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Temperature-mediated microhabitat choice and development time based on the *pgm* locus in the yellow dung fly *Scathophaga stercoraria*

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Temperature can affect the performance of insects through its influence on enzyme function. We report a series of laboratory and field experiments investigating the putatively adaptive temperature-dependent effects of phosphoglucomutase (*pgm*) genotype on development time, a central life-history fitness component, as mediated by the microhabitat choice of ovipositing female yellow dung flies (*Scathophaga stercoraria*). The allozyme corresponding to the most common *pgm* allele 3 (approximate 90% natural frequency) had the highest Q_{10} and showed higher *in vitro* activity at warmer temperatures (17–27 °C), whereas the allozyme corresponding to allele 4 (4.5%) showed relatively higher activity at the lowest temperature (7 °C), and the allozyme corresponding to allele 2 (2.5%) showed lower activity throughout. A laboratory experiment revealed that egg development time for allele 3 was shortest (i.e. best) at all temperatures tested, although egg-to-adult development time was longest for offspring derived from field females bearing allele 3. Importantly, over 3 years in the field, allele 4 did not increase in frequency late in the season as the temperatures dropped. Although females augmented their proportion of eggs laid on the warmer south slope of a dung pat (adaptive response), the development times of their offspring consistently increased towards the end of the season (maladaptive response). Regardless, females did not systematically bias the *pgm* composition of eggs laid on the north versus south slopes, as was expected from previous work hypothesizing that females exert microhabitat choice based on the *pgm* locus. We conclude that, although PGM allozymes differ in activity and the *pgm* genotype does differentially affect development time, these effects are inconsistent, and not predictable from *in vitro* allozyme assays, and therefore difficult to reconcile with an adaptive framework of cryptic female choice. *Biological Journal of the Linnean Society*, 2012, **107**, 686–696.

ADDITIONAL KEYWORDS: allozymes – cryptic female choice – electrophoresis – enzyme activity – insect – natural selection – oviposition.

INTRODUCTION

Allozyme polymorphisms have been extensively used as putatively neutral markers in population genetic studies in the past. Nevertheless, it has long been appreciated that many of these enzymes are far from neutral because they serve important catalytic functions central to the metabolism of most organisms (Eanes, 1999). Indeed, the maintenance of polymorphisms at some allozyme loci has been successfully linked to divergent natural selection across environmental gradients (Watt, 1977; Eanes, 1999; Galindo *et al.*, 2009). Such studies lie at the heart of evolutionary biology because, ideally, a direct link can be

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made between a gene, its physiological function, and some fitness effect at the whole-organism life-history level in terms of, for example, growth, fecundity or survival under different environmental conditions (Watt, 1985). This is distinct from more complex but often functionally unclear fitness effects mediated by multilocus heterozygosity (Mitton & Grant, 1984; Teska, Smith & Novak, 1990).

Because of the general temperature dependence of all catalytic processes, temperature is the prime environmental variable exerting selection on the performance of terrestrial ectotherms via its influence on enzyme function (Huey & Kingsolver, 1989, 1993; Angilletta, 2009). For example, in their comprehensive studies of *Colias* butterflies, Watt and co-workers (Watt, 1977, 1985, 1992; Watt, Cassin & Swan, 1983) found that different phosphoglucose isomerase (PGI) genotypes vary in their metabolic efficiency and hence their flight capacity under different temperature conditions, ultimately affecting dispersal and survival. Such studies directly connect allozyme polymorphisms with adaptation to local climate (Watt, 1985; Verrelli & Eanes, 2001; Rank & Dahlhoff, 2002; Sezgin *et al*., 2004; Saastamoinen & Hanski, 2008). Most free-living organisms, from protists to vertebrates, encounter and tolerate a wide range of environmental conditions within and across populations. Behavioural, morphological, biochemical or physiological responses to fluctuations in temperature are therefore expected and common, and often have a genetic component, such that they can evolve in response to changes in environmental conditions (Huey & Kingsolver, 1989, 1993; Angilletta, 2009).

