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Potential life-history costs of parasitoid avoidance in *Drosophila melanogaster*

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ABSTRACT

Pupal parasitoids are a common natural enemy of *Drosophila*. As *Drosophila* pupae do not have an immunological defence against pupal parasitoids, they have to avoid being attacked. As a first step to identifying the costs of avoidance of parasitism by pupal parasitoids, we explored three traits that potentially influence the probability of *D. melanogaster* pupae to survive attack by *Pachycrepoideus vindemiae*. We found that larvae pupating on the food source had a higher probability of avoiding parasitism, but that the distance that larvae pupate away from the food had no effect on survival probability when exposed to pupal parasitoids. We also found no indication that the thickness of the puparial wall influences risk of parasitism. Pupal size, however, was correlated with the probability of surviving parasitoid attack, with smaller pupae having a higher survival probability. If pupal size is indeed the key trait influencing risk of parasitism of *D. melanogaster* pupae by *P. vindemiae*, the potential life-history costs of parasitoid avoidance are smaller adult size, leading to lower general fitness.

Keywords: cost of resistance, *Drosophila*, life-history evolution, *Pachycrepoideus*, pupal parasitoid, trade-offs.

INTRODUCTION

Parasitoids are common natural enemies of many insect species. As by definition their successful attacks are invariably fatal, the host is expected to be under strong selection pressure to evolve means of defending itself. When the parasitoid larva feeds internally in the still-living host (a koinobiont endoparasitoid), the most common form of defence is through the insect's cellular immune system, which causes the encapsulation and death of the parasitoid egg or larva (Salt, 1968; Vinson, 1990; Strand and Pech, 1995). However, this defence is not generally available to hosts that are attacked by ectoparasitoids which feed externally (Godfray, 1994; Quicke, 1997; but see Wilson *et al.*, 2001, for a case of an immune reaction to an ectoparasitoid) and often kill or paralyse their host at oviposition (idiobionts). In these cases, defence must involve avoidance of parasitism either by escaping detection or through preventing oviposition.

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Selection for defence will be tempered by any trade-offs between resistance and other components of the host's fitness. Evidence for the existence of costs of defence in insects against their parasites and parasitoids is mounting (reviewed in Kraaijeveld *et al.*, 2002; Schmid-Hempel and Ebert, 2003). *Drosophila melanogaster* and its larval parasitoids have been used extensively as a model system to investigate the evolution of host resistance against parasitoids (Kraaijeveld *et al.*, 1998; Fellowes and Godfray, 2000). Larval parasitoids of *Drosophila* belong to the families Braconidae and Figitidae (= Eucoilidae) and are all endoparasitoids (Carton *et al.*, 1986). When replicate populations of hosts are exposed to high levels of parasitism over several generations, they evolve higher cellular immunity, but at the cost of reduced larval competitive ability (Kraaijeveld and Godfray, 1997; Fellowes *et al.*, 1998a).

Pupae of *Drosophila* are attacked by several parasitoids in the families Pteromalidae and Diapriidae, of which the pteromalid *Pachycrepoideus vindemiae* is the most common (Nøstvik, 1954; van Alphen and Thunnissen, 1983; Carton *et al.*, 1986). Pteromalid parasitoids of *Drosophila* lay their eggs inside the host's puparium, but outside the actual pupa, and hence are ectoparasitoids. There has been some study of how housefly pupae may defend themselves against parasitoids (see Discussion), although avoidance and resistance mechanisms in *Drosophila* pupae have received very little attention. Melanization of pupal parasitoid eggs has never been reported and once a pupa has been parasitized, it rarely survives to become an adult fly (van Alphen and Thunnissen, 1983). This strongly suggests that *Drosophila* pupae have no effective resistance mechanism once oviposition has taken place. Work by Sokolowski and co-workers (Sokolowski *et al.*, 1986; Rodriguez *et al.*, 1991, 1992) has shown that there is variation within and between natural populations of *D. melanogaster* in the pupation site of the larvae, in particular whether pupation took place on or away from the fruit. This polymorphism in pupation site choice is strongly linked to a polymorphism in larval foraging behaviour (Sokolowski, 1980; de Belle and Sokolowski, 1987; Osborne *et al.*, 1997). 'Rovers' move around while feeding and pupate away from the fruit, while 'sitters' stay in one place while feeding and pupate on the fruit. Although this polymorphism is usually linked to heterogeneity in food availability, soil humidity and parasitism by larval parasitoids (Graf and Sokolowski, 1989; Rodriguez *et al.*, 1991; Carton and Sokolowksi, 1992), it is possible that parasitism by pupal parasitoids plays an additional role.

