les environnements (c'est-à-dire, la variation génétique dans la plasticité des EGI). De plus, les changements dans les EGD et les EGI entre les environnements étaient fortement et positivement corrélés (c'est-à-dire qu'il y avait une interaction EGD × EGI × environnement), bien que la confirmation de ce résultat dans d'autres études soit justifiée étant donné que l'effet n'était pas significatif ($P = 0.10$), en partie due aux grandes incertitudes découlant en partie des petites composantes de variance. En tant que système pour quantifier les EGI dans la territorialité chez la drosophile, mon approche est prometteuse, mais il y a des problèmes à résoudre, notamment l'automatisation des mesures de comportement. De plus, l'humidité pendant les essais pourraient entraîner une expression accrue de certains comportements territoriaux.

Acknowledgements

I would like to thank my supervisors, Dr. Vincent Careau and Dr. Howard Rundle, for their constant support and encouragement on this project. The pandemic and shutdowns made laboratory work difficult, but my supervisors showed me how to weather the storm and find enjoyment in the thesis process. I am grateful for their sophisticated teaching efforts and the diverse problem solving and analysis approaches they shared with me over the past two years. I am thankful for our Lab's funding sources, without whom this project would not be possible.

In addition, I would like to thank my committee members Dr. Rosyln Dakin and Dr. Julien Martin for their excellent feedback and suggestions. Their research insights have been extremely valuable to this thesis. I would also like to thank my lab members Cameron Kendrick, Paul Agnani, Tia Chen, Will Jarvis, Caroline Maloney, Mathieu Videlier, Chris Angell, Natalie Kermany, Tom Lorenz, Li Yun, Merlin Caron-Lévesque, Jeremie Bouffard, Nicolas Bonin, Wyeth Blumberg and Kehinde Osijo. Their assistance in troubleshooting cameras, arena construction, fly painting and other tasks has been invaluable.

Finally, I would like to acknowledge Algonquin Anishinabe people. The University of Ottawa is situated on the unceded territory of the Algonquin Anishinabe. I am grateful to have the opportunity to conduct research in this territory.

Table of Contents

Introduction

Indirect Genetic Effects

One of the goals of research in quantitative genetics is to understand the genetic architecture of phenotypes (i.e., traits) within populations. Quantifying and partitioning phenotypic variance into underlying components, such as environmental and genetic (additive and non-additive), can provide insight into the nature of genetic variation and its maintenance, the consequences of non-random forms of mating like inbreeding and outbreeding, and is crucial to predicting the response to selection and quantifying constraints on the evolutionary processes (Lynch & Walsh 1998). The genetic architecture of some traits can be complex, involving additional components of variation beyond straightforward environmental and genetic. Indirect genetic effects (IGEs) are one example of this, and evidence suggests they are an important for many traits (Wolf et al., 1998, Moore et al., 1997).

IGEs describe the effect of a social interaction with one or more conspecifics on the phenotype of a focal individual. When an individual engages in a social interaction with a conspecific, the phenotype they exhibit may not only be affected directly by the organism's genotype (i.e., direct genetic effects, DGEs), but it can also be indirectly affected by traits, and hence the underlying genotype, of their social partner (i.e., the individual with which they are interacting). In a sense, this causes a component of the social environment to be "heritable" and capable of itself evolving, and this can impact the evolutionary response of the phenotype of interest (Wilson et al., 2009). IGEs have been observed in mussels, for example, whereby an organism genetically predisposed to have an increased growth rate limited the growth of neighbouring individuals by reducing available food resources (Brichette et al., 2001).

IGEs first gained prominence in agricultural research, where they can represent a distinct challenge to farmers. Genetic variants with a positive DGE on a focal individual's phenotype can also negatively impact nearby livestock, and may reduce total productivity and yield of the herd. For instance, phenotypes displaying increased aggression are often harmful to nearby conspecifics (Dickerson 1947, Camerlink et al., 2013). Early research into IGEs often sought to maximize productivity by lessening the effects of harmful IGEs within agricultural environments, and hence was focused on growth or productivity traits such as body mass or egg production in farm animals. In more recent years, researchers have quantified IGEs in a wider range of traits and organisms including anemones, crickets, and burying beetles (Wilson et al., 2009, Santostefano et al., 2017, Lane et al., 2020). Given the prevalence of social interactions, opportunities exist for IGEs in many taxa. By disregarding IGEs and assessing only direct genetic effects, previous studies on complex phenotypes may have often failed to capture potentially key components of variance (Wilson et al., 2009).

Covariance between IGEs and DGEs

An additional layer of complexity exists because of the potential for positive or negative covariances between DGEs and IGEs. A positive covariance arises if genotypes that increases the expression of a trait in focal individuals (a DGE) also tend to elicit the expression of that trait in conspecifics with which the focal individual interacts (an IGE). In contrast, a negative covariance would occur if alleles that increase expression of a trait in focal individuals also tend also to inhibit that trait in social partners. A positive DGE-IGE covariance has been demonstrated in aggression in deer mice, for example, whereby aggressiveness in one individual elicited increased aggressiveness in their opponent (Wilson et al., 2009). Negative covariances in

aggression have similarly been observed in Mediterranean field crickets, where a genotype predisposed to aggression tended to inhibit aggression in nearby conspecifics (Santostefano et al., 2017).

A DGE-IGE covariance can have important implications for the response to selection; a positive covariance will contribute to a more rapid response (Wilson et al., 2009, Santostefano et al., 2017). For example, consider that selection favours increased aggression and a genotype that produces a more aggressive individual. The increased aggression this individual exhibits towards conspecifics will, in turn, elicit increased aggressive responses from interacting individuals. In this situation, the positive covariance drives the evolution of a social environment in which further aggression is elicited. A negative covariance, in contrast, will tend to constrain the response to selection (Wilson et al., 2009). For example, if an individual has a genotype that encourages rapid growth under competition, this individual may also limit the available food, and thus potential growth, of nearby conspecifics. While selection may favour increased growth, the negative covariance reduces the response to selection because increased expression of the trait drives the evolution of a social environments that tends to suppresses the expression of the trait.

IGEs and Behaviour

While IGEs may arise in any phenotype, behavioural traits are usually highly plastic and are expressed in a variety of social contexts, meaning that they are particularly likely to feature strong IGEs (Wolf et al., 1998). My interest here is with male aggression, expressed within the context of territoriality, which is a phenotype that is only expressed in a social context (i.e.,

during encounters with rival males). IGEs in aggression have received some attention in a few cases, as previously mentioned, and have been shown to create social environments that can favour increased aggression (positive covariance between IGEs and DGEs; Wilson et al., 2009) or ones that suppress it (i.e., where increased aggression in an individual discourages aggression in nearby conspecifics; Santostefano et al., 2017).

There are practical challenges to quantifying IGEs, including that a large number of individuals need to be phenotyped and their genetic relatedness must be known. This can be done via a pedigreed population, or via a classic breeding design (e.g., a paternal half-sibling in which multiple dams are mated to a given sire and offspring are phenotyped). Behavioural traits are also more difficult to quantify than fixed morphological traits, and specific criteria outlining what constitutes a behaviour are essential for consistent scoring. Additionally, this task becomes more complex when environment is taken into account, as behavioural traits can be highly sensitive to environmental variation, both physical and social (Dobzhansky et al., 1974, Rooke et al., 2020). Ideally, the environment must be controlled, or uncontrolled variation must not be confounded with factors of interest (e.g., sire identity).