The yellow dung fly *Scathophaga stercoraria* (Diptera: Scathophagidae; sometimes *Scatophaga*), the classic model species for investigations of sperm competition and sexual selection (Simmons, 2001), inhabits temperate regions of the entire northern hemisphere, favouring cooler climates at high altitudes (e.g. the Swiss Alps) and high latitudes as far north as Iceland and Spitzbergen (Blanckenhorn *et al*., 2010). Hot temperatures appear to limit the distribution of flies in the south, where they occur only at higher elevations such as the Sierra Nevada in Spain or North America. Both adults and larvae are killed by temperatures exceeding 27 °C (Ward & Simmons, 1990; Blanckenhorn, 1998; Blanckenhorn *et al*., 2001). This fly therefore should show adaptations to avoid heat.

Phosphoglucomutase (enzyme: PGM; coding locus: *pgm*) is a highly polymorphic enzyme important in glycolysis. It catalyzes the internal transfer of a phosphate group from C-3 to C-2, converting 3-phosphoglycerate (3PG) to 2PG, and thus is central to the mobilization and storage of glucose into glycogen (Bailey, 1975; Sacktor, 1975; Eanes, 1999). Several lines of evidence indicate that *pgm* genotype affects yellow dung fly performance and is presumably under natural selection. *Pgm* genotypes have been shown to vary in larval growth and development depending on temperature and light conditions in the laboratory, producing adults of different sizes (Ward, 1998). Adult body size is a key fitness component conferring clear advantages to both males and females (Blanckenhorn, 1998; Jann, Blanckenhorn & Ward, 2000). Yellow dung fly eggs are also nonrandomly distributed with respect to *pgm* genotype in the field according to shading conditions (Ward *et al*., 2002), suggesting that females might place different genotypes into different microhabitats. Further, *pgm* genotypes vary systematically over the season, with the most common allele (approximate 90% natural frequency) being more frequent in the spring and autumn than in the summer (Ward, Jann & Blanckenhorn, 2004). Finally, out of six allozyme loci tested, *pgm* was the only one showing geographical population differentiation within Switzerland (Kraushaar, Goudet & Blanckenhorn, 2002), although a recent population genetic study (Demont *et al*., 2008) revealed no evidence for a latitudinal *pgm* cline in Europe (cf. Eanes, 1999; Verrelli & Eanes, 2001; Sezgin *et al*., 2004). In total, these results are consistent with reported cryptic female choice in yellow dung flies based on environment-dependent *pgm* genotype performance (Ward, 1998, 2000), referring to the phenomenon that females may somehow choose among stored sperm from different males (Birkhead, 1998). However, a direct functional link between the oviposition choice of individual female yellow dung flies under field conditions and the temperature-dependent performance of their offspring possessing particular *pgm* genotypes has not been examined.

In the present study, we report a series of laboratory and field experiments investigating the putatively adaptive temperature-dependent effects of the *pgm* genotype on juvenile development in the yellow dung fly. First, we assessed the *in vitro* activity at different temperatures of the allozymes corresponding to the three most common *pgm* alleles [Slow (S) = 2, approximate 2.5% natural frequency; Medium (M) = 3, approximate 90% natural frequency; and Fast (F) = 4, approximate 4.5% natural frequency; i.e. the alleles called 3, 5 and 7 in Ward, Jann & Blanckenhorn, 2004, respectively], testing individuals homozygous for these alleles bred in the laboratory. Assuming the *in vitro* and *in vivo* enzyme activities are congruent (e.g. as is the case in *Colias* or *Drosophila*: Watt *et al*., 1983; Freriksen *et al*., 1994; Verrelli & Eanes, 2001), we could then derive predictions regarding consequent effects on a central juvenile fitness trait: development time (Ward, 1998). Thus, a higher *in vitro* activity of an allozyme variant at a