Our long-term aims are to identify avoidance or resistance mechanisms against pupal parasitoids in *Drosophila*, and look for trade-offs and costs associated with a non-immunological defence mechanism. As a first step towards this goal, we identify three possible traits that may influence survival of pupae under attack by *P. vindemiae*. Because trade-offs are often only revealed when resources are limiting, we investigate these traits in two different circumstances: when the larvae have abundant or limited food. We investigate whether there is phenotypic variation in these traits, and whether this is correlated with the probability of surviving parasitism. With the proviso that we are exploring these issues in a laboratory setting, and that we are looking at phenotypic rather than genotypic variation, such correlations are a prerequisite for avoidance or resistance to be selected. The three traits and their expected association with surviving parasitoid attack are:

1. *Pupation site*: larvae pupating further away from the larval food source may have a lower probability of being found by a searching parasitoid female (in the field, parasitoid females are attracted to the larval food source; personal observation).

2. *Pupal size*: smaller pupae may have a lower probability of being attacked, either because they are less likely to be encountered or are actively rejected by the wasp (many parasitoid species, when offered a range of host sizes, prefer larger hosts; Godfray, 1994).
3. *Puparium thickness*: as the parasitoid female has to drill through the puparial wall, a thicker puparial wall may deter oviposition.

MATERIALS AND METHODS

Insects

The *D. melanogaster* strain used in the cultures and experiments was originally collected near Lyon, France, and had been kept as an outbred population in the laboratory for 7 years. The flies were cultured in a $30 \times 30 \times 30$ cm perspex cage in 150 ml bottles containing yeast/sugar medium and live baker's yeast as food and oviposition substrate; the cage and bottles were kept at 20 ± 1°C and in a 16 : 8 light–dark regime.

The *P. vindemiae* were collected at Silwood Park, Berkshire, England, and had been in laboratory culture for over 10 years. For culturing, parasitoid females were added to bottles from the *D. melanogaster* culture which contained 1-day-old pupae (5 females per bottle). The bottles were incubated at 25 ± 0.5°C and in a 16 : 8 light–dark regime. Emerging parasitoids were kept in jars with an agar layer and honey as food, at 20 ± 0.5°C and in a 16 : 8 light–dark regime, until required for culturing or experimentation. Parasitoid females were always used when less than 1 week old.

Pupation site/pupal size experiment

In the first experiment, we allowed larvae to chose their pupation site in a laboratory setting that was designed to mimic aspects of the natural environment. We exposed pupae to parasitoids and tested whether the probability of surviving parasitism was correlated with pupation site and pupal size. We performed the experiments under two different competition regimes: with abundant and limited food for the larvae. In each replicate of the first (low competition) treatment, we filled four large Petri dishes (14 cm diameter) with wet vermiculite. In the centre of each of these dishes, we placed a 1.5 cm thick slice of banana (leaving the peel in place), which had been soaked in a dilute suspension of live baker's yeast for 1 h before the experiment. We put 100 second-instar larvae on each banana slice and placed the four dishes in a $30 \times 30 \times 30$ cm perspex cage inside an incubator (25 ± 0.5°C and a 16 : 8 light–dark regime). Using such a set-up, rather than artificially manipulating pupal distance, the larvae were allowed to pupate at a site of their choice and therefore created a more natural distribution of pupation distances. Two days later, we released 10 parasitoid females in the cage. When the developing adult flies were fully sclerotized and about to emerge from the pupae (usually 4 days after parasitoid release), we carefully sorted through the Petri dishes and separated the fly pupae in each Petri dish into three categories: pupae that were in contact with what remained of the banana slice ('on'), pupae not touching the banana and not further than 5 cm from the centre of the dish ('near'), and pupae that were more than 5 cm away from the centre of the dish ('far'). Pupae from each of these three concentric areas were incubated in separate vials with a layer of agar at 25 ± 0.5°C and in a 16 : 8 light–dark regime (giving a maximum of 12 vials per replicate).

