Defense traits of larval *Drosophila melanogaster* exhibit genetically based tradeoffs against different species of parasitoids

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Abstract

Populations of *Drosophila melanogaster* face significant mortality risks from parasitoid wasps that use species-specific strategies to locate and survive in hosts. We tested the hypothesis that parasitoids with different strategies select for alternative host defense characteristics and in doing so contribute to the maintenance of fitness variation and produce trade-offs among traits. We characterized defense traits of *Drosophila* when exposed to parasitoids with different host searching behaviors (*Aphaereta sp.* and *Leptopilina boulardi*). We used host larvae with different natural alleles of the gene *Dopa decarboxylase* (*Ddc*), a gene controlling the production of dopamine and known to influence the immune response against parasitoids. Previous population genetic analyses indicate that our focal alleles are maintained by balancing selection. Genotypes exhibited a trade-off between the immune response against *Aphaereta sp.* and the ability to avoid parasitism by *L. boulardi.* We also identified a trade off between the ability to avoid parasitism by *L. boulardi* and larval competitive ability as indicated by differences in foraging and feeding behavior. Genotypes differed in dopamine levels potentially explaining variation in these traits. Our results highlight the potential role of parasitoid biodiversity on host fitness variation and implicate *Ddc* as an antagonistic pleiotropic locus influencing larval fitness traits.

Keywords

adaptation; pleiotropy; parasitism; genetic variation; behavior; polymorphism

The composition of species in communities not only shapes ecological interactions it also influences the genetic characteristics of interacting populations. The evolutionary importance of these interactions has been very well demonstrated in plant-herbivore (Lankau and Strauss 2008, Agrawal 2011, Winde and Wittstock 2011) and host-parasite systems (Gandon and Van Zandt 1998, Lively and Dybdahl 2000, Gandon et al. 2008) where the fitness of the attacking species (herbivore or parasite) is completely dependent on the ability to find and use a suitable host. In these systems we expect strong selection on traits that both

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reduce the probability of attack and the damaging effects of the attacker. From the perspective of the host, defense against attack is especially challenging when different species must be defended against (Futuyma and Mitter 1996). Futuyma and Mitter outline two potential scenarios that can result from these interactions. If similar defenses are effective against a range of potential attackers, then positive genetic correlations among defense traits are expected. Alternatively, if one defense trait is useful against one attacker, but decreases defense against another, then negative correlations among such traits are expected (Futuyma and Mitter 1996). Thus, the biodiversity of species attacking hosts in a community can have important influences on the genetic characteristics of host species, potentially resulting in correlations among traits.

Genetic correlations are one of the more important genetic characteristics that influence evolution (Rose 1982), Lande and Arnold 1983, Rose 1985, Arnold 1992, Price et al. 1993, Scarcelli et al. 2007, Gratten et al. 2008). When genetically correlated traits influence fitness they can influence phenotypic evolution in at least three ways. First, selection on one trait can cause evolutionary changes in traits that are not the targets of selection (Leroi et al. 2005, Roff and Fairbairn 2007). Indeed, this could explain many correlated patterns of morphological, behavioral and life history diversification among populations and species (Armbruster 1991, Promislow 1995, Schluter 1996, Steppan et al. 2002, Baker and Wilkinson 2003 but see Baer and Lynch 2003). Second, genetic correlations can act as a constraining force in evolution, limiting the adaptive independent evolution of traits, or at a minimum, constrain the rate and/or evolutionary trajectory of interrelated traits (Lande 1982, Arnold 1992, Schluter 1996, Houle 2001, Roff and Fairbairn 2007 but see Conner et al. 2011). Third, correlations can contribute to the maintenance of variation in fitness through antagonistic pleiotropy (Rose 1982, 1985) or through the general effects of balancing selection acting on traits influenced by pleiotropic loci (Turelli and Barton 2004).

While theoretical models of the evolutionary consequences of genetic correlations are well developed, fundamental questions remain that only empirical data can address. Which traits are genetically correlated and so might be expected to exhibit correlated evolutionary changes? What are the fitness effects of allelic variation at pleiotropic loci and how is this variation maintained? What is the nature of selection acting on correlated traits (and by extension pleiotropic genes) and how do the combined effects of selection and correlation influence phenotypic evolution? While numerous studies provide evidence for the importance of pleiotropy in evolution (Williams 1957, Rose and Charlesworth 1981, Harshman and Hoffmann 2000, Bucan and Abel 2002, Featherstone and Brodie 2002, Anholt and Mackay 2004, Hughes and Reynolds 2005, Kenney-Hunt et al. 2006, Roff and Fairbairn 2007), studies of the pleiotropic effects of natural allelic variation at individual genes (Leroi et al. 2005, Carbone et al. 2006, Scarcelli et al. 2007, Tellier et al. 2007, Anderson et al. 2012) are needed to address the above questions (Leroi et al. 2005).

The host parasitoid interactions of *Drosophila melanogaster* provide an excellent system to address these questions for three main reasons. First, a number of different species of parasitoids use *Drosophila melanogaster* as hosts in natural populations (Carton et al. 1986, Allemand et al. 2002, Mitsui et al. 2007). Because many parasitoids use different strategies to locate and survive in hosts (Kraaijeveld and Godfray 2009, Lee et al. 2009), this sets up the conditions that could produce trade-offs in host defense traits (Futuyma and Mitter 1996). Second, parasitoids impose a significant mortality risk for Drosophila larvae, with larval mortality from parasitoids exceeding 50% in some populations (Janssen et al. 1988, Fleury et al. 2004). Thus parasitoids are potentially important agents of natural selection. Finally, as Drosophila are model genetic organisms, we have the potential to identify the genes influencing these traits including those that give rise to trade-offs.

In this study we ask if larval defense traits of *D. melanogaster* exhibit genetic correlations when confronted with two species of parasitoids, *Leptopilina boulardi* and *Aphaereta sp.*, that use different behaviors to locate their hosts. In addition, we used a set of fly stocks derived from a natural population to examine the potential pleiotropic effects of polymorphism in the gene *Dopa decarboxylase*, (*Ddc*) on larval traits. *Ddc* encodes DOPA decarboxylase (DDC) an important enzyme in the catecholamine biosynthesis pathway that produces two neurotransmitters, dopamine and serotonin (Christensen et al. 1972, Livingstone and Tempel 1983, Coleman and Neckameyer 2005). Dopamine and serotonin are key players in reproductive, developmental, behavioral, and immune processes in vertebrates and invertebrates (Neckameyer 1998, Goodson et al. 2009, Schweitzer and Driever 2009, Strell et al. 2009). De Luca et al. 2003 found evidence for balancing selection at this locus, predominantly between two haplotypes associated with longevity. These haplotypes are formed via three naturally occurring single nucleotide polymorphisms (SNPs), a T/C polymorphism in the promoter region, C/A polymorphism in exon 2 and T/G polymorphism in intron 3. Assuming that selection is not acting directly on life span (Hamilton 1966, we hypothesized that balancing selection is acting on one or more traits that are genetically correlated with life span and that may also be influenced by polymorphism in *Ddc*.