particular temperature should lead to higher (i.e. better) *in vivo* activity and, ultimately in this case, to faster development at that temperature. Towards the end of the season, developing yellow dung fly larvae are regularly time constrained because they have to reach the pupal stage to successfully overwinter, leading to documented natural selection for faster growth and development, with biologically relevant differences in the range of a few days (Blanckenhorn, 1997, 1998; Blanckenhorn *et al*., 2001; Teuschl, Reim & Blanckenhorn, 2007). If different PGM allozymes perform better at different temperatures according to their *in vitro* activity, we can derive general expectations as to which PGM genotype the offspring produced by an ovipositing female in the field at a given temperature or time of season should have. Second, we performed a laboratory experiment aiming to assess the joint effects of *pgm* genotype and temperature on egg as well as subsequent larva-to-adult development times. This experiment investigated whether different *pgm* genotypes indeed mediate variation in development time before and after larval hatching, and whether this variation interacts with temperature. Third, we investigated the joint effects of *pgm* genotype and temperature on egg-to-adult development time as mediated by female oviposition behaviour in the field during the autumn fly season over 3 years (2003–2005). This experiment explored female oviposition according to microclimate and time of season (which correlates with temperature) in relation to *pgm* genotype, as well as the ensuing *pgm*-dependent development times in the laboratory of the offspring produced. With this set of experiments, we thus test the plausibility of the global hypothesis that yellow dung fly females might exert cryptic female choice (e.g. by microhabitat or sperm selection) based on the effects that the *pgm* locus presumably has on their life history (Ward, 1998, 2000; Ward *et al*., 2002, 2004; Bussière *et al*., 2010). We explicitly note that we do not actually test whether the flies exert cryptic female choice *per se*, only whether such choice would confer development or growth advantages should it indeed occur.

MATERIAL AND METHODS

Study organism

Yellow dung flies are very common around cow pastures in north-central Europe. In lowland Switzerland, as in other central European countries, each year has a spring (March to June) and an autumn season (September to November), with temperatures near freezing in early spring and late autumn, and mean temperatures of around 20 °C at the end of spring and at the beginning of autumn. During the hot midsummer (July and August), the flies largely disappear from the pastures because of their heat sensitivity (Parker, 1970; Ward & Simmons, 1990; Blanckenhorn *et al*., 2001, 2010). We studied a yellow dung fly population on a dairy farm in Fehraltorf, near Zürich, Switzerland (N47°23′, E8°44′).

Yellow dung fly females spend most of their time foraging for nectar and prey in the vegetation surrounding the pasture. Every 3–10 days, they come to the dung to oviposit a clutch of eggs. Males are already waiting on and around the dung pats to copulate with the incoming females. Females show little behavioural choice or resistance in response to male mating attempts, such that they mate at least once every time they lay a clutch. Therefore, the typical female has stored sperm from several males in her three spermathecae, although she receives most sperm from her most recent mate (Bussière *et al*., 2010). The paternity share of the last male to copulate (P2) varies but averages approximately 80% (Simmons, 2001). Because the operational sex ratio is highly male-biased, there is strong male–male competition, and selection usually favours large body size in males and females (Jann *et al*., 2000). After copulation, which lasts approximately 30–40 min, the female lays approximately 30–70 eggs partially submerged into the dung while the male is guarding her; thereafter, the female leaves the pasture, whereas the male typically stays to obtain other mates. The larvae hatch after 24–48 h to feed on the fresh dung and eventually pupate in the ground near the dung pat.

Pgm lines

Six *pgm* lines, two for each of three homozygote alleles 2, 3, and 4, were established in the laboratory at approximately 20 °C by retrospective selective breeding and held for several generations for another study (Ward, 2000). The flies originally stemmed from our field population in Fehraltorf. From these lines, individuals were taken for some of the experiments described below at various generations.

In vitro enzyme activity

We performed a coupled *in vitro* enzymatic assay on PGM activity of homozygote flies with glucose-6-phospate dehydrogenase as the indicator enzyme. At generation 4, seven female flies were randomly chosen per line and frozen at –80 °C (i.e. total $N = 14$ per allele). Then the thorax of each frozen fly was separated and homogenized in 400 µl of a potassium phosphate buffer (0.01 M KH_2PO_4, 1 mM ethylenediaminetetraacetic acid, pH 7.4; Verrelli & Eanes, 2001). The homogenate was centrifuged for 5 min at 10 000 r.p.m., and aliquots of 30 µl of the supernatant

were frozen in liquid nitrogen and stored at –80 °C. To estimate and control for body size (see below), the total soluble protein content of fly homogenates was determined by a Bradford assay on a multi-well spectrophotometer (SpectraMax 340PC; Bucher Biotec) at 595 nm and room temperature.