The second (high competition) treatment of the first experiment was similar, except that we used one-quarter of the banana slice per Petri dish. In preliminary trials, this amount had been shown to result in considerable competition. As larvae grow slower under competition, we released parasitoids in the cage after 5 (instead of 2) days and again separated the pupae just before the onset of fly emergence. Five weeks after the replicate was set up, we checked all the pupae and scored whether a fly or parasitoid had emerged (a fly emerges through the hinged operculum, leaving the puparium intact, whereas a parasitoid gnaws a large emergence hole in the puparial wall) or whether nothing had emerged from the pupa (in which case it was scored as dead). We then measured the length (not including the pupal horns and anal papillae) and maximum width of each pupa with an ocular micrometer at 20× magnification and calculated its volume, assuming a pupa to be an ellipsoid. As some pupae could not be detached from the vermiculite without damaging them, a small number could not be measured. Each of the two treatments was replicated 15 times, and for each replicate we pooled the results of the four dishes within the cage. Data for dishes from which no parasitoid emerged were discarded, as it was assumed that these dishes had not been visited by a parasitoid female.

Puparium thickness experiment

The second experiment was designed to test the effect of puparium thickness. We continuously observed parasitoid females as they searched in a Petri dish (9 cm diameter) containing twenty 2-day-old pupae, which were randomly picked from the fly culture. When a female investigated a pupa with her antennae and walked away from it without attempting to oviposit, the pupa was taken from the dish and scored as 'rejected for oviposition'. Pupae in which a female attempted to oviposit but failed to drill through the puparial wall were taken from the dish and scored as 'accepted for oviposition – drill failure'. Finally, when a female's ovipositor did break through the puparial wall, she was immediately removed from the pupa with a paint brush and the pupa was removed from the dish and scored as 'accepted for oviposition'. We observed 15 females and their encounters with each of 10 pupae. Pupae in the three categories were incubated in three separate vials per female with a layer of agar at 20 ± 0.5°C and in a 16 : 8 light–dark regime. After emergence of the adult fly, we dried the empty pupae in air for several days, weighed them on an ultramicrobalance and subsequently measured their length and maximum width as described above. Not all pupae gave rise to an adult fly, reducing the final sample size to 102. We divided weight by surface area (again assuming pupae to be ellipsoid), a method that has been used previously (Fellowes *et al.*, 1998b; Morris and Fellowes, 2002). The resulting measure, hereafter referred to as 'puparium thickness', captures both the thickness of the puparial wall and its denseness, both of which may influence the ease with which females can drill through the puparial wall. Preliminary trials had shown that removing the parasitoid immediately after the ovipositor broke through the puparial wall never resulted in parasitism, and that the emergence rate of adult flies from those pupae was similar to that of unattacked pupae.

To analyse the data, we used general linear modelling techniques with binary response variables. In the pupation site/pupal size experiment, we included 'cage' (with 15 levels), 'dish' (with 4 levels) and 'site' (with 3 levels) as factors and fitted 'dish' nested within 'cage' before exploring the effects of pupation site and pupal size. In the puparium thickness experiment, we included 'female' (with 15 levels) as a factor.

RESULTS

Pupation site/pupal size experiment

In the treatment with low competition, 19.6% of the larvae died before pupating. Of the larvae that made it to pupation, 58.7, 37.5 and 3.8% were found in the 'on', 'near' and 'far' sections, respectively; pupal mortality (i.e. pupae from which no insect emerged) was 22.9%. In the series with high competition, larval mortality was 61.5% and the percentages of pupae in the 'on', 'near' and 'far' categories were 75.6, 20.2 and 4.1%, respectively; pupal mortality was 61.6%.

Because the response variable is in fact trinomial (i.e. each pupa gives rise to a fly, a parasitoid or no insect), we first analysed the effect of pupation site on the probability that an insect (fly or parasitoid) emerged. In the low competition treatment, pupation site was not significant after fitting 'dish' within 'cage' ($n = 3848$, change in deviance = 0.5 with 2 degrees of freedom, $P = 0.5$; upper left panel in Fig. 1). In the high competition treatment, pupation site was highly significant ($n = 1278$, change in deviance = 33.4 with 2 degrees of freedom, $P < 0.00001$; lower left panel in Fig. 1), a result of the lack of any adult insects emerging from the 'far' category. Next, we analysed the effect of pupation site on the probability that a fly or parasitoid emerged. In the low competition treatment, pupation site