There is good reason to suspect that polymorphism in *Ddc* could affect immunological and non-immunological larval traits in Drosophila. Immunological defense against parasitoids involves the encapsulation and melanization of parasitoid eggs by circulating blood cells of Drosophila larva (Vass and Nappi 2000), killing the developing wasp (Vass and Nappi 2000, Nappi and Christensen 2005). Dopamine is a precursor to melanin production in invertebrates (Nappi and Christensen 2005, Hodgetts and O'Keefe 2006) and indeed, variation in *Ddc* expression affects the melanotic encapsulation response against the eggs of parasitoid wasps (Nappi et al. 1992, Schlenke et al. 2007). As a result we hypothesized that *Ddc* polymorphism could affect the melanotic encapsulation of wasp eggs, potentially by altering dopamine levels.

The non-immunological defense of fly larvae is to avoid being attacked and many aspects of fly larval behavior, such as foraging, rolling, and digging influence the likelihood of parasitoid attack (Carton and David 1983, Carton and Sokolowski 1992, Kraaijeveld and van Alphen 1995). As a neurotransmitter, dopamine affects locomotory behavior (Pendleton et al. 2002, Pendleton et al. 2005, Jordan et al. 2006, Vermeulen et al. 2006) so we hypothesized that *Ddc* polymorphism may also contribute to variation in fly larval behaviors under selection by parasitoids.

Artificial selection experiments have revealed genetically based tradeoffs between immunological and non-immunological traits like the ones mentioned above. Flies that have been artificially selected for increased parasitoid resistance against *Asobara tabida* and *L. boulardi* have reduced larval competitive ability under low resource conditions compared to unselected control populations (Kraaijeveld and Godfray 1997, Fellowes et al. 1998). Given that immune response and competitive ability are negatively correlated, we hypothesized that if *Ddc* polymorphism affects the immune response against parasitoids, it may also contribute to the trade-off between melanotic encapsulation ability and larval competitive ability. To test this hypothesis we measured the feeding rate of the different *Ddc* genotypes because feeding rate has repeatedly been shown to be indicative of competitive ability in Drosophila (Sewell et al. 1975, Burnet et al. 1977, Joshi and Mueller 1988, 1996).

Materials and Methods

FLY LINES

We selected six *Chromosome II* (*CII*) extraction lines derived from the natural population of *D. melanogaster* in Raleigh, NC (creation of lines are described in detail in De Luca et al. 2003). This extraction procedure resulted in homozygous lines that were genetically identical for genes on the first and third chromosomes but which differed by the origin of the 2nd chromosome.

Three of the lines chosen had a *Ddc* haplotype that was associated with long-lived flies (CAT) and the other three lines had the alternative SNP haplotype associated with shorter lifespan (TCG). We selected these two haplotypes because, of the seven *Ddc* haplotypes found segregating in this population, these two were at high frequency and were the only two haplotypes found at significantly higher frequencies than expected under neutrality (De Luca et al. 2003). We used the offspring of crosses between these lines to measure several larval fitness traits, two of which (larval foraging and melanotic encapsulation ability) are related to defense against parasitoids that commonly parasitize larval *D. melanogaster* in Maryland, *L. boulardi* and *Aphaereta sp.* (Hodges, unpublished data).

CROSSING DESIGN TO CREATE EXPERIMENTAL GENOTYPES

We carried out six reciprocal homozygote crosses (each line served as both sire and dam) crossing each of the three lines of each *Ddc* haplotype to each other in a crossing design similar to that of Geiger-Thornsberry and Mackay (2002). We created heterozygous larvae by crossing two of the lines with the long-lived (CAT) *Ddc* haplotype to two lines with the short-lived (TCG) *Ddc* haplotype, also in a reciprocal crossing design. We found no significant maternal or paternal (dam or sire) effect for any trait measured, so we used the pooled data from each reciprocal cross for analyses of the data.

We used the offspring of crosses among the lines rather than testing the inbred lines to minimize the influence of inbreeding depression while at the same time allowing us to examine the average effects of different *Ddc* genotypes on fitness traits in otherwise outbred backgrounds (Geiger-Thornsberry and Mackay 2002).

We performed phenotypic assays with 2nd instar larvae because this is the developmental stage most efficiently parasitized by parasitoids (van Alphen and Drijver 1982).

PARASITOID COLLECTION AND MAINTENANCE

We established laboratory colonies of *Aphaereta sp.* and *L. boulardi* from wasps collected at two local sites, Boordy Vineyards (Hyde, MD) and Boyer Farms (Severn, MD). Wasp colonies were maintained using larvae of an inbred *D. melanogaster* laboratory strain (Samarkand). Adult wasps were fed a 30% honey solution and maintained as large populations (> 300 individuals) in 12" X 12" X 12" plexiglass cages at 18°C on a 12-hour light/dark cycle.

PARASITOID IDENTIFICATION

The identity of *L. boulardi* was determined morphologically (and confirmed by Matthew Buffington, Smithsonian Institution). To identify *Aphaereta sp.*, we sequenced the D2 region of the 28S ribosomal RNA (rRNA). We obtained the D2 sequence from three different individual wasps using PCR conditions and primers from Gimeno et al. 1997 (Gimeno et al. 1997): 5'AGAGAGAGTTCAAGAGTACGTG3' (forward primer) and 5'TTGGTCCGTGTTTCAAGACGGG3' (reverse primer). Sequences were obtained using an ABI Prism 3100 Genetic Analyzer. Sequences were aligned to thirty-four 28S D2 rRNA

sequences from Genbank (including those from the Alysiinae, Exothecinae and Opiinae subfamilies and *Gnamptodon pumilo*) and a 28S sequence of *Aphaereta genevensis* provided by Peter Mayhew (University of York, York, UK). Sequences were initially aligned using Sequencher 4.6 (Gene Codes Corp., Ann Arbor, Mich.) and further manually aligned using Mega 4.0 (Tamura et al. 2007). We used 381 base pairs of this sequence to construct a pairwise distance matrix among species using Mega 4.0. The D2 sequence from our samples of *Aphaereta* did not match any of the published sequences. The species most closely related to our *Aphaereta sp.* was *A. minuta* (evolutionary distance of 0.6%) and *A. genevensis* (evolutionary distance of 0.9%). The next closest relative to our *Aphaereta sp.* was *Phaenocarpa sp.* (evolutionary distance of 2.3%). Thus, to the best of our knowledge, this species has either not yet been described or its D2 sequence has not been published.

PARASITOID EXPOSURE ASSAY: LABORATORY

For each homozygote and heterozygote *Ddc* cross, we placed 30 2nd instar F1 larvae on a Petri dish containing 1% agar covered with a thin layer of standard fly food. This food layer allowed the larvae to evade visual inspection by the wasps. We exposed larvae on each plate for a four-hour period to a single female wasp with prior ovipositioning experience. A different female wasp was used for each exposure assay, with only one individual wasp exposed to each plate. Following exposure, *D. melanogaster* larvae were placed in incubators at 25°C or 29°C to test the effects of temperature on melanotic encapsulation efficiency. We found no effects of temperature on this process so we pooled the data from both temperature treatments for analysis. Two days after exposure we dissected the 3rd instar *D. melanogaster* larvae, scoring each for the presence of a wasp larva or a melanized encapsulated egg (both indications of parasitism). The ability to avoid parasitism was determined for each replicate plate and defined as the proportion of fly larvae on a plate that had not been parasitized in the four-hour period. The exposure assay was repeated for each cross until at least 30 larvae per cross per temperature had been parasitized. Melanotic encapsulation ability was scored as the proportion of parasitized larvae on a plate that had a melanotic encapsulated wasp egg. A total of 5,519 3rd instar larvae from 208 assays were dissected. In each of these cases the plate was the unit of replication for the statistical analyses (discussed below).