Enzyme activity of each fly was measured with a ultraviolet/visible spectrophotometer (Ultraspec 2000; Pharmacia Biotech) at each of five temperatures: 7, 12, 17, 22, and 27 °C, in duplicate. We followed the protocol of Verrelli & Eanes (2001). Our assay contained 12 µl of fly homogenate and 204 µl of reagent mix (0.83 mM glucose-1-phospate, 0.5 mM NADP, 1.0 mM $MgCl_2$, 3.1 units mL^{-1} glucose-6-phospate dehydrogenase in 20 mM Tris-HCl, pH 7.4; Verrelli & Eanes, 2001). An increase of absorbance as a result of NADPH reduction was detected at 340 nm (Stam & Laurie-Ahlberg, 1982). PGM activity was calculated from the slopes in the steady-state, where the amount of reduced NADPH (ε_{340} NADPH = 6.27 $mM^{-1}\,cm^{-1}$) corresponds to the amount of consumed glucose-1-phosate. The unit of PGM activity was finally expressed as µM glucose-1-phosphate per minute (per milligram of soluble protein). Because PGM is a monomer, there are no interactions between protein subunits, and so differences in enzyme activity are straightforward and heterozygotes should be intermediate.

Enzyme activity was analyzed using repeated-measures analysis of covariance with (homozygous) *pgm* genotype (alleles 2, 3 or 4) as a crossed factor, temperature (7, 12, 17, 22, and 27 °C) as a repeated factor (because material from the same female fly was tested at all temperatures), and fly protein content as a covariate to correct for body size. No data transformation was necessary. The corresponding Q_{10} for the three allozymes were calculated from log-transformed data because the relationships are curvilinear (Fig. 1).

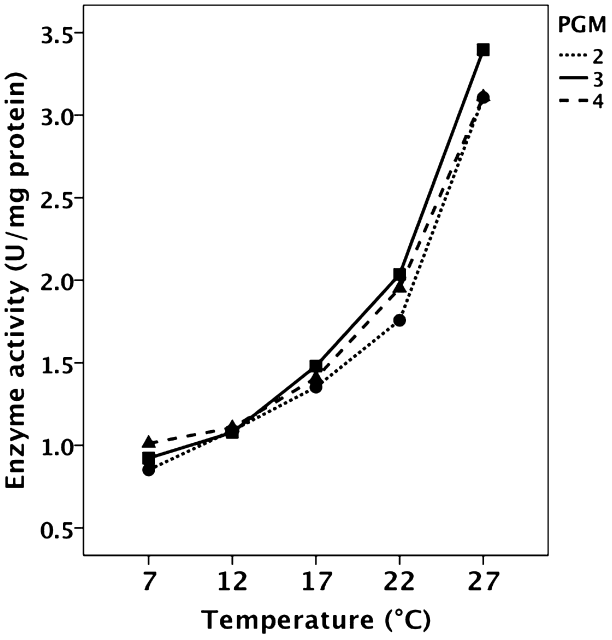

Figure 1. Size-controlled *in vitro* activity of three homozygous allozymes for the yellow dung fly *Scathophaga stercoraria* at five temperatures ($N = 14$ females per allozyme). The corresponding Q_{10} for the three allozymes is 1.845 [95% confidence interval (CI) = 1.710–1.990] for PGM-2, 1.928 (95% CI = 1.791–2.076) for PGM-3, and 1.752 (95% CI = 1.636–1.875) for PGM-4.

Laboratory assessment of temperature- and *PGM*-dependent egg development time

A laboratory experiment was conducted using homozygous and (presumably intermediate) heterozygous individuals derived from the 2, 3, and 4 *pgm* lines described above. From each clutch of eggs, a female laid after having mated with one male (henceforth called family; total $N = 30$ families), five eggs were distributed to each of five temperatures (12, 17, 22, 27, and 32 °C) to measure egg development time as a function of *pgm* genotype. By contrast to adults and even larvae, which can behaviourally respond to heat stress, eggs have to regularly endure extreme temperatures on the exposed dung pat, such that higher temperatures had to be tested here. These temperatures are well within the range of shade temperatures measured in the field over three spring and autumn seasons, which ranged from 9 °C in mid March 2003 to 24 °C in September 2004, whereas sun temperatures (not measured) are considerably higher. The eggs were transferred on a piece of wet filter paper, which was then placed into a small plastic vessel containing a superabundant amount of at least 5 g of fresh cow dung so that larval hatching could be scored. Larval hatching was checked three times a day (every 8 h). Eggs were left in their respective temperature until the larvae hatched, whereupon the dung container with the hatched larvae was transferred to constant 20 °C, a benign and optimal temperature environment, until the adult offspring flies emerged. *Pgm* genotype was then scored. We thus specifically tested for carry-over effects on overall development time of temperatures endured during the egg stage only.