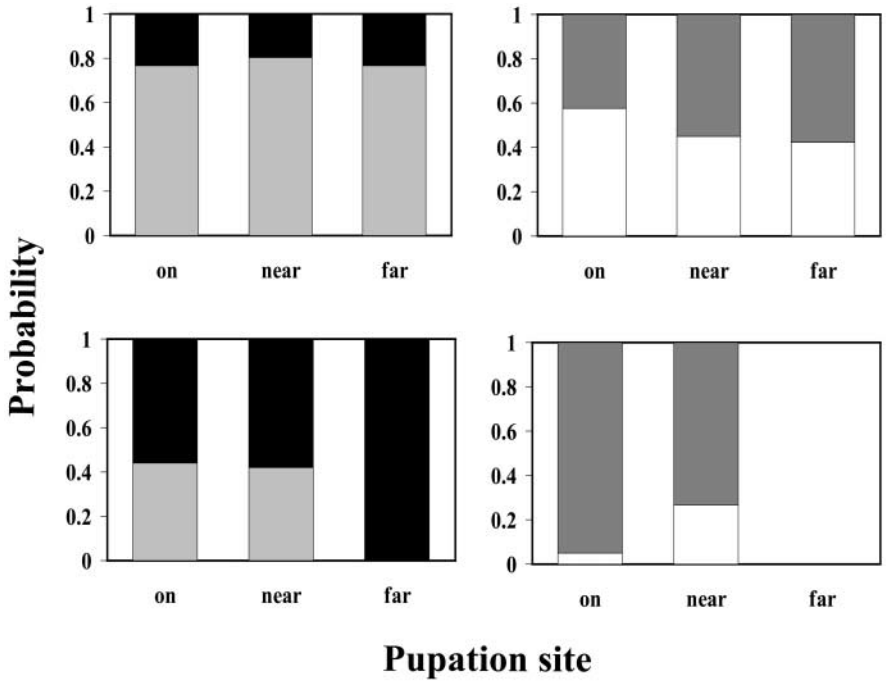

Fig. 1. Effect of pupation site on the probability of fly or parasitoid emergence. Left-hand panels show the effect of size on the probability of survival of an adult insect (light grey; black = pupae from which no insect emerged). Right-hand panels show the probability of a fly (white) or a parasitoid (dark grey) emerging. The upper panels show the results of the 'low competition' treatment, the lower panels those of the 'high competition' treatment. See text for further details.

was highly significant ($n = 3001$, change in deviance = 23.7 with 2 degrees of freedom, $P < 0.00001$; upper right panel in Fig. 1). Further model simplification showed no difference in fly survival between the 'near' and 'far' category (change in deviance = 1.0 with 1 degree of freedom, $P = 0.3$). In the high competition treatment, there was no significant effect of pupation site ($n = 541$, change in deviance = 0.05 with 1 degree of freedom, $P = 0.83$; lower right panel in Fig. 1).

Analysis of the effect of pupal size was made on a subset of the previous data set, as not all pupae could be measured accurately. We visualized the effect of pupal size on survival probability by performing logistic regressions of the fate of the pupa on its size (Fig. 2). As before, we first analysed the effect of size on the probability that an adult insect (fly or parasitoid) emerged (left half of Fig. 2). The upper half of Table 1 gives the results of these analyses (after fitting 'dish' nested within 'cage' and fitting the effect of pupation site before assessing the effect of pupal size; not surprisingly, the effect of pupation site on survival probabilities was very similar to that found previously). Pupal size has a significant effect on survival probability, with larger pupae more likely to give rise to an adult insect both in the 'low competition' and 'high competition' treatment. The effect is the same in the three distance categories. Subsequently, we analysed the effect of pupal size on the probability of avoiding parasitism in the same way as described above (right half of Fig. 2 and lower half of Table 1). Smaller pupae have a lower probability of being parasitized in the 'low