Environmental Variance and IGEs

Although there is no doubt IGEs exist for a variety of traits in various organisms, and they are sometimes of sufficient magnitude to be evolutionarily relevant (Wilson et al., 2009, Santostefano et al., 2017), little attention has been given to factors affecting their relative importance and nature (e.g., the sign and strength of covariance with DGEs). For example, considerable phenotypic variation is often attributed to variation in the physical environment that organisms experience, but few studies have investigated possible ways in which the environment

can modulate IGEs and their covariance with DGEs for a given focal trait. Changing the size of a resource over which males compete has been shown to impact the expression of territorial behaviours in *Drosophila*, as has changes in density/group size (Hoffman and Cacoyianni, 1990). Environmental variation can not only result in direct changes in an individual's behavioural, but could also change how individuals impact the expression of the trait in others (i.e., IGEs). Specifically, there is potential for an "IGE × E" interaction, where the environment affects the magnitude of IGEs and the directionality of the DGE-IGE covariance.

Study overview & objectives

Here, I quantify IGEs in territorial interactions in *Drosophila melanogaster*, a powerful model system for behavioural ecology and quantitative genetics due to their short generation time and relatively simple care requirements that allow large populations to be easily maintained and breeding designs to be conducted, with large sample sizes (i.e., many individuals phenotyped). Multiple studies have examined territoriality and aggressive behaviours in *Drosophila* (Hoffman, 1987, White and Rundle, 2015, Tremblay et al., 2021). Two species (*Drosophila melanogaster, Drosophila Serrata)* in which territoriality has been studied have been shown to exhibit a resource defense mating system whereby a male will defend a small territory containing a food resource as a means of gaining access to females. Females are attracted to this food resource, likely as a means of sustenance and/or as a high-quality oviposition site (Hoffman, 1987). Males with an increased propensity to display antagonistic behaviours (e.g., leg kicks, lunges, chases), allowing them to control a territory, tend to be more successful in terms of reproduction (White and Rundle, 2015; Hoffman, 1987; Tremblay et al., 2021). To assess territoriality in *D. melanogaster*, a standard territoriality assay was performed that involved two males in a petri

dish with a food resource at the center. The males competed for control of the food, allowing quantification of aggressive behaviours. Using a similar setup, a recent study demonstrated that indirect effects on territoriality were negatively and significantly correlated with body mass, indicating that larger males tended to suppress territorial behaviours in those they interacted with (Tremblay et al., 2021). Although this study revealed "opponent effects" in territorial behaviours, indirect effects were quantified only at the among-individual level, as repeated measures were taken but a breeding design was not performed.

While IGEs have been identified previously in various species (Wilson et al., 2009, Santostefano et al., 2017, Lane et al., 2020), factors impacting them have not been addressed. One prime candidate for investigation of IGEs in territoriality is the size of the food resource. The size of the food resource could impact how aggressively an individual defends it (e.g., by altering the value of the resource to them and/or their ability to defend it), and it also could alter the effect their aggression has on that of interacting individuals, thereby affecting IGEs (e.g., by changing the value to the interacting individual and/or the likelihood of this individuals taking control of it). By directly manipulating resource size, IGEs can be characterized as a function of this aspect of the environment.

In partitioning variation in male territoriality in *Drosophila*, I have two main objectives. The first is to quantify DGEs, IGEs, and their covariance for a behavioural trait that has direct ecological relevance in a competitive context. This is important because previous research concerning the genetic architecture of behavioural phenotypes has tended to focus on DGEs alone (Wilson et al., 2008). The second, and more novel, is to gain insight into how DGEs, IGEs, and their covariance change as a function of a key aspect of the environment: the size of the resource. By extending the capacity of this established model system for behavioural

quantification in this context, I provide some of the first data addressing ecological causes underlying variation in IGEs and I extend the capabilities of this influential model system, opening new opportunities for studying the quantitative genetics of behaviour.

Methods

Study population

A *D. melanogaster* population was established in February 2016 from a laboratory stock population first collected in Dundas, ON in 2006 (MacLellan et al., 2012). The population was maintained via non-overlapping generations in 64 vials at 25˚C at 50% relative humidity with a 12-hour light and 12-hour dark photoperiod. Flies were fed a cornmeal-based food that consisted of 90 g/L cornmeal, 100 g/L turbinado sugar, 40 g/L yeast, and 12 g/L agar. The population was maintained via a life cycle that includes a 4-day mating period in a 'complex' environment consisting of glass bottles filled with 75 ml of food with plastic dividers separating the surface of the food into six equal segments. Two curled pipe cleaners were also inserted into the foam stopper of the bottle to increase structural complexity (compared to standard *Drosophila* vials/bottles). Flies were kept at reduced population density during the 4d in these mating bottles (10 males and 10 females per bottle; 64 bottles total), after which males were discarded and females were moved to standard vials (28.5 mm × 95 mm) to lay eggs for 24 hours. Females were discarded following this. Offspring were collected after 9 days, mixed among vials, and groups of 10 males and 10 females were allocated to 64 new bottles for the 4-day mating phase. This protocol was repeated for subsequent generations. The increased structural complexity and reduced population density were used to promote the expression of territorial behaviours.

Specifically, males may be better able to establish and defend a territory compared to standard, high-density rearing conditions.

Breeding design

A classic paternal half-sibling breeding design was used in which each sire was mated to four dams and six offspring were collected using light CO_2 anesthesia (I aimed to phenotype four but collected six to have backups). In total, 128 sires and 512 dams made up the breeding design, and 1,236 offspring were phenotyped from 618 dyadic territorial trials, as described see below. This was done in four separate blocks of 32 sires and 128 dams per block over 8 generations of the stock. Following their collection, offspring of each dam were marked with a dot of orange or green acrylic paint (DecoArt Fluid Art, READY TO POUR) on the thorax to allow for identification during trials. These offspring were then kept in vials with stock flies to maintain a controlled social environment prior to phenotyping (three 'focal' males – i.e., sons from the breeding design – were held together with seven stock males and 10 stock females). Territoriality assays began 48 hours later. Dyads were determined systematically such that offspring from a given sire interacted with offspring from multiple other sires without ever being paired with the same sire more than once (Table 1). Two offspring of a given sire were also never paired in the same trial.

Territorial assay

The territorial assays involved placing two differently colour-marked males (offspring from the breeding design) into a circular plastic arena (diameter = 8 cm). Within each arena, a roughly

circular food item of given size (see below) was placed at the center. An apparatus was constructed in order to start multiple trials simultaneously. This apparatus featured 16 separate arenas in which 16 unique dyads could be placed (Fig. 1). It featured a sliding mechanism whereby opposite sections of each arena could be moved between two positions. The first was a "loading" position, whereby a single fly could be aspirated, without CO_2, into a separate holding well, preventing each from interacting with the other or with the food in the central compartment. Once all the flies were loaded, the apparatus would be shifted into the "trial" position, which would align all compartments into a single 8 cm circular arena, thus introducing each pair of males into their respective arena simultaneously.

The sides of each individual arena were coated with Bioquips's fluon, a product intended to prevent insects from climbing surfaces. The arenas were raised 12 cm above the tabletop and four LED under-cabinet light bars (Cnsunway Lighting, 48W 3600LM High Bright) were placed underneath to illuminate the arenas. A light diffuser was included between the lights and the arenas to equalize lighting and prevent glare. Two LED light bars were also placed 30cm above the arena at 45-degree angles to illuminate the tops of flies and improve the visibility of the colour marks. An array of 16 raspberry pi cameras (Raspberry Pi Zero W computer with a Raspberry Pi Camera Module 2, Raspberry Pi) were mounted, one above each arena, to record each individually. These cameras were remotely controlled so that they could be started simultaneously, at trial start.