PARASITOID EXPOSURE ASSAY: FIELD

For each homozygote and heterozygote *Ddc* cross, we placed 30 2nd instar F1 larvae on a Petri dish containing 1% agar covered with a thin layer of slightly decayed homogenized peaches which had been colonized with natural bacteria and yeast from our field site. Plates containing larvae were transported in a Styrofoam container to Boyer Farms and positioned near baits that had been put out three days prior to the field assay to attract flies and wasps. We exposed 10 plates of larvae at a time (one plate of larvae from each cross), allowing a total exposure time of 40 "wasp-minutes" per plate (defined below), measured from the time that the first wasp landed and started probing for hosts on the plate. Preliminary experiments in the field indicated that a single wasp could parasitize about half of the larvae on a plate in 40 minutes (much more quickly than in the laboratory). Often more than one wasp would land on a plate and begin to probe for hosts in the field experiment. To standardize the time of larval exposure to parasitoids on each plate, we recorded the number of wasps parasitizing a single plate and the time that individual wasps began to probe for hosts. The amount of time that a plate was exposed to wasp(s) varied depending on the number of wasps searching on a plate. If only one wasp was searching, then the exposure time was forty minutes. If there were two wasps, then the time was adjusted to 20 minutes, and so on. The maximum number of wasps on a plate observed at one time was three. At the end of an exposure period for a plate, the wasp(s) were aspirated from the plate and placed in ethanol for identification (all were determined to be *L. boulardi* based on visual inspection and the

fact that none of the fly larvae melanotically encapsulated the eggs). Fly larvae were returned to the lab, placed in an incubator for 48 hours at 25°C and then dissected and scored for parasitism and melanotic encapsulation. The total number of replicates ranged from 6 to 10 plates per cross and a total of 2,165 3rd instar larvae were dissected.

Because our laboratory experiments indicated that the Drosophila genotypes differed only in the probability of parasitism from *L. boulardi* and not *Aphaereta sp.* (see Results), we confined our field experiment to late summer, when *L. boulardi* is the dominant parasitoid. All field experiments were carried out from August through October.

DROSOPHILA LARVAL FORAGING BEHAVIOR ASSAY

For each homozygote and heterozygote *Ddc* cross, we measured foraging behavior of thirty 2nd instar F1 larvae using the method described in Sokolowski et al. (Sokolowski et al. 1997). Briefly, we placed a single larva in the center of a circular well (0.5 mm deep with an 8.25 cm radius) containing a 2:1 water:yeast mixture and then covered the well with a Petri dish. After five minutes of acclimation, we allowed larvae to forage on the plate for five minutes. The foraging path was then traced on the lid. We measured foraging behavior in a 25°C temperature-controlled room. Paths were photographed using a digital imager and path lengths measured using Metamorph software version 7.0.

DROSOPHILA LARVAL FEEDING RATES

For each cross, we measured the feeding rates of thirty 2nd instar F1 larvae using the number of cephalopharyngeal retractions in a 30 second period as an estimate of feeding rate (Joshi and Mueller 1988, Fellowes et al. 1999a). For each larva, we covered a petri dish containing 1% agar with a layer of 10% water:yeast mixture. We gently transferred the larva to the petri dish. After a 30-sec acclimatization period, we counted the number of cephalopharyngeal contractions made by the larva during a 30-second period. We measured feeding rates in a 25°C temperature-controlled room.

HPLC ANALYSIS OF DOPAMINE LEVELS

For each cross, we collected three samples containing 30–40 mg of 2nd instar larvae. Larvae were treated with 5% meta-phosphoric acid (5uL/mg) and then frozen at −80°C. For analysis of dopamine levels samples were allowed to thaw for 5–10 min, sonicated for 10 min while floated over chilled water in a sonicating bath, and vortexed (3 cycles). The samples were then loaded into spinX tubes and centrifuged at 12,900xg for 10 min and then transferred to tapered hplc vials. Dopamine was quantified on a LC-2010CHT HPLC (Shimadzu, Columbia, MD) equipped with an auto-sampler with Peltier temperature control set to 4°C and an 8-channel ESA CoulArray Detector (model 5600 A; ESA Laboratories, Chelmsford, MI) by the method of Takeda (Takeda 1997) with minor modifications. Briefly, samples (20 μL) were separated on a Luna, reverse phase, C18(2), 150x 4.60 mm column (Phenomenex, Torrance, CA) provided with a Phenomenex guard column (ODS, 4-mm length, 3.0-mm ID) with an isocratic mixture of 50 mM sodium phosphate buffer pH 3.2 containing 200 μM sodium octylsulfonate, and methanol (90:10) (Sigma-Aldrich, St. Louis, MO) at a flow rate of 0.6 ml/min. The analyte was typically analyzed at 350 mV and ratios to signals from leading and/or trailing potentials were used to control for interference of possible co-eluting substances. All samples and standards were run in duplicate, and the results were averaged. The collected data were analyzed with ESA CoulArray software (ESA).

DATA ANALYSES

We tested for differences among the genotypes in melanotic encapsulation ability and ability to avoid parasitism using logistic regression as implemented by SAS (SAS V.9.0, SAS

Institute, Cary, NC). The phenotype being analyzed was the response variable and the genotype (as referenced by the known genotype at the *Ddc* locus, CAT/CAT, CAT/TCG, TCG/TCG) was the predictor. The best fit was a logistic regression with binomial errors, a logit link, and overdispersion parameter.

We tested for differences in foraging behavior, feeding rates, and dopamine levels among the genotypes by ANOVA using Proc GLM. We used CONTRAST statements to compare genotypes in post hoc analyses (SAS V.9.0, SAS Institute, Cary, NC). We transformed the foraging path length data to natural logs to satisfy assumptions of ANOVA.

We tested for an association between the genotype of larvae and the number of wasps attacking a plate of larvae (in the field component of this experiment) with Fishers exact test using Proc Freq (SAS V.9.0, SAS Institute, Cary, NC).

Results

ABILITY OF FLY LARVAE TO AVOID PARASITISM DEPENDS ON GENOTYPE

There were no differences among genotypes in their ability to avoid parasitism by *Aphaereta sp.* ($\chi^2 = 0.46$; $P = 0.79$) (Figure 1). However, there was a significant difference among *Ddc* genotypes on the ability to avoid parasitism by *L. boulardi* ($\chi^2 = 8.87$; $P = 0.01$) (Figure 1), with the rank order of avoidance being TCG homozygotes > CAT homozygotes > heterozygote. Larvae homozygous for the TCG polymorphism were 18% better at avoiding attack than the CAT homozygous larvae (CAT, $\chi^2 = 3.60$; $P = 0.06$) and 21% better than heterozygote larvae (CAT/TCG, $\chi^2 = 8.00$; $P = 0.005$). So in the case of avoidance of parasitism, larvae homozygous for TCG are best able to avoid parasitism in the laboratory as compared with the heterozygotes and CAT homozygotes.