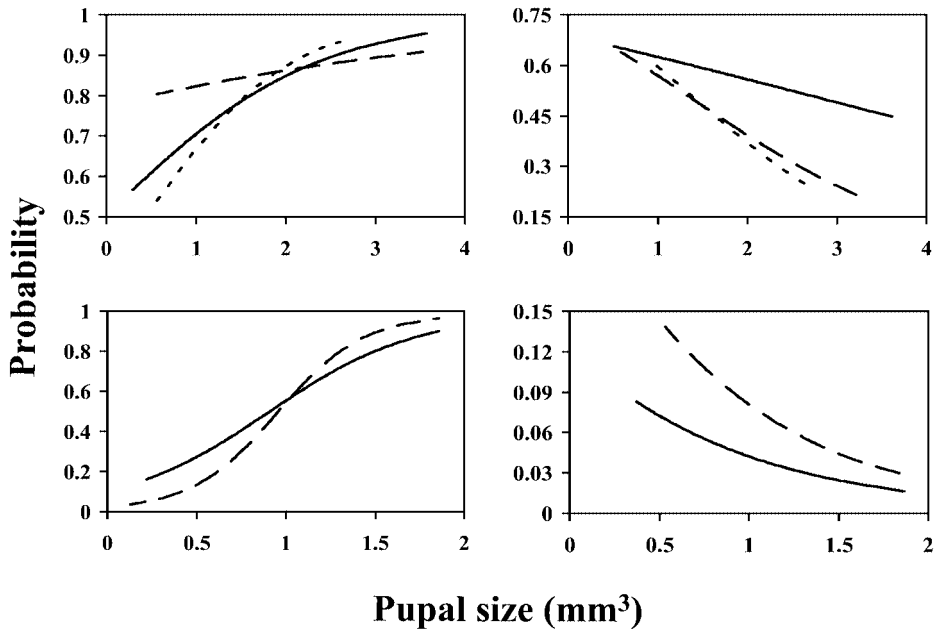

Fig. 2. Results of logistic regressions of the fate of a pupa on its size. Left-hand panels show the effect of size on the probability of survival of an adult insect; right-hand panels show the effect of size on probability of avoiding parasitism. The upper panels show the results of the 'low competition' treatment, the lower panels those of the 'high competition' treatment. Solid lines are for pupae 'on' the food, dashed lines for pupae 'near' the food and dotted lines for pupae 'far' from the food. See text for further details.

Table 1. Results of GLIM analyses on the effect of pupal size on survival probability (see text for further details)

	Change in deviance	d.f.	*P*-value
Comparison of live and dead pupae			
Low competition treatment ($n = 3591$)			
Pupation site	3.34	2	0.19
Pupal size	8.58	1	0.0034
Site × size interaction	2.77	2	0.25
High competition treatment ($n = 1053$)			
Pupation site	13.70	2	0.001
Pupal size	140.34	1	> 0.00001
Site × size interaction	0.01	2	0.99
Comparison of parasitoid and fly pupae			
Low competition treatment ($n = 2912$)			
Pupation site	22.06	2	> 0.00005
Pupal size	8.72	1	0.0032
Site × size interaction	0.63	2	0.73
High competition treatment ($n = 487$)			
Pupation site	0.15	2	0.70
Pupal size	0.60	1	0.44
Site × size interaction	0.61	2	0.74

competition' treatment; further model simplification showed that the relationship for the pupae 'on' the food is different from that in the 'near' or 'far' category (change in deviance = 4.67 with 1 degree of freedom, $P = 0.03$). In the 'high competition' treatment, the trend is in the same direction, but far from significant. Again, any effect does not depend on distance from the banana slice.

Puparium thickness experiment

In total, 59.2% of the encountered pupae were rejected, whereas 40.8% were accepted and would have been parasitized had we not prevented oviposition (in only three pupae did the female fail to drill through after accepting the pupae for oviposition). Females differ in their acceptance behaviour as fitting female as a factor first significantly reduced deviance (change in deviance = 37.04 with 14 degrees of freedom, $P = 0.001$). Subsequently, no effect of puparium thickness was detected (change in deviance = 0.43 with 1 degree of freedom, $P = 0.51$) and there was no significant interaction between female and puparium thickness (change in deviance = 7.55 with 14 degrees of freedom, $P = 0.91$).

DISCUSSION

As a first step in our goal of determining whether there are trade-offs and costs associated with a non-immunological defence mechanism, we explored how three traits – pupation site, pupal size and puparium thickness – may be correlated with the probability of survival of

D. melanogaster after exposure to searching *P. vindemiae* females. We found that larvae pupating on the food source had a higher probability of avoiding parasitism than larvae pupating away from the food source, but that distance from the food did not influence survival probability. The probability that an adult insect emerges from a pupa is positively correlated with pupal size, a well-known phenomenon in *D. melanogaster* (Bakker, 1959, 1961). More importantly, pupal size was associated with differences in the probability of being attacked, with larger individuals more likely to be parasitized. This effect disappeared when larval food was limited, although the trend was in the same direction. Whether larger pupae have a higher probability of being parasitized because they are more likely to be found or as a result of active preference by searching females is not clear.