Prior to trials, a circular section of moist filter paper was placed at the bottom of the arena to prevent desiccation of the flies. On top of the filter paper, a disc of standard food (i.e. that used during stock maintenance; see above), either small (d ~1.2 cm) or large (d ~3.5 cm), was placed. Food resource sizes were alternated between trials in a given arena. Once the 32 flies

were loaded in the apparatus, it was moved underneath the camera array. The cameras would start recording and then immediately afterwards the apparatus would be moved from the "loading" position to the "trial" position. Video-recording lasted 20 minutes, after which the arenas were cleaned with water prior to the next trial, where the procedure was repeated with a new set of 32 males. This was done for four blocks; each block took place over a two-week period, with one week of offspring preparation and one experimental week. Each experimental week had three days during which trials were run, except for block 3 which only had two trial days. Each trial day had 5 separate trials (i.e., the apparatus was run with new flies five times) for a total of 15 trials per block.

To quantify aggression, agonistic behaviours were manually scored by separately viewing each trial video in VLC media player. Footage was viewed at reduced speed as necessary to identify behaviours of interest. The total number of occurrences of each of four previously recognized behaviours was quantified for each male: lunges, chases, kicks, and faceoffs (Jacobs 1960, Hoffman 1987, Hoyer et al., 2008). Any trial where a fly escaped, was injured or had damaged wings, or had any sort of obstruction impeding view of the camera, was omitted.

Four behaviours were recorded: kicks, lunges, chases, and faceoffs. Kicks were defined as a swift motion towards the torso of another individual with the front leg. This was to distinguish from a "probing" behaviour where a fly will extend its legs outward and gently "feel" nearby objects. A lunge was counted when a fly reared upon its hindlimbs and thrusted its body towards the opposing individual. This behaviour is more akin to a short jump at an opposing individual rather than a simple leg motion as with kicking behaviours. A chase was counted when one fly followed the path of another individual while in "touching distance". A chase was

not counted if a fly was more than one body length away from the other individual. This was to prevent instances where one fly simply follows a similar path from being counted as a chase. Lunges, kicks and chases were each scored for the individual that performed the behaviour. The fourth behaviour, faceoffs, occurred when two individuals would "lock" forelimbs while facing each other directly usually followed by a struggle where flies would push at each other. Faceoffs typically last until one fly backs away from its opponent. Faceoffs were counted for the victor of the encounter only. Lunges, kicks, chases, and faceoff wins were then compiled into a single "aggressiveness" variable, which was used for further analysis. This was done by summing the instances of each aggressive behaviour, for both the focal and opponent males separately, in a given dyadic territoriality assay.

Statistical analyses

Aggressiveness was analyzed with a random regression model in ASReml-R version 4 (Butler et al., 2018). Since the residuals of the raw aggressiveness scores were not normally distributed (Fig. 2A), I ran a box-cox transformation procedure to find the power transformation that yielded the most normally distributed model residuals. Multiple random regression models (see structure below) were fitted with aggressiveness scores raised to different values ranging from 0.15 to 0.85 (in 0.05 increments). Residuals were extracted from each model and a Shapiro-Wilk normality test was conducted to find which power transformation yielded the highest W-statistic. The power 0.4 returned the best W statistic and therefore retained for the results (Fig. 2B). Although the Shapiro-Wilk test indicated the residuals were not normally distributed ($P < 0.05$), the deviation from normality was minor (Fig. 2C-D) and mixed mixed-effects models are reasonably

robust to sizeable deviations from normality (Schielzeth et al., 2020). After the power transformation, the aggressiveness score was re-scaled to a mean of zero and a variance of one.

The random regression model included fixed effects of measurement block (4 levels), arena (16 levels), colour marking (green vs red), time of day, and resource size (small vs large), which allowed me to test for an overall effect of food size on aggressiveness while controlling for nuisance variables. The significance of fixed effects was determined using conditional Wald *F*-statistic with the denominator degrees of freedom determined following Kenward and Roger (Kenward et al., 1997). To account for the non-independence due to the fact that aggressiveness was recorded on both flies in each trial (i.e., we obtained two observations per dyadic trial), I included dyad as a random effect and specified its variance component (V_{dyad}) as unconstrained during estimation (observations within dyads may positively or negatively covary; Bijma, 2014). The model also included random effects of focal dam and opponent dam to control for direct and indirect common environmental variance and potential non-additive genetic effects. Finally, the model included day (11 levels) as a random effect to control for possible differences in behaviour across days within each block.

Direct and indirect genetic effects, and changes in their magnitude according to the size of the food source, were modelled using a reaction norm approach (Fig. 3). The reaction norm approach entails estimating overall strength of DGEs and IGEs as the variance components associated with focal and opponent sire respectively by fitting these as intercept random effects. Moreover, changes in IGEs and DGEs as function of an environmental variable, in this case, the size of the food source (small = -0.5, large = 0.5) were modelled as focal and opponent sire random effect slopes. The focal and opponent sire variance components (intercepts and slopes)

were modeled in a 4×4 correlation matrix. Significance of the correlation estimates were assessed using likelihood ratio tests that compared models with and without correlation of interest.

Results

Fixed Effects

I quantified aggressiveness in a total of 1,236 males during 618 trials. There were no significant differences in measurement block, color marking (green vs. red) or arena. However, food size was significant (Table 2). Specifically, flies were also more aggressive, on average, in the presence of larger compared to smaller food sizes (Table 2; Fig. 4).

Random Effects

Intercept variance estimates for both focal and opponent sires were small and nonsignificant, suggesting little direct and indirect genetic variance in aggressiveness respectively (Table 3). However, reaction norms for focal and opponent sires show substantial variation (Fig. 5), suggesting plasticity in direct and indirect effects and warranting caution in interpreting the overall DGE and IGE estimates (i.e., the intercept variances for focal and opponent sires) as these effects vary depending on the food size. Focal and opponent sire intercepts and slopes as well as their correlations, are summarized in Fig. 6. While the slope variance estimate for focal sire was relatively small and non-significant, that for opponent sire was larger, although non-significant (0.102 ± 0.061; Table 3, Fig. 6). There was also a strong and positive correlation (0.930 ± 0.500) between focal sire and opponent sire slopes that approached significance (likelihood ratio test: $P = 0.10$; Table 3; Figs. 5C, 6).

Discussion

Although, IGEs have been shown to exist in a variety of traits (Moore et al., 1997, Wolf et al., 1998), little is known about environmental effects on them, including how their magnitude may change with aspects of the physical environment. Here, by varying the size of a defendable resource, I demonstrated that a larger food size tended to elicit more aggressive behaviours from individuals than did a smaller food size. I also found significant genetic variation in how the IGE changed between the two food sizes, implying plasticity in opponents' effects on focal male aggressiveness. In addition, there was evidence of an IGE × DGE × environment effect, where variation in focal and opponent sire slopes were correlated. Given that IGE × DGE covariances can have profound impacts on the evolutionary response to selection (Wilson et al., 2009), environmental impacts on them could also be important.

Population-level effect of food size on aggression

Average aggressiveness was higher in trials with the large food size in comparison to the small food size (Table 2; Fig 4). This suggests that individuals were more inclined to compete for larger resources, perhaps because these were perceived to be more valuable. An alternative, non-exclusive interpretation is that the larger resource may simply take more effort to defend, and hence require more aggressive behaviours to control, even if they are perceived as being no more valuable. The increase in average aggressiveness I observed with my resource sizes coincides with results from similar studies on *Drosophila* that found males were more likely to defend mid-sized food resources when compared to smaller food resources (Hoffman and Cacoyianni, 1990). These studies also found an "upper limit" on the size of food resource that a male was

able to defend. Specifically, males expended more energy defending a two-centimeter food resource compared to a four-centimeter food resource (Hoffman and Cacoyianni, 1990). My large food resource (d = 3.5 cm) was in-between the large and medium sizes of Hoffman and Cacoyianni (1990); it tended to elicit more frequent aggressive behaviours than my small food resource (d = 1.2 cm), which was slightly larger than the small food size present in Hoffman and Cacoyianni (1990). The observation that average aggression varies non-linearly with resource size, being highest at intermediate sizes, raises questions about how the genetic components underlying variation in aggressiveness change across a broader range of resource sizes than the two I used.