All flies were stored at –80 °C for subsequent horizontal cellulose acetate gel electrophoresis of PGM in accordance with standard procedures defined by Hebert & Beaton (1989) with slight modifications (Demont *et al*., 2008), scoring the relative mobility of the different enzymes. *Pgm* alleles were designated

numbers, allele 2 having the slowest relative mobility of the three alleles considered here, and 4 the fastest.

We analyzed square root-transformed egg development times (time from egg laying to hatching of larvae in hours) using analysis of variance (ANOVA) with offspring sex, temperature treatment, and *pgm* genotype as fixed factors and family as a random effect (to remove quantitative genetic differences in development times among families). *Pgm* genotypes included allele 2, 3, and 4 homozygotes plus 2/3 and 3/4 heterozygotes (2/4 heterozygotes were rare and excluded from the analysis).

Field study of female oviposition and seasonal *PGM*-dependent development time

We investigated female oviposition in relation to temperature and *pgm* genotype over the autumn fly seasons (September to November) 2003–2005 in Fehraltorf. We caught copulating dung fly pairs in the field and carefully moved them individually into an aired plastic container with a small dung pat artificially shaped to feature a north and a south slope (Ward, Foglia & Blanckenhorn, 1999). After oviposition, eggs laid on each slope were counted and transferred for laboratory breeding into two separate containers with more than 2 g of dung per larva. The pair and the breeding containers were then transported to the laboratory where all offspring could develop at constant 18 °C and 60% humidity so that the *pgm* genotype and development time of all individuals could be scored and compared under standard laboratory conditions. In 2003 and 2004, randomly field-caught pairs were investigated, whereas, in 2005, we additionally brought laboratory-reared males derived from our *pgm* selection lines to the field as mates to increase the overall frequency of the rare alleles 2 and 4. Field shade temperatures were taken on all days during the time of observation of any particular female, from which a mean for every particular oviposition period was computed.

We analyzed individual development times of all offspring hatched from eggs laid by field females over three autumn seasons 2003–2005 using ANOVA with *pgm* genotype, north/south exposure (N/S), sex, and year as fixed factors, family as a random effect, and Julian day in the season as a continuous covariate, including all relevant interactions. All nonsignificant higher-order interactions were dropped from the final model.

RESULTS

In vitro enzyme activity

Enzyme activity expectedly increased with temperature ($F_{4,156} = 2.89$, $P = 0.018$) and protein content (i.e. body size; $F_{1,38} = 12.30$, $P = 0.001$), although mean enzymatic activity did not differ among *pgm* genotypes (main effect: $F_{2,38} = 1.61$, $P = 0.212$). However, there was a significant interaction between temperature and *pgm* genotype ($F_{8,156} = 3.31$, $P = 0.002$): the allozyme PGM-3 for the most common allele 3 showed higher activity at the warmer temperatures (17–27 °C), whereas the activity of PGM-4 was relatively higher at the lowest temperature (7 °C), with PGM-2 showing lower activity throughout (Fig. 1). Nevertheless, paired comparisons between PGM-3 and PGM-4 were not significantly different at any temperature except 27 °C ($P < 0.05$). The Q_{10} for the three allozymes are 1.928 [95% confidence interval (CI) = 1.791–2.076] for PGM-3, 1.752 (95% CI = 1.636–1.875) for PGM-4, and 1.845 (95% CI = 1.710–1.990) for PGM-2. The Q_{10} for PGM-3 and PGM-4 are significantly different because their means are outside the range of the CI of the other enzyme. Assuming that *in vivo* enzyme activity reflects *in vitro* activity, we thus expected that females should preferentially produce offspring bearing allele 4 as temperatures drop towards the end of the season.