VARIATION AMONG GENOTYPES IN IMMUNE RESPONSE IS SPECIES-SPECIFIC

We found significant differences in the melanotic encapsulation ability among the genotypes against the parasitoid, *Aphaereta sp.* ($\chi^2 = 20.39$, $P < 0.0001$) (Figure 2) with the rank order being heterozygotes > CAT homozygotes > TCG homozygotes. This is a large effect, notable because more larvae were able to avoid parasitism by *Aphaereta sp.* (compared to *L. boulardi*) and so we had reduced power to detect differences in encapsulation ability in this test. Heterozygotes (CAT/TCG) and homozygous CAT larvae had 17% and 10% higher melanotic encapsulation abilities, respectively, compared to TCG homozygotes. While the average melanotic encapsulation ability was greater in heterozygotes it was not formally significantly different compared with the CAT homozygous larvae ($\chi^2 = 3.25$, $P = 0.07$). However, this is approaching significance, suggesting that there may be heterozygote advantage (at least with respect to this trait) that could contribute to the maintenance of variation (and by extension *Ddc*).

We found no difference among the *Ddc* genotypes in melanotic encapsulation ability against *L. boulardi* ($\chi^2 = 4.13$, $P = 0.13$) (Figure 2). In fact very few larvae (< 4%) melanotically encapsulated eggs from this species. This low ability to melanotically encapsulate eggs combined with the fact that fewer larvae were able to avoid being attacked by *L. boulardi* indicates that this species is probably a more potent agent of selection on larval defense traits than *Aphaereta sp.* Whether or not this is true will require us to carry out a long-term regional assessment of the prevalence of these parasitiods in field populations.

VARIATION AMONG GENOTYPES INFLUENCES THE ABILITY OF LARVAE TO AVOID PARASITISM BY *L. BOULARDI* IN NATURAL POPULATIONS: A FIELD TEST OF THE LABORATORY RESULTS

The number of wasps attacking a single plate ranged from 1–3, and more wasps attacking a plate resulted in more hosts being parasitized in spite of our attempts to standardize exposure time ($\chi^2 = 5.72$, $P = 0.02$). A three-way contingency table analysis indicated that the number of wasps attacking a plate did not differ among genotypes ($\chi^2 = 1.3622$, $P = 0.8507$). As a result, the positive relationship between the number of wasps attacking a plate and the number of hosts attacked should not affect our conclusions about differences among genotypes in their ability to avoid attack reported below.

Results in the field were in broad agreement with the laboratory results; there were significant differences among genotypes in their ability to avoid parasitism by *L. boulardi* ($\chi^2 = 6.86$; $P = 0.03$) (Figure 1), with TCG homozygous larvae being much better at avoiding parasitism than the CAT homozygous larvae ($\chi^2 = 5.56$; $P = 0.02$) or heterozygotes ($\chi^2 = 5.09$; $P = 0.02$). Dissections of the field-parasitized larvae two days after exposure revealed that none of the larvae were able to melanotically encapsulate the eggs of *L. boulardi.*

GENOTYPES DIFFER IN LARVAL FORAGING BEHAVIOR

We found significant differences in foraging behavior among genotypes; TCG homozygotes moved 60% more while foraging than CAT homozygous larvae ($F_{1,290} = 88.4$, $P < 0.0001$), and 62% more than heterozygotes (CAT/TCG, $F_{1,290} = 99.7$ $P < 0.0001$) (Figure 3).

GENOTYPES DIFFER IN FEEDING RATE

We found significant differences among genotypes in feeding rates. TCG homozygotes fed 10% faster than CAT homozygous larvae ($F_{1,290} = 57.6$, $P < 0.0001$) and heterozytes ($F_{1,290} = 72.3$, $P < 0.0001$) (Figure 4).

GENOTYPES DIFFER IN DOPAMINE LEVELS

To evaluate the potential that the polymorphism in *Ddc* contributed to phenotypic differences observed among the genotypes, we measured dopamine levels in 2nd instar larvae from each of the *Ddc* crosses. *Tyrosine hydroxylase* (encoded by the *pale* gene in Drosophila) is thought to control the rate limiting step in the production of dopamine (Wright 1987). However, because the *pale* gene is on the third chromosome in Drosophila it should be homozygous in our extraction lines and so not contribute to variation in dopamine levels among genotypes.

The rank order of the *Ddc* haplotypes in the average dopamine levels (Figure 5) matches that of melanotic encapsulation ability of the different genotypes against *Aphaereta sp.* (Figure 1): heterozygote > CAT homozygotes > TCG homozygotes. This suggests that variation in dopamine levels may contribute to the observed differences in melanotic encapsulation ability.

Statistically, the analyses were able to separate the genotypes with the most extreme differences in dopamine level (heterozygotes had higher dopamine levels than short-lived homozygotes) but could not distinguish the adjacent genotypes. Heterozygous larvae (CAT/TCG) had significantly higher levels of dopamine than homozygous TCG larvae ($F_{1,20} = 4.93$, $P = 0.04$) but were not significantly different from dopamine levels in CAT homozygotes ($F_{1,20} = 2.46$, $P = 0.13$) (Figure 5). There were no significant differences in dopamine levels between TCG and CAT genotypes ($F_{1,20} = 0.37$, $P = 0.54$) (Figure 5).

Discussion

PARASITOID COMMUNITY COMPOSITION AND THE EVOLUTION OF NEGATIVE GENETIC CORRELATIONS AMONG LARVAL TRAITS

We found that larval defense traits exhibit negative genetic correlations when confronted with two different species of parasitoids, *Aphaereta sp.* and *L. boulardi*. Genotypes that have higher melanotic encapsulation abilities against the eggs of *Aphaereta sp.* face a higher risk of parasitism by *L. boulardi*. Our results provide support for the predictions of Futuyma and Mitter (1996) that negative correlations between defense traits are expected if different trait values are favored against different potential attackers. However, our results are more complicated than the simplest version of their hypothesis. The most straightforward interpretation is that the tradeoff would involve a single trait. In the context of our work we might have expected that genotypes that were better able to avoid attack by one species of parasitoid would be more vulnerable to attack by a different parasitoid (if each species used a different strategy to locate hosts). Instead what we saw was a trade-off between two different types of defense traits, one immunological (melanotic encapsulation ability) and the other non-immunological (presumably larval behavior). This more complicated scenario of the Futuyma and Mitter hypothesis is expected when traits are connected by pleiotropic loci whose allelic effects produce antagonistic relationships between the traits which is the pattern we observed here.

In our experiment, we found that the genotypes that were more active foragers (those with the TCG *Ddc* alleles) were better able to avoid parasitism by *L. boulardi* (which uses mainly ovipositor searching), than the less active foragers (those homozygous for the CAT *Ddc* polymorphism). These results match the findings of earlier work on the *foraging* gene (*for*), showing that less active larvae suffer higher rates of parasitism from *L. boulardi* (Carton and Sokolowski 1992). In that study, natural polymorphism in the *foraging* (*for*) gene in Drosophila produces differences in larval behavior (Sokolowski 1980) and these differences affect the probability of parasitism by different species of parasitoids, depending on the search strategy that parasitoids use to find a host. A potential explanation for our results is that more active larvae are either harder to locate, harder to successfully parasitize, and/or require longer handling times and incur higher energy costs for successful parasitism. Thus, all else being equal, this would reduce the number of larvae that can be parasitized in a given amount of time. Disentangling these mechanisms is a promising area for future work.