IGE × E interaction

Although there was limited variance in opponent sire intercept when considered across environments, the opponent sire slope estimate was substantial relative to its standard error (0.11 ± 0.064), implying an IGE × environment interaction (Table 3; Fig. 6). This is visible in Fig. 5B as the variation in the slopes (but not intercepts) of the opponent sire reaction norms. This suggests that there is genetic variation in how the environment impacts opponent effects on focal individual aggressiveness; i.e., genetic variation in environmental plasticity of IGEs. An IGE × E interaction mirrors conceptually the more easily understood G × E interaction in which genetic variation in a trait differs across environments; i.e., genotypes vary in how they respond to a change in environment (Roff and Wilson 2014). G × E are common and have previously been identified in *Drosophila* in response to changes in both physical and social environments (Burns et al., 2012, Kent et al., 2009, Dukas et al., 2003; Saltz et al., 2011). G × E interactions indicate

genetic variation in plasticity in terms of direct genetic effects on focal individuals' traits. By contrast, an IGE × E interaction indicates genetic variation in plasticity in terms of indirect effects of interacting individuals on focal individuals' traits. I am not aware of any prior estimates of IGE × E interactions, although their existence is implied in some studies (e.g., Danielson-François, 2007), including in aggressiveness in *Drosophila* (Saltz, 2013).

I varied an aspect of the physical environment (i.e., food resources), but IGEs could also arise from variation in social environment. For instance, theoretical work suggests that group size may be an important axis of variation in social environment that may impact IGEs (Heidaritabar, et al., 2019). Other aspects of social environment would also be interesting to explore, including density, sex ratio, and even number and identity of heterospecific interacting individuals (e.g., coevolution between species; see De Lisle et al., 2022). IGEs are a widely applicable framework for exploring the effects of interactions on phenotypes and the evolutionary impacts of variation in physical and social environments. My results suggest that there is plasticity in IGEs in response to an axis of environmental variation. When IGEs vary with environment, then the heritability of the social environment may change and this could facilitate or constrain the evolutionary response to selection (Wilson et al., 2009).

DGE × IGE × environment

When IGEs exist, their correlation (or not) with DGEs is of special interest given its potential to impact the response to selection. I found little evidence of a DGE × IGE interaction (Table 3, Fig 6). This was not because DGE were independent of IGEs, however, as changes in DGE and IGEs across environments were strongly and positively correlated (i.e., there was a DGE × IGE ×

environment correlation). In my model, this association was quantified as the slope × slope correlation; i.e. the extent to which variation in focal sires slopes was associated with variation in opponent sire slopes (Fig. 4), and this correlation was strongly positive (0.93 ± 0.50; Fig 5C) and approached significance (Table 3). This suggests that sires that produced offspring with greater aggressiveness in large compared to small food patches also tended to produce offspring that elicited greater aggressiveness in their opponent in large compared to small food patches. In other words, the strong slope-slope correlation suggests that genetic variation affecting how DGEs change across environments is almost entirely shared with genetic variation in how IGEs change across environments. As noted, DGE × IGE correlations can have important impacts on the response to selection, and when this correlation itself varies by environment, such impacts will be environment-specific. Quantifying impacts on the response to selection would be complex as it would involve estimating selection on individual aggressiveness, individuals' impacts on others, and plasticity in these.

Finally, some caution is warranted in the interpretation of this correlation in my case. Although the point estimate was strongly positive, uncertainty was large. This likely reflects, at least in part, the small variances from which the correlation was calculated; small changes in these would have a large impact on the resulting correlation. In addition, despite a large dataset, individual behaviours were often rare and, during model fitting, one estimate was bounded to 1 and did not have a standard error, potentially indicating issues with model fitting. It would therefore be useful to confirm this result in a future study.

Future Directions

The quantitative genetic partitioning of phenotypic variance into numerous and complex components requires extensive data. The present assay design has potential as a means of high-throughput data collection for the study of aggressive behaviours in *Drosophila*, but there were challenges that hampered data collection. One of the issues was that most of the behaviours were infrequent. Specifically, of the behaviours scored, chases, lunges, and face-offs were rare, or absent, in most assays. Only kicks were present frequently. Sparse data is likely to contribute to greater uncertainty in estimates, and it prevented me from analyzing individual traits.

Altering the design of the assay could address this by increasing the frequency of these behaviours. For instance, considerable time was needed to place flies into all 16 arenas, and the holding wells into which they were loaded had no filter paper. Variation in the time an individual spent in their respective holding well may have generated varying degrees of desiccation stress, and drying out of the filter paper in the main arena may have also contributed to this. These issues could potentially be addressed by increasing the humidity of the experimental environment. In addition, the assay environment included 6 LED light bars (see Methods), and this was brighter than the laboratory environment. Light levels have been shown to alter exploratory and reproductive behaviours in *Drosophila* (Qiu et al., 2018, Spieth 1950), so this may have impacted territoriality in my experiment. Dimming experimental lights may make the flies harder to see, in particular their legs. An acclimation period could be useful, although I am not aware of any evidence in support of this. Flies were also observed to spend a substantial amount of time exploring the periphery of their arena rather than engaging with opponents. Reducing the size of the overall petri dish could increase territorial interactions. The current

arena size (d = 8 cm) was determined based on previous studies in *Drosophila* territoriality which used slightly larger plates (d = 10 cm; Hoffman et al., 1987a).

Another issue that limited the high-throughput assay design was slow behavioural phenotyping. Manually scoring videos proved to be a time-consuming process, with many hours of footage recorded. Originally, I had planned to automate the identification of behaviours using a machine learning program, but difficulty arose with the fly tracking system. The program I used, CTRAX (Branson et al., 2009), had difficulty tracking flies when they moved within close proximity of each other, sometimes even switching the marks between them. This will require further troubleshooting, including the possibility of updated tracking software. Marks were difficult to identify at certain points in the video due to the lighting system. My setup including backlighting that was useful in distinguishing individual limbs for fine behaviour identification, but this also made dorsal marks more difficult to discern. This may have contributed to the problems experienced with the fly tracking software as backlighting was reduced or absent in earlier studies that used this software (Branson et al., 2009). An alternate marking technique might be useful to address this. I used dorsally-applied acrylic paints because they had been previously used with success in *Drosophila* (e.g., Chen et al., 2002), but they were often difficult to see with backlighting. Wing clipping is one possibility as an alternative marking technique, but potential impacts of this on aggressive behaviours are unknown and would hence need to be explored.

Conclusion

My study provides some of the first evidence for plasticity of IGEs, demonstrating genetic variation in how opponent effects on male aggressiveness in *D. melanogaster* change with environment. I also present some evidence of a positive correlation between focal and opponent sire variances in slope, suggesting a shared genetic basis to changes in DGE and IGE with environment. As a model system to study the quantitative genetics of behaviour, future research should seek to increase the frequency of behaviours and the number of trials, possibly through automating behavioural scoring and altering arena design. There is much potential of this system, given that it is possible to control and manipulate a broad range of physical and social environments, and to explore the effects of variation in social environments as well.