Laboratory assessment of temperature- and *PGM*-dependent egg development time

Egg development times strongly decreased with temperature ($F_{4,333} = 9150.8$, $P < 0.001$) and varied unsystematically among families ($F_{29,333} = 9.50$, $P < 0.001$) but not according to offspring sex ($F_{1,333} = 0.11$, $P = 0.752$). Crucially, egg development time typically was shortest for individuals possessing allele 3 and longest for 2 and 4 homozygotes (*pgm* main effect: $F_{4,333} = 3.29$, $P = 0.012$; as indicated by the U-shaped distribution of each group of five *pgm* genotypes in Fig. 2, top). This was more or less similar at all temperatures, and so the temperature by *pgm* genotype interaction was not significant ($F_{16,333} = 1.87$, $P = 0.116$; Fig. 2, top). All other interactions were not significant and not of interest, and therefore were dropped from the model.

When analogously analyzing the subsequent time from larval hatching to adult emergence after all larvae were moved to constant 20 °C, a lasting systematic effect of temperature experienced during the egg stage remained in that development times increased with temperature ($F_{4,333} = 30.34$, $P < 0.001$; Fig. 2, bottom). In addition, the unsystematic among-family variation remained ($F_{29,333} = 2.08$, $P = 0.001$), and the well-known development time difference between the sexes became apparent ($F_{1,333} = 569.86$, $P < 0.001$; males are larger and hence develop for longer time; Blanckenhorn, 1998). Importantly, *pgm* genotype no longer systematically influenced overall development time ($F_{4,333} = 1.21$, $P = 0.309$; Fig. 2,

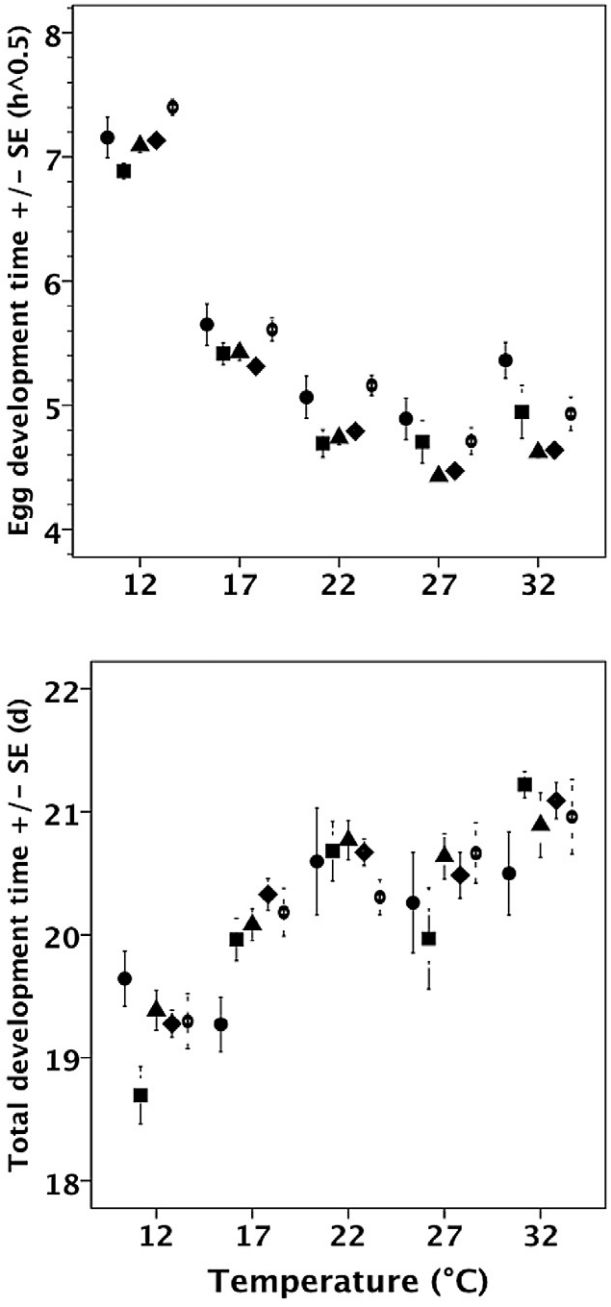

Figure 2. Mean ± SE (top) egg development time (√h) at five temperatures and (bottom) subsequent larva-to-adult (total) development time at a constant laboratory temperature of 20 °C for five *pgm* genotypes [left to right: 2/2 (circle), 2/3 (square), 3/3 (triangle), 3/4 (diamond), 4/4 (oval); $N = 388$ eggs from 30 females].

bottom), nor was the genotype by temperature interaction significant ($F_{16,333} = 1.17$, $P = 0.291$). This shows that, although *pgm* genotype apparently affects egg development times, this effect does not carry over to total larva-to-adult development time, at least not when offspring are subsequently raised in the same (constant) temperature environment after having experienced diverse temperatures during egg development only. Thus allele 2 and 4 homozygotes after hatching were able to compensate for their initially retarded egg development.