We should note that the *Ddc* and *for* genes are on the same arm of the second chromosome (2L) but are over 1Mb away from each other, so SNPs in these genes are not likely to be in linkage disequilibrium. However, because the polymorphism(s) in the *foraging* gene that contribute to the rover-sitter phenotype are not known (Marla Sokolowski pers. comm.) we cannot test this hypothesis.

In our experiment, larvae that were homozygous for the TCG *Ddc* polymorphism moved more while foraging but were not parasitized at a higher rate by *Aphaereta,* a genus characterized as a vibrotaxis searcher (Vet and van Alphen 1985). These results seem at odds with the relationship between larval foraging behavior and searching behavior of parasitoids established in the studies of the *for* gene. In previous studies of the rover-sitter phenotypes of the *for* gene, larvae with the rover phenotype faced a higher risk of parasitism from parasitoids that use vibrotaxis searching to locate their hosts (*Ganaspis xanthopoda*; *Asobara tabida*) (Sokolowski and Turlings 1987, Kraaijeveld and van Alphen 1995, Hughes and Sokolowski 1996). While behavioral observations indicate that the species of *Aphaereta* used in our experiment uses vibrotaxis, it is possible that this species uses additional cues to locate hosts (e.g., kairomones: Vet et al. 1993).

We also found that larvae that were more active foragers were less able to encapsulate the eggs of *Aphaereta sp.* This result also differs from the positive correlation between larval *Drosophila* foraging behavior and melanotic encapsulation ability found in a study of the rover and sitter phenotypes of the *for* gene (Hughes and Sokolowski 1996). Hughes and Sokolowski (1996) found that rovers were better able to melanotically encapsulate the eggs of *Asobara tabida* than sitters. However, the phenotypic association between these traits is not always observed (Green et al. 2000) and the ability of *Drosophila* to encapsulate eggs of parasitoids varies among parasitoid species (reviewed in (Kraaijeveld and Godfray 2009). Considering this and the fact that encapsulation ability and foraging behavior are likely to be polygenic traits, potentially influenced by pleiotropic and non-pleiotropic loci, we need more detailed information on the genetic basis of variation in these traits to resolve the influence of pleiotropy on their evolution.

In our study larvae with the CAT allele are better at melanotically encapsulating the eggs of *Aphaereta sp.* but have lower feeding rates than larvae homozygous for the TCG allele. This result is consistent with earlier work (Fellowes et al. 1999b) and so supports a trade-off between melanotic encapsulation ability against parasitoids and larval competitive ability.

Pleiotropic Effects of Natural Polymorphism in *Ddc?*

We found evidence that *Ddc* is a pleiotropic locus that contributes to tradeoffs among larval fitness traits. Three independent studies have identified *Ddc* as a locus influencing the melanotic encapsulation defense against parasitoids (Nappi et al. 1992, Orr and Irving 1997, Schlenke et al. 2007) although the QTL region identified by Orr and Irving was later fine mapped to a small region with large effect on this trait that did not contain *Ddc* (Hita et al. 1999). In addition, in our study genotypes with alternative haplotypes at the SNP locus exhibit differences in dopamine levels that match the patterns of melanotic encapsulation abilities. These independent lines of evidence provide further support for the hypothesis of antagonistic pleiotropy influenced by *Ddc.*

An important caveat to this conclusion is that *Ddc* is surrounded by a dense cluster of 17 other functionally-related genes with many of the genes, such as *Catsup*, *Dox-A2*, and *amd*, involved in catecholamine metabolism (Stathakis et al. 1995, Wright 1996, Stathakis et al. 1999). It is possible that the *Ddc* polymorphism is linked to polymorphisms in a nearby gene that produce the phenotypic variation observed in our experiments. Further studies are needed to validate the effects of the SNPs in *Ddc* that we observed on the phenotypes evaluated in this study.

Antagonistic Pleiotropy and the Maintenance of Variation at *Ddc*

The potential antagonistic pleiotropic effects of polymorphism at *Ddc* have important implications for the evolution of these traits in natural populations of *D. melanogaster.* Could the antagonistic effects that we measured be enough to maintain variation at *Ddc*? Based on the models of Curtsinger et al. 1994 and Hedrick 1999, it is not likely. Our experimental design allowed us to evaluate evidence for two hypotheses that could contribute to the maintenance of genetic variation, heterozygote advantage and antagonistic pleiotropy. While antagonistic pleiotropy itself can maintain polymorphism, the conditions under which it does so are restrictive (Curtsinger et al. 1994, Hedrick 1999, Van Dooren 2006). One condition that facilitates the maintenance of polymorphism by antagonistic pleiotropy is a beneficial reversal of dominance. In this case, when two alleles segregate at a locus, the allele that confers a trait value with higher fitness is dominant, with both alleles exhibiting dominance when it confers higher fitness for different traits. We found no evidence for a beneficial reversal of dominance or consistent heterozygote advantage across the traits. Of course, given the central importance of this gene in the production of neural

transmitters and its influence on pigmentation (Hodgetts and O'Keefe 2006), a number of other traits could be affected by the polymorphism in *Ddc*. If so, the dominance relationships of these haplotypes on other affected phenotypes would need to be measured. This information, combined with measurements of the relative contribution of these traits to fitness, would provide a more thorough test of the role of antagonistic pleiotropy in maintaining variation at *Ddc*.

If antagonistic pleiotropy is not important for maintaining variation, what is? To recap, flies with the TCG genotype have the lowest dopamine levels and the lowest encapsulation rates but have the highest feeding rates, move more while foraging and have the best ability to avoid attack by the species of wasp (*L. boulardi*) that attacks at the highest rate and is the most lethal (summarized in Table 1). One potential explanation that offers a promising avenue for further work is that the community composition of parasitoids is known to differ geographically and temporally (Fleury et al. 2009). Geographic variation in the community composition of parasitoids selecting against alternative traits combined with gene flow among populations could be an important contributor to variation in these traits (Levene 1953, Dempster 1955). To the best of our knowledge there is no published information on geographic variation in parasitoid community composition along the eastern coast of the U.S. Such data would be useful in this context. We do have five years of field data from our two local sites in Maryland that suggest regular seasonal changes in parasitoid community composition. *Aphaereta sp.* are common early in the breeding season but not later in the summer, when parasitoids in the genus Leptopilina are dominant (Hodges, unpublished data). Therefore, regular temporal changes in selection by parasitoids may help preserve genetic variation in these traits (Ellner and Hairston 1994, Ellner and Sasaki 1996). Our field data also suggest that fly density increases as the summer breeding season progresses. This provides the potential for variation in the strength and direction of selection for density dependent competitive ability. Detailed information on the role of geographic and temporal variation of parasitoid community composition on larval trait evolution is needed. This, combined with data on geographic and temporal variation in density dependent selection on larval traits in Drosophila are needed to reveal the relative role of these ecological factors on the evolution and maintenance of variation in larval fitness traits.