References

Bijma, P. 2014. The Quantitative Genetics of Indirect Genetic Effects: A Selective Review of Modelling Issues. Heredity. 112:61–69.

Branson, K., A. Robie, J. Bender, P. Perona, and M. Dickinson. 2009. High-Throughput Ethomics in Large Groups of *Drosophila*. Nat Methods. 6:451–57.

Brichette, I., M.I. Reyero, and C. García. 2001. A Genetic Analysis of Intraspecific Competition for Growth in Mussel Cultures. Aquac. 192:155–169.

Burns, J.G., N. Svetec, L. Rowe, F. Mery, M. Dolan, W.T. Boyce, and M.B. Sokolowski. 2012. Gene-Environment Interplay in *Drosophila Melanogaster*: Chronic Food Deprivation in Early Life Affects Adult Exploratory and Fitness Traits. PNAS. 109:17239–44.

Butler, D., B.R. Cullis, A.R. Gilmour, D.J. Gogel, and R. Thompson. 2018. ASReml-R reference manual Release 4. Hemel Hempstead (UK): VSN International Ltd.

Camerlink I., S.P. Turner, P. Bijma, and J.E. Bolhuis. 2013. Indirect Genetic Effects and Housing Conditions in Relation to Aggressive Behaviour in Pigs. PLoS ONE. 8:e65136.

Carter M.J., A.J. Wilson, A.J. Moore, and N.J. Royle. 2019. The Role of Indirect Genetic Effects in the Evolution of Interacting Reproductive Behaviors in the Burying Beetle, *Nicrophorus Vespilloides*. Ecol Evol. 9:998–1009.

Chen S., A.Y. Lee, N.M. Bowens, R. Huber, and E.A. Kravitz. 2002. Fighting Fruit Flies: A Model System for the Study of Aggression. PNAS. 99:5664–68.

Danielson-François A.M., Y. Zhou, and M.D. Greenfield. 2008. Indirect Genetic Effects and the Lek Paradox: Inter-Genotypic Competition May Strengthen Genotype × Environment Interactions and Conserve Genetic Variance. Genetica. 136:27–36.

De Lisle S.P., D.I. Bolnick, E.D. Brodie III, A.J. Moore, and J.W. McGlothlin. 2022. Interacting Phenotypes and the Coevolutionary Process: Interspecific Indirect Genetic Effects Alter Coevolutionary Dynamics. Evolution. 76:429–44.

Dickerson, G.E. 1947. Composition of hog carcasses as influenced by heritable differences in rate and economy of gain. Res bull. 354:492-524.

Dobzhansky, T. 1974. Genetic Analysis of Hybrid Sterility Within the Species *Drosophila Pseudoobscura*. Hereditas. 77:81-88.

Dukas, R., and A.Ø. Mooers. 2003. Environmental Enrichment Improves Mating Success in Fruit Flies. Anim Behav. 66:741–49.

Falconer D.S., and T.F.C. Mackay. 1996. Introduction to quantitative genetics. Addison Wesley Longman, Harlow.

Heidaritabar, M., P. Bijma, L. Janss, C. Bortoluzzi, H.M. Nielsen, P. Madsen, B. Ask, and O.F. Christensen. 2019. Models with Indirect Genetic Effects Depending on Group Sizes: A Simulation Study Assessing the Precision of the Estimates of the Dilution Parameter. Genet Sel. 51:24–24.

Hoffmann, A.A. 1987. A Laboratory Study of Male Territoriality in the Sibling Species *Drosophila Melanogaster* and *D. Simulans*. Anim Behav. 35:807–18.

Hoffmann, A.A., and Z. Cacoyianni. 1990. Territoriality in *Drosophila melanogaster* as a Conditional Strategy. Anim Behav. 40:526–37.

Hoyer, S.C., A. Eckart, A. Herrel, T. Zars, S.A. Fischer, S.L. Hardie, and M. Heisenberg. 2008. Octopamine in Male Aggression of *Drosophila*. Curr Biol. 18:159–67.

Jacobs, M.E. 1960. Influence of Light on Mating of *Drosophila Melanogaster*. Ecol. 41:182–88.

Kent, C.F., T. Daskalchuk, L. Cook, M.B. Sokolowski, and R.J. Greenspan. 2009. The *Drosophila* Foraging Gene Mediates Adult Plasticity and Gene-Environment Interactions in Behaviour, Metabolites, and Gene Expression in Response to Food Deprivation. PLoS Genetics. 5:e1000609–e1000609.

Kenward, M.G., and J.H. Roger. 1997. Small Sample Inference for Fixed Effects from Restricted Maximum Likelihood. Biometrics. 53:983–97.

Lane, S.M., A.J. Wilson, and M. Briffa. 2020. Analysis of Direct and Indirect Genetic Effects in Fighting Sea Anemones. Behav Ecol. 31:540–47.

Lynch, M., and B.Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland.

MacLellan, K., L. Kwan, M.C. Whitlock, and H.D. Rundle. 2012. Dietary Stress Does Not Strengthen Selection Against Single Deleterious Mutations in *Drosophila Melanogaster*. Heredity. 108:203–10.

Moore, A.J., E.D. Brodie III, and J.B. Wolf. 1997. Interacting Phenotypes and the Evolutionary Process: I. Direct and Indirect Genetic Effects of Social Interactions. Evol. 51:1352–62.

Qiu, S., and C. Xiao. 2018. Walking Behavior in a Circular Arena Modified by Pulsed Light Stimulation in *Drosophila Melanogaster* W^{1118} Line. Physiol Behav. 188:227–38.

Roff, D.A., and J.W. Alastair. 2014. Quantifying Genotype-by-Environment Interactions in Laboratory Systems. Genotype-by-Environment Interactions and Sexual Selection, John Wiley & Sons.

Rooke, R., A. Rasool, J. Schneider, and J.D. Levine. 2020. *Drosophila Melanogaster* Behaviour Changes in Different Social Environments Based on Group Size and Density. Commun Biol. 3: 304-305.

Saltz, J.B. 2011. Natural Genetic Variation in Social Environment Choice: Context-Dependent Gene-Environment Correlation in *Drosophila melanogaster*: Natural Genetic Variation in Social Environment Choice. Evol. 65:2325–34.

Saltz, J.B. 2013. Genetic Composition of Social Groups Influences Male Aggressive Behaviour and Fitness in Natural Genotypes of *Drosophila Melanogaster*. Proc. Royal Soc. B. 280:20131926–20131926.

Santostefano, F., A.J. Wilson, P.T. Niemelä, and N.J. Dingemanse. 2017. Indirect Genetic Effects: a Key Component of the Genetic Architecture of Behaviour. Sci Rep. 7:10235–10235.

Schielzeth, H., N.J. Dingemanse, S. Nakagawa, D.F. Westneat, H. Allegue, C. Telitsky, D. Réale, N.A. Dochtermann, L.Z. Garamszegi, Y.G. Araya-Ajoy, and C. Sutherland. Robustness of linear mixed-effects models to violations of distributional assumptions. Methods Ecol. Evol. 11:1141-1152.

Spieth, H.T., and T.C. Hsu. 1950. The Influence of Light on the Mating Behavior of Seven Species of the *Drosophila Melanogaster* Species Group. Evol. 4:316–25.

Tremblay, M., H.D. Rundle, M. Videlier, and V. Careau. 2021. Territoriality in *Drosophila*: Indirect Effects and Covariance with Body Mass and Metabolic Rate. Behav Ecol. 32:679–85.

White, A.J. and H.D. Rundle. 2015. Territory Defense as a Condition-Dependent Component of Male Reproductive Success in *Drosophila Serrata*. Evol. 69:407-18.