Location is known to influence risks of parasitism in other systems, for example in aphids attacked by secondary parasitoids (Brodeur and McNeil, 1992; Müller *et al.*, 1997). In *Drosophila*, Carton and Sokolowski (1994) showed that *D. melanogaster* pupae embedded in agar substrate had a higher probability of being parasitized by *P. vindemiae* than pupae exposed on the surface. Here we found indications for an opposite effect, with the more exposed pupae in the vermiculite having a lower survival probability than those on the banana slice. Presumably, this is due to many of the pupae on the banana being hidden in cracks between the fruit and the peel or under the slice. Also, the higher probability of survival for pupae with the same size on the banana slice compared with those in the vermiculite is probably also caused by this difference in degree of exposure.

The lack of an association between puparium thickness and probability of surviving parasitism is perhaps less surprising, as *P. vindemiae* attacks a wide range of cyclorrhaphous dipterans (Nøstvik, 1954), some of which are much larger than *D. melanogaster* and almost certainly have much thicker puparial walls. Morris and Fellowes (2002) found no difference in the handling time of *P. vindemiae* females attacking *D. melanogaster* and the much larger *Musca domestica* pupae, even though the thickness of the puparial wall of *M. domestica* pupae is about three times that of *D. melanogaster* pupae. If there are costs in time and energy in drilling through larger, thicker puparia, the wasp may be compensated by the better quality of the host.

Given that *D. melanogaster* is attacked by both larval and pupal parasitoids, a cost of resistance to one type of parasitoid may be increased vulnerability to the other. The few available data do not point to this being the case. Pupae from lines that were selected for increased resistance to the larval parasitoid *Asobara tabida* were as susceptible to the pupal parasitoid *P. vindemiae* as pupae from the unselected control lines (Green, 2000; Kraaijeveld *et al.*, 2002). Also, Delpuech *et al.* (1994) found no correlation among isofemale lines for resistance to larval and pupal parasitoids. However, Fellowes *et al.* (1998b) found that larvae which had successfully encapsulated an egg from a larval parasitoid were more likely to be attacked by a pupal parasitoid after pupation, showing that a cost of *actual* defence against larval parasitoids is increased susceptibility to pupal parasitoids.

The only major study of resistance to pupal parasitoids of which we are aware is that of Pimentel and colleagues on house flies (*M. domestica*) and the parasitoid *Nasonia vitripennis* (Pimentel and Al-Hafidh, 1965; Pimentel and Stone, 1968; Olson and Pimentel, 1974; Zareh *et al.*, 1980). Populations exposed to high rates of parasitism over several generations evolve heavier puparia and shorter pupal durations. Zareh *et al.* (1980) suggested that heavier puparia have stronger puparial walls, and that a shorter pupal period reduces exposure to parasitism. With regard to potential costs of resistance to pupal parasitoids, Zareh *et al.* (1980) observed that the house flies exposed to parasitism had lower fecundity. This

decreased fecundity could be an indirect result of the observed reduction in pupal period, if that reduction led to smaller flies. There is a large body of literature on artificial selection for a range of life-history traits in *D. melanogaster*, including development time, but no attempt has yet been made specifically to select for shorter or longer pupal periods. However, selection for faster overall development leads chiefly to a reduction in the larval period, but also to some shortening of the pupal period (Prasad *et al.*, 2001). Selection for faster development also results in smaller adult fly size (Zwaan *et al.*, 1995; Nunney, 1996; Chippendale *et al.*, 1997; Prasad *et al.*, 2001), whereas selection for slower development leads to an increase in body size (Zwaan *et al.*, 1995).

Might a population exposed to intense pupal parasitism by *P. vindemiae* evolve smaller pupal size? For this to occur there must be additive genetic variation in the trait, and the costs must not outweigh the benefits. Pupal and adult size are tightly (and positively) linked in *D. melanogaster* (Bakker, 1959, 1961, 1969) and it is possible to select for an increase or decrease in adult body size (Hillesheim and Stearns, 1992; Partridge and Fowler, 1993), showing that additive genetic variation for this trait exists. Adult size is positively correlated with fecundity, mating prowess, longevity and dispersal ability in *Drosophila* (Ewing, 1961; Hillesheim and Stearns, 1992; Partridge and Fowler, 1993; Nunney, 1996) and other insects (van den Assem *et al.*, 1989; Visser 1994; Via and Shaw, 1996; West *et al.*, 1996; Ellers *et al.*, 1998; Ryder and Siva-Jothy, 2000). Thus there may be major costs of reducing parasitism by a decrease in pupal size.

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