Acknowledgments

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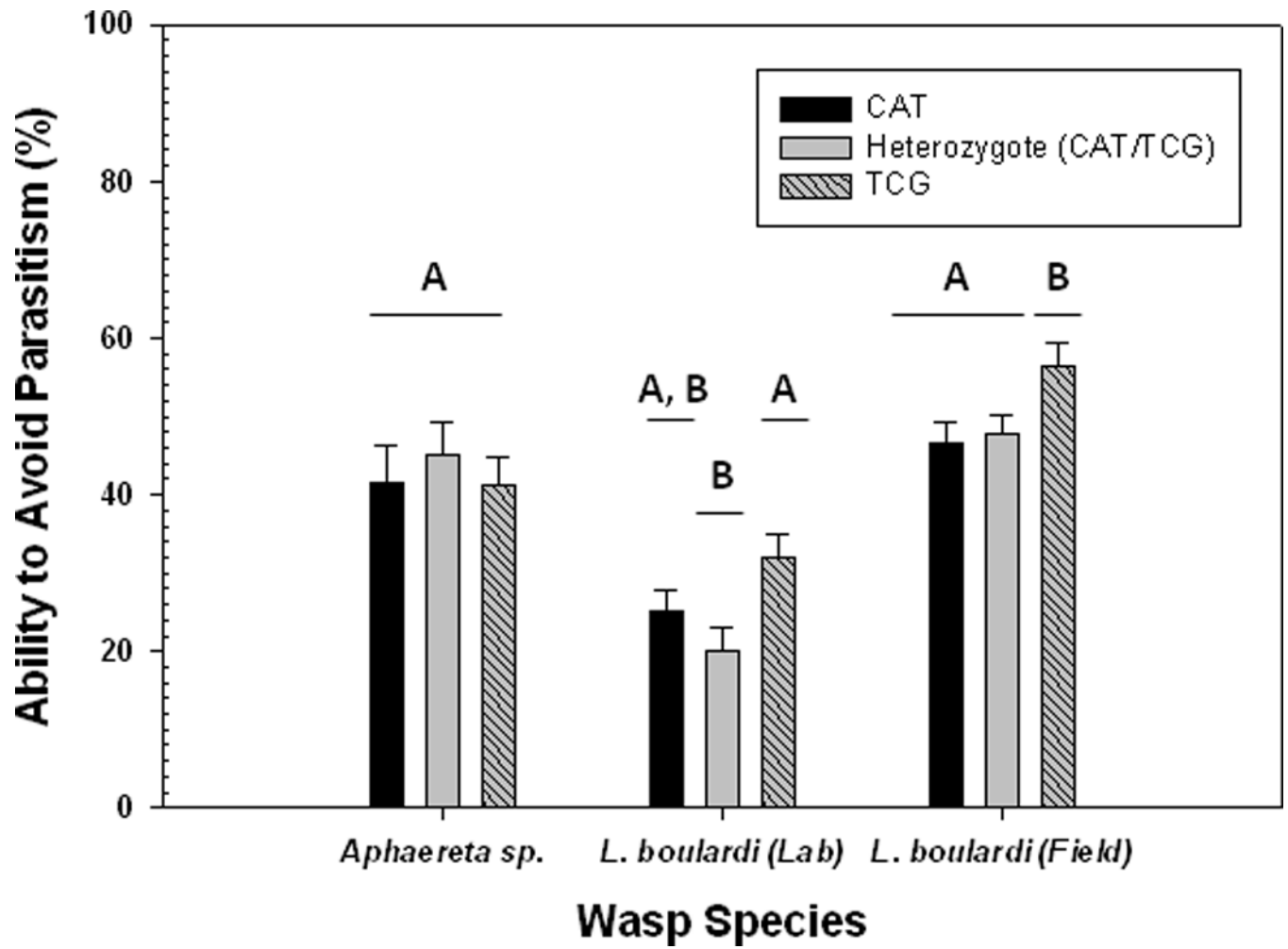
Figure 1.
Ability to avoid parasitism by *Aphaereta sp., L. boulardi* (lab), and *L. boulardi (field)* for different *Ddc* genotypes. Shown are mean values (± 1 s.e.m.). Different uppercase letters indicate significant differences between *Ddc* genotypes.