Wilson, A.J., U. Gelin, M.C. Perron, and D. Réale. 2009. Indirect Genetic Effects and the Evolution of Aggression in a Vertebrate System. Proc. Royal Soc. B. 276:533–41.

Wolf, J.B., E.D. Brodie III, J.M. Cheverud, A.J. Moore, and M.J. Wade. 1998. Evolutionary Consequences of Indirect Genetic Effects. Trends Ecol Evol. 13:64–69.

Tables and Figures

Table 1. Pairing analysis table within a block. Each sire was assigned an ID number between 1 and 32. Offspring from these sires were then paired for trials. Pairings avoided any repeat matchups and attempted to represent sires evenly.

	Day 1					Day 2					Day 3					Day 4				
Arena	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
1	1-2	1-32	24-25	1-31	1-4	4-7	1-5	5-9	1-6	6-11	1-7	7-13	1-8	8-15	1-9	9-17	1-10	10-19	1-11	11-21
2	22-23	2-3	2-4	3-5	2-5	5-8	2-6	6-10	2-7	7-12	2-8	8-14	2-9	9-16	2-10	10-18	2-11	11-20	2-12	12-22
3	7-8	4-5	5-7	4-6	24-30	6-9	3-7	7-11	3-8	8-13	3-9	9-15	3-10	10-17	3-11	11-19	3-12	12-21	3-13	13-23
4	24-27	6-7	6-8	7-9	7-10	10-13	4-8	8-12	4-9	9-14	4-10	10-16	4-11	11-18	4-12	12-20	4-13	13-22	4-14	14-24
5	9-10	8-9	9-11	8-10	8-11	11-14	9-13	13-17	5-10	10-15	5-11	11-17	5-12	12-19	5-13	13-21	5-14	14-23	5-15	15-25
6	11-12	10-11	10-12	11-13	9-12	12-15	10-14	14-18	11-16	16-21	6-12	12-18	6-13	13-20	6-14	14-22	6-15	15-24	6-16	16-26
7	13-14	12-13	13-15	12-14	13-16	16-19	11-15	15-19	12-17	17-22	13-19	19-25	7-14	14-21	7-15	15-23	7-16	16-25	7-17	17-27
8	15-16	14-15	14-16	15-17	14-17	17-20	12-16	16-20	13-18	18-23	14-20	20-26	15-22	22-29	8-16	16-24	8-17	17-26	8-18	18-28
9	17-18	16-17	17-19	16-18	15-18	18-21	17-21	21-25	14-19	19-24	15-21	21-27	16-23	23-30	17-25	24-32	9-18	18-27	9-19	19-29
10	19-20	18-19	18-20	19-21	19-22	22-25	18-22	22-26	15-20	20-25	16-22	22-28	17-24	24-31	18-26	1-21	19-28	7-27	10-20	20-30
11	21-22	20-21	21-23	20-22	20-23	23-26	19-23	23-27	21-26	26-31	17-23	23-29	18-25	25-32	19-27	2-22	20-29	8-28	21-31	4-29
12	23-24	3-4	22-24	23-25	21-24	5-6	20-24	24-28	22-27	27-32	18-24	3-6	19-26	7-30	20-28	3-23	21-30	9-29	22-32	5-30
13	25-26	1-3	25-27	24-26	25-28	28-31	25-29	1-30	23-28	1-29	25-31	1-24	20-27	8-31	21-29	4-24	22-31	10-30	8-32	6-31
14	27-28	26-27	26-28	27-29	26-29	29-32	26-30	2-31	24-29	2-30	26-32	2-25	21-28	9-32	22-30	5-25	23-32	11-31	1-26	7-32
15	29-30	28-29	29-31	28-30	27-30	2-24	27-31	4-26	25-30	7-29	9-31	3-26	5-28	1-25	23-31	6-26	5-29	12-32	2-27	1-27
16	31-32	30-31	30-32	2-32	1-23	3-25	28-32	5-27	6-28	8-30	10-32	4-27	6-29	2-26	3-27	4-28	6-30	7-31	3-28	2-28

Table 2. Estimates (±se), numerator and denominator degrees of freedom (df_{num} and df_{den}), conditional Wald *F*-statistic, and *P*-values associated with each fixed effect included in the random regression model of aggressiveness (raised to a power of 0.4, then scaled to a mean of 0 and variance of 1) in 1,236 male *D. melanogaster* during staged dyadic territorial assays.

	estimate	±	se	df_{num}	df_{den}	*F*	*P*
Intercept	-0.085	±	0.148	1	7.3		
Measurement block				3	7.3	1.62	0.2669
Arena				15	458	1.43	0.1270
Time of day	0.000	±	0.000	1	526.2	1.29	0.2572
Color mark$_{[red]}$	0.026	±	0.043	1	541.6	0.35	0.5525
Food size$_{[small]}$	**-0.187**	**±**	**0.080**	**1**	**52.7**	**5.51**	**0.0228**

Table 3. A) Variance and B) correlation estimates from the random regression model of aggressiveness (raised to a power of 0.4, then scaled to a mean of 0 and variance of 1) in 1,236 male *D. melanogaster* during staged dyadic territorial assays. In B, likelihood ratio tests were used to test for significance of each correlation between focal and opponent sire intercept and slope variance components (components 1 to 4 in A).

		component	±	SE	χ^2(df = 1)	*P*
A) variance components						
1	focal sire	0.0084	±	0.0115		
2	focal sire × env	0.0110	±	0.0149		
3	opponent sire	0.0476	±	0.0513		
4	opponent sire × env	0.1021	±	0.0616		
	focal dam	0.0000	±	NA		
	opponent dam	0.0036	±	0.0281		
	trial dyad	0.3216	±	0.0455		
	day	0.0383	±	0.0268		
	residual	0.5583	±	0.0435		
B) correlations between focal and opponent sire						
	2 vs 1	0.998	±	NA	1.63	0.202
	3 vs 1	0.139	±	1.345	0.14	0.712
	3 vs 2	0.360	±	1.011	0.16	0.69
	4 vs 1	0.486	±	0.688	0.73	0.392
	4 vs 2	**0.930**	**±**	**0.500**	**2.70**	**0.10**
	4 vs 3	0.332	±	0.774	0.24	0.626