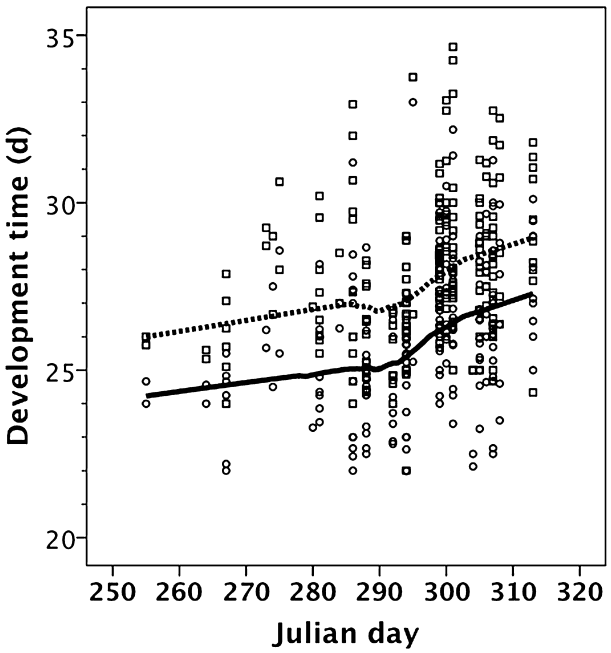

Figure 3. Mean egg-to-adult development times (at 18 °C in the laboratory) of female (circles and solid nonparametric regression line) and male (squares and hatched line) offspring produced per family ($N = 169$) over the autumn seasons 2003–2005 (Julian day 252 = 1 September).

Field study of female oviposition and seasonal *PGM*-dependent development time

Development times of flies collected in the field as eggs varied between years ($F_{2,2619} = 81.29$, $P < 0.001$) and between the sexes ($F_{1,2619} = 332.00$, $P < 0.001$; Fig. 3). Interestingly, development times at a constant laboratory temperature of 18 °C systematically increased as offspring were produced later in the season as temperatures dropped (effect of Julian day: $F_{1,2619} = 321.59$, $P < 0.001$; Fig. 3). Importantly, development times varied among the five *pgm* genotypes (three homozygotes plus two heterozygotes: $F_{4,2619} = 2.79$, $P = 0.025$), being slightly longer for individuals possessing alleles 3 and 4 and shorter for those bearing allele 2 (Fig. 4). Finally, north versus south exposure did not affect development times, and did also not interact with Julian day or *pgm* genotype (all $F_{1,2619} < 2.34$, $P > 0.1$). All other interactions were not significant and were dropped from the model.

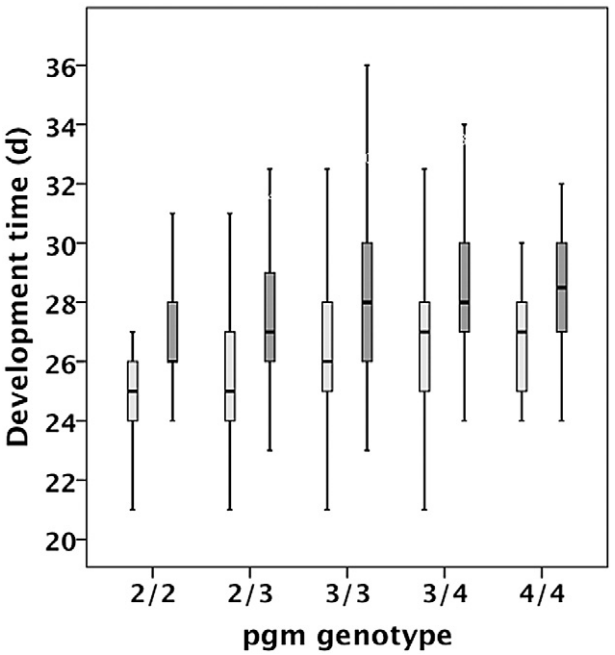

Figure 4. Box-plot of egg-to-adult development times at constant 18 °C in the laboratory of 2726 offspring females (light grey) and males (dark grey) produced by 169 mothers in the field according to *pgm* genotype (controlled for the seasonal trend in Fig. 3).