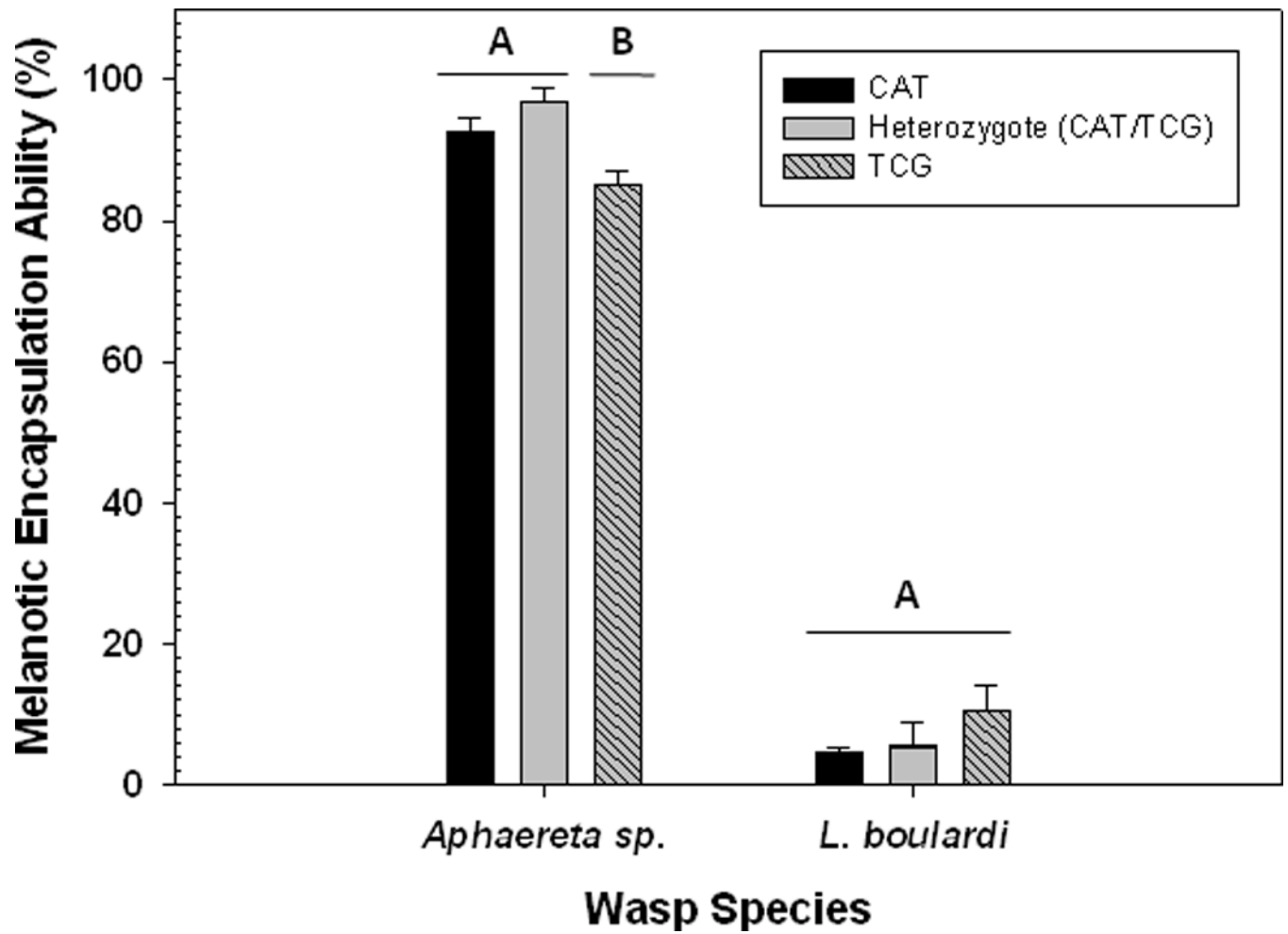
Figure 2.
Melanotic encapsulation abilities of 2nd instar larvae with different *Ddc* genotypes against
Aphaereta sp. and *L. boulardi*. Shown are mean values (± 1 s.e.m.). Different uppercase
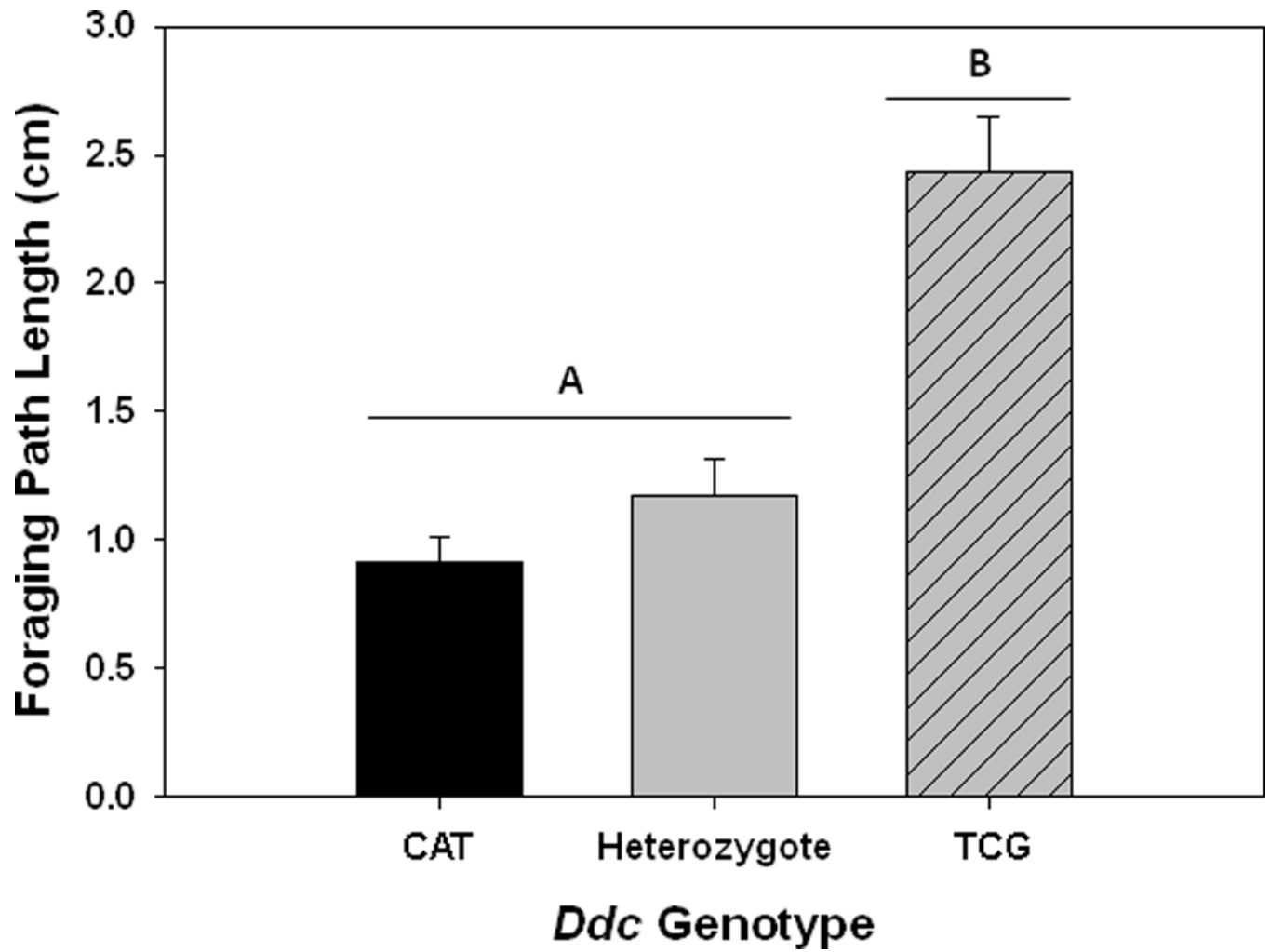letters indicate significant differences between *Ddc* genotypes.