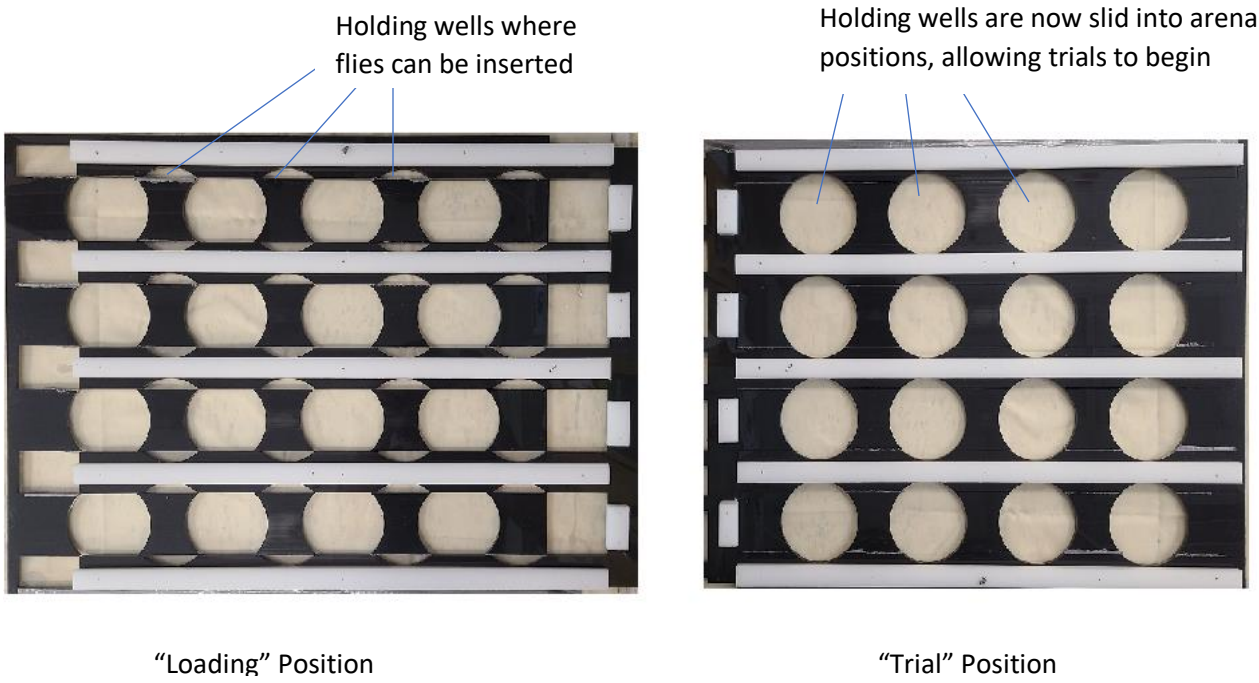

Figure 1. Diagram of arena in both loading and trial positions. When in loading position, flies can be inserted into semicircular holding wells. After a fly is inserted in each holding well a transparent cover is slid over the arena to prevent flies from escaping. Once all flies are loaded, arena is moved into trial position, introducing the pair of flies to each other and to the arena, and recording begins.

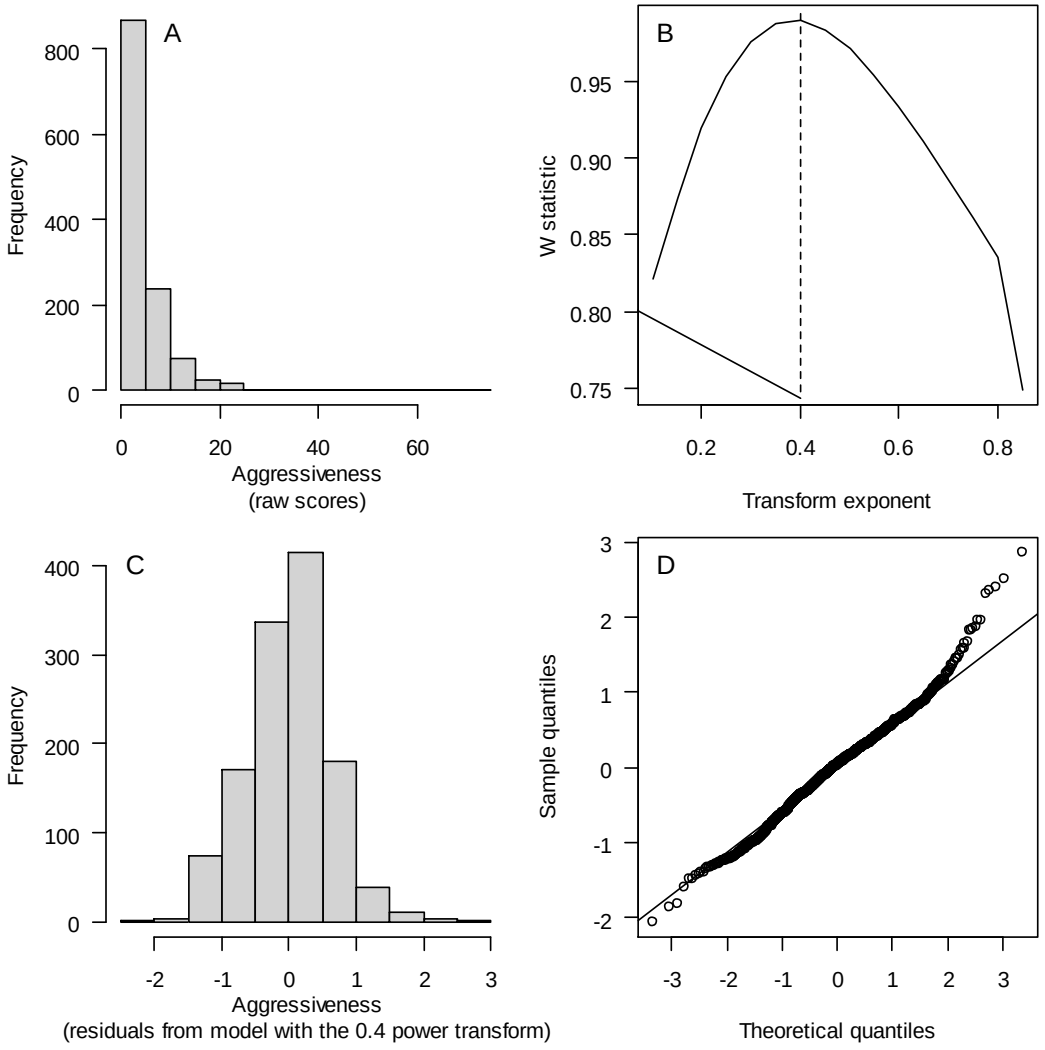

Figure 2. A) Distribution of the raw aggressiveness scores in 1,236 male *Drosophila melanogaster* during staged dyadic territorial assays. B) W-statistic from the Shapiro-Wilk tests on the residuals from the random regression model run on aggressiveness scores raised to various power values. C) Frequency distribution and D) normal quantile plot of the residuals from the model using a 0.4 power transform.

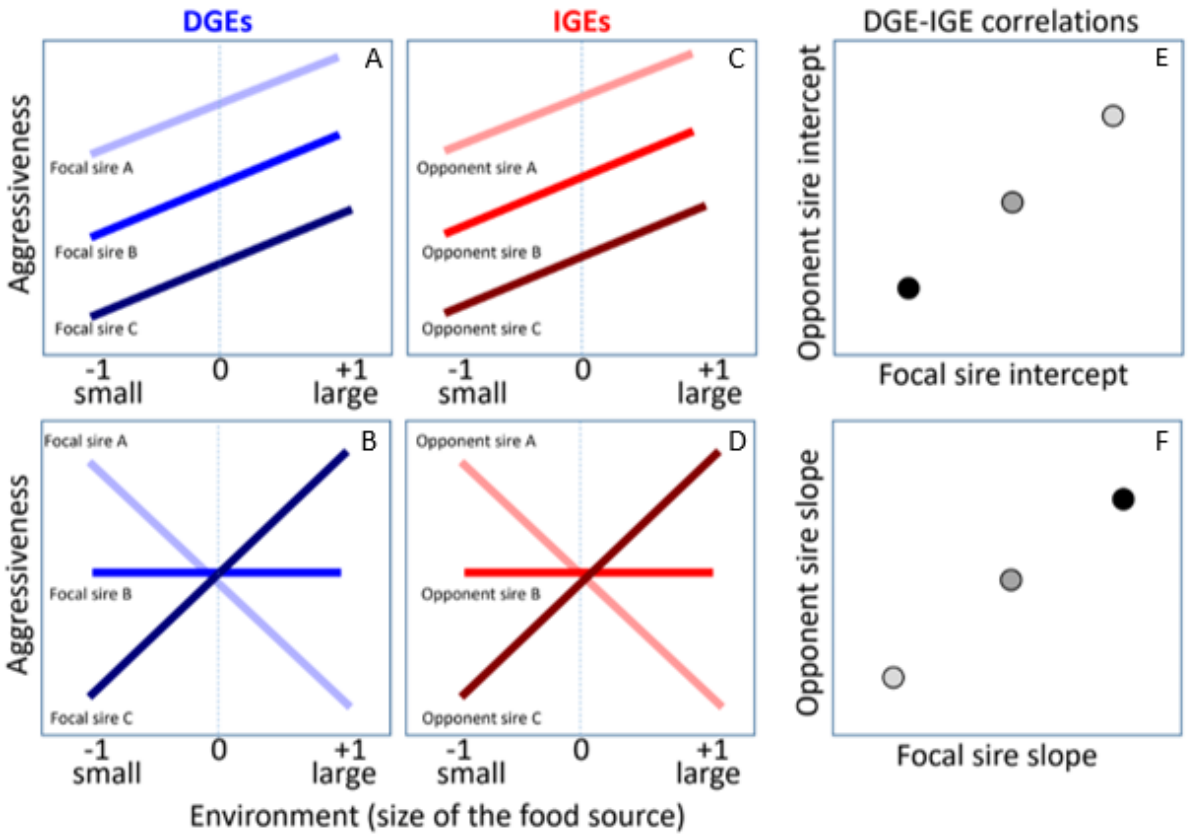

Figure 3. A depiction of the reaction norm approach used to estimate overall direct genetic effects (DGEs; differences among focal sire intercepts), indirect genetic effects (IGEs; differences in opponent sire intercepts), and changes in IGEs and DGEs as function of an environmental variable (size of the food source). In A, DGEs are shown as variation in intercept among focal sires. In B, focal sires vary in their slope, indicating changes in DGEs with the size of the food resource. Similarly, in C IGEs are illustrated as intercept variance among opponent sires, whereas in D changes in IGEs with the environment are illustrated as slope variation among opponent sires. In E, a positive DGE × IGE correlation is illustrated (i.e., sires A/B/C have high/medium/low intercepts as focal and opponent sires). In F, a DGE × IGE × environment is illustrated (i.e., sires A/B/C have negative/nil/positive slopes as focal and opponent sires).

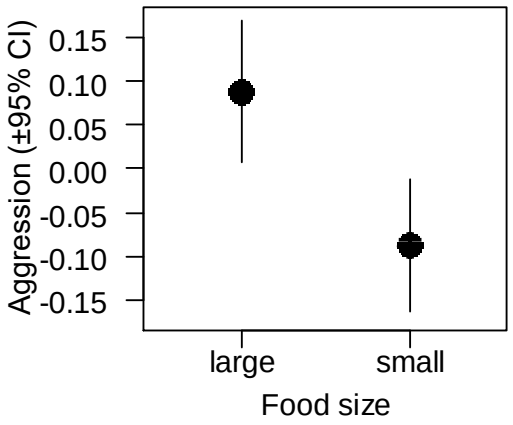

Figure 4. Average aggression (sum of lunges, kicks, chases, and faceoff wins; raised to a power of 0.4, and then scaled to a mean of 0 and variance of 1) expressed by male *Drosophila melanogaster* as function of the size of the food source during staged dyadic territorial trials.

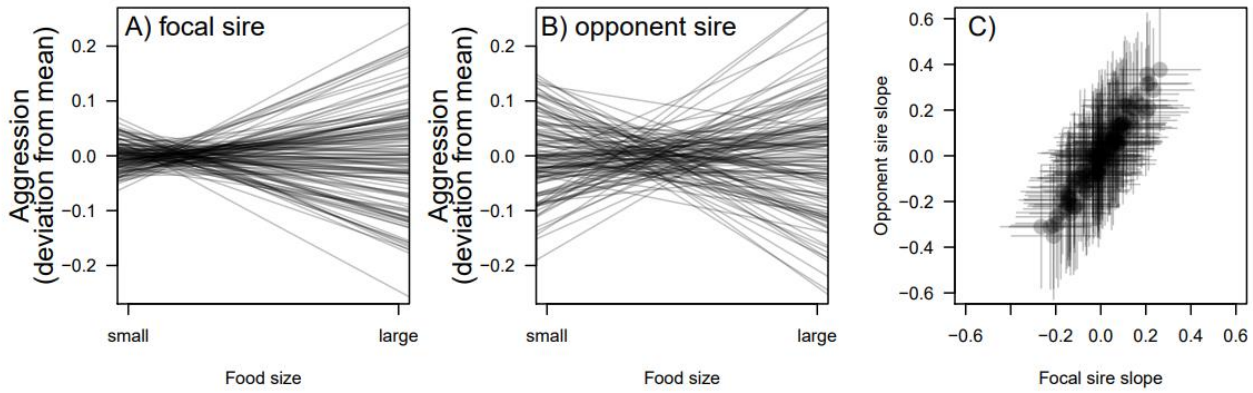

Figure 5. Aggressiveness (deviation from population mean) as function of the size of the food source in male *D. melanogaster* interacting in territoriality assays (conceptually, this figure follows the format outlined in Fig. 3). Each line represents the reaction norm of one of 128 sire based on the best linear unbiased predictors for the A) focal and B) opponent sire intercepts and slopes random effects. C) Focal and opponent sire slopes were plotted showing a significant correlation, as previously described in Table 3 (4 vs. 2 correlation).

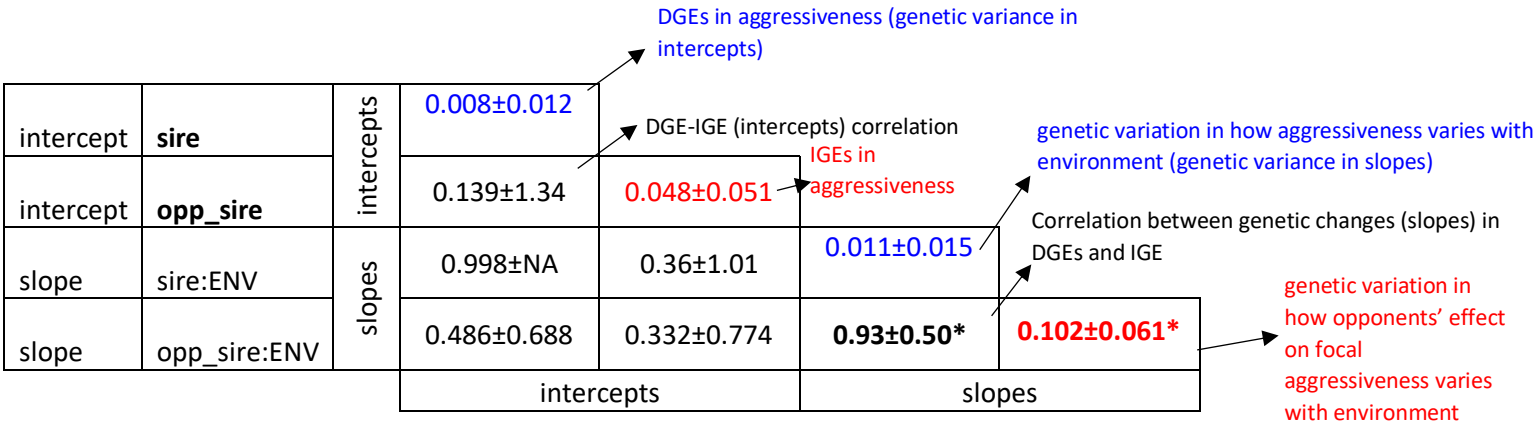

Figure 6. Correlation matrix of random regression analysis of male aggressiveness, with variance components (±se) along the main diagonal and correlations (±se) in the off-diagonal.