Figure 3.
Foraging path lengths of 2nd instar larvae for different *Ddc* genotypes. Shown are mean values (± 1 s.e.m.). Different uppercase letters indicate significant differences between *Ddc* genotypes.
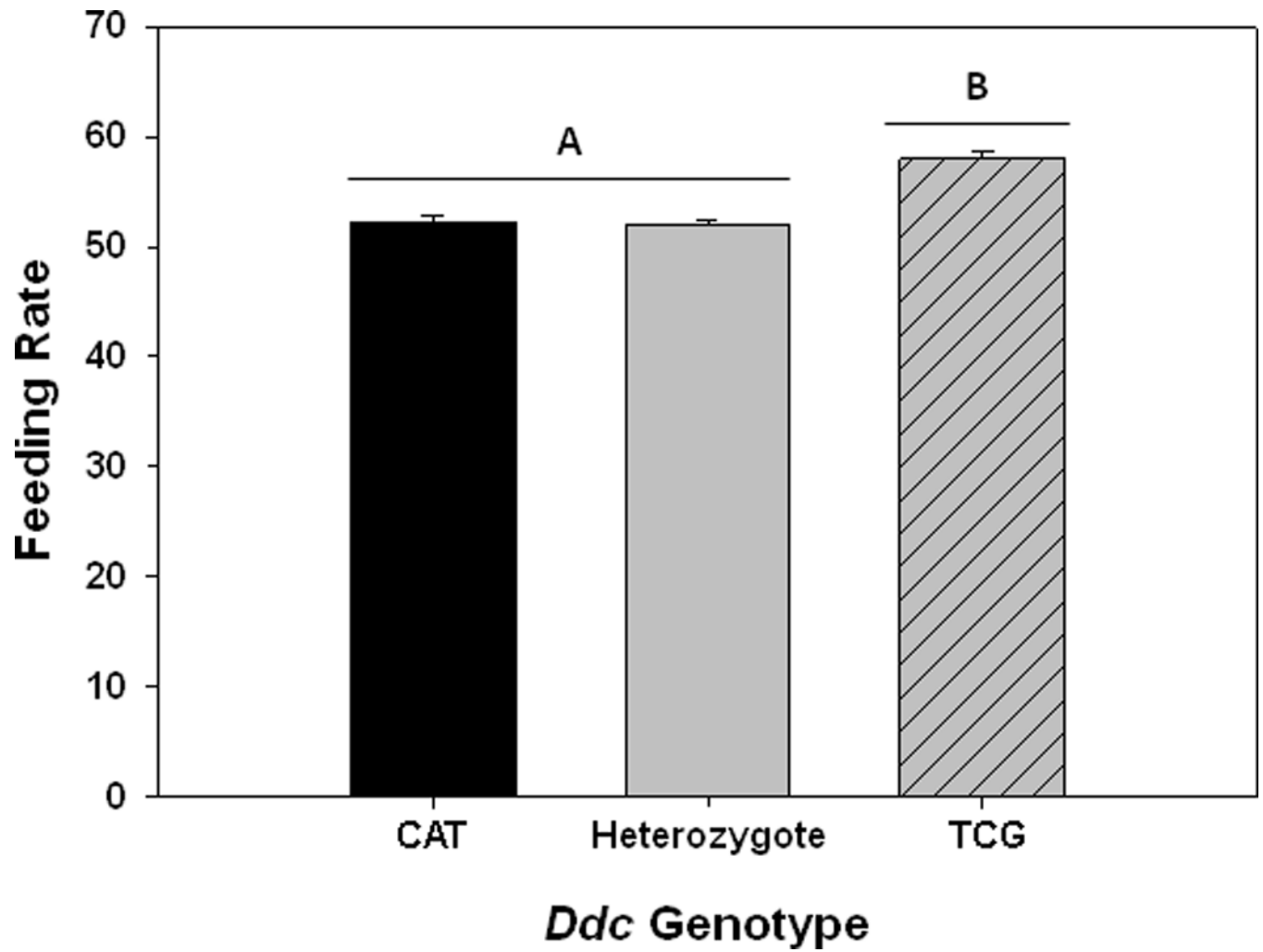
Figure 4.
Feeding rates of 2nd instar larvae for different *Ddc* genotypes. Shown are mean values (± 1 s.e.m.) of the number of cephalopharyngeal retractions in a 30 second period. Different uppercase letters indicate significant differences between *Ddc* genotypes.
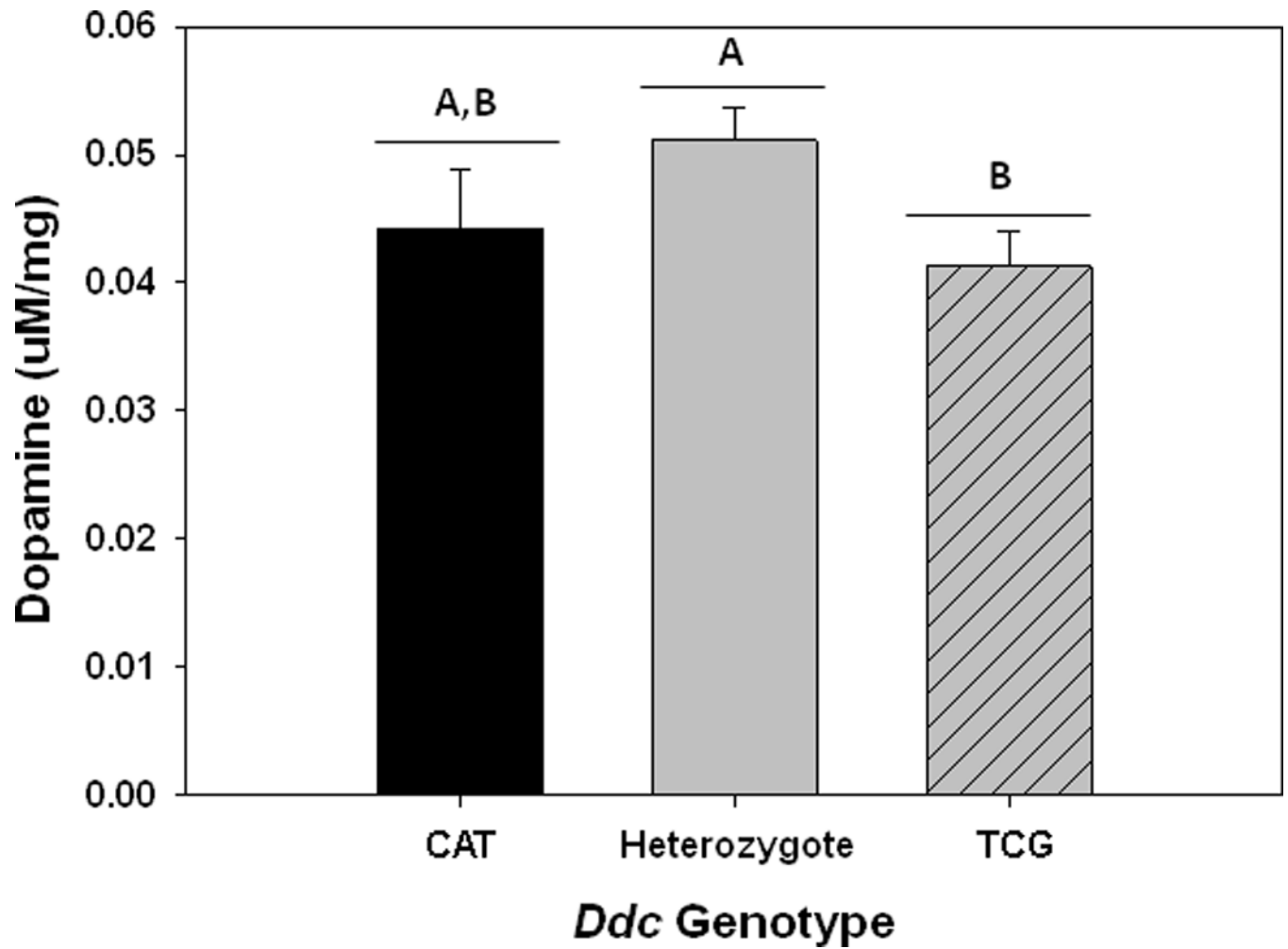
Figure 5.
Dopamine levels (uM/mg) of 2nd instar larvae for different *Ddc* genotypes. Shown are mean values (± 1 s.e.m.). Different uppercase letters indicate significant differences between *Ddc* genotypes.

Table 1

Relative differences among *Ddc* genotypes for fitness traits assayed as well as life span measured by De Luca et al. 2003.

Trait	*Ddc* Genotype		
	CAT/CAT	**Heterozygote**	**TCG/TCG**
Life span	Longer	Longer Not measured	Shorter
Immune response against *Aphaereta sp.*	Higher	Higher	Lower
Immune response against *L. boulardi*	NS	NS	NS
Ability to avoid parasitism by *Aphaereta sp.*	NS	NS	NS
Ability to avoid parasitism by *L. boulardi* (L: Lab, F: Field)	L: NS F: Lower	L: Lower F: Lower	L: Higher F: Higher
Distance move while foraging	Shorter	Shorter	Longer
Feeding rate	Lower	Lower	Higher
Dopamine levels	NS	Higher	Lower

Boxes with NS indicates no significant difference with the other *Ddc* genotypes