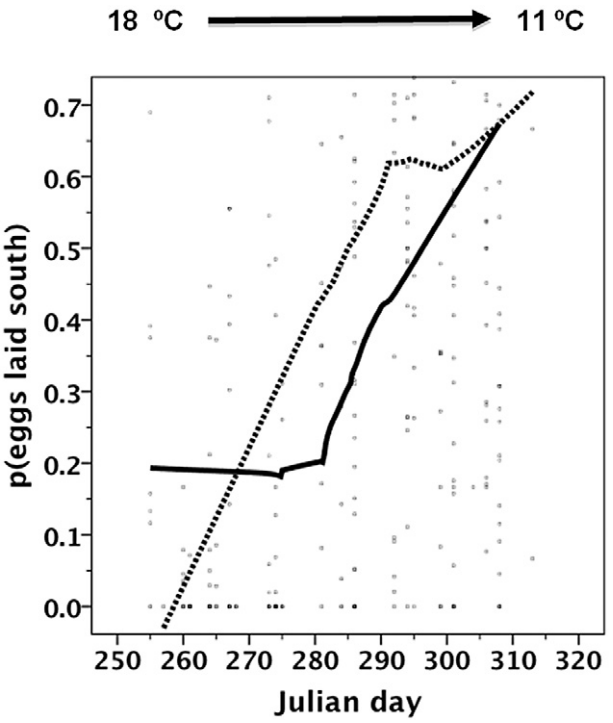

Figure 5. Proportion of eggs laid on the (warmer) south-facing slope of a dung pat by 30 individual females over the autumn seasons in 2003 (solid nonparametric regression line) and 2004 (dotted line; Julian day 252 = 1 September). The approximate mean shade temperatures during the experiments decreased over the season as indicated.

Nevertheless, the proportion of eggs laid on the warmer south slope by individual females in 2003 and 2004 (when they had a choice) strongly increased as the winter approached (ANOVA: $F_{1,349} = 78.63$, $P < 0.001$; Fig. 5). Given that females show such apparently adaptive microhabitat choice with regard to temperature, we finally tested for biases in the *pgm* composition between eggs laid on the north and south slopes, in accordance with suggestions made by Ward and colleagues (Ward, 1998, 2000; Ward *et al*., 2002, 2004) that females might exert cryptic microhabitat choice based on the *pgm* locus. We expected offspring bearing allele 4 to be more commonly placed on the cooler north slope because, at cool temperatures PGM-4, showed the highest *in vitro* activity of all allozymes (Fig. 1). To maximize the probability of detecting an effect if it exists, we used various tests. For the 2003 and 2004 data sets, where individual females had the choice to oviposit north and/or south, we calculated the proportion of offspring possessing allele 4 (i.e. the heterozygous genotype 3/4 because no homozygous genotype 4/4 offspring were produced by any female as a result of this allele being naturally rare) for the north and south slopes and compared these paired data using parametric *t*-tests, Wilcoxon tests and Sign tests (all $P > 0.1$; one-tailed because we had a directed prediction). Second, we computed a mean genotype for all the eggs laid per female (genotypic values 2–4, with heterozygotes given intermediate values) on the north and south slopes and similarly compared those paired means using the same tests, expecting a greater mean for the north slope (all $P > 0.1$, one-tailed test; mean ± SE genotypic difference north–south: 0.025 ± 0.024). Of the originally 169 females tested, we ended up with a final sample size of only 30 females because most females produced offspring homozygous for allele 3 and some additional females did not lay eggs on one or the other slope. For the (unpaired) 2005 data set, where females were randomly allocated to lay either on the north or the south slope, we had more usable data ($N = 84$) because field females were supplemented with laboratory males bearing the rare alleles 2 and 4 as last mates. Nevertheless, ANOVA of the mean genotype (as above) with north/south as fixed factor and Julian day as covariate showed no significant differences [genotypic mean ± SE: 3.081 ± 0.035 (north) versus 3.072 ± 0.048 (south), one-tailed $P > 0.3$] before or after dropping the nonsignificant interaction from the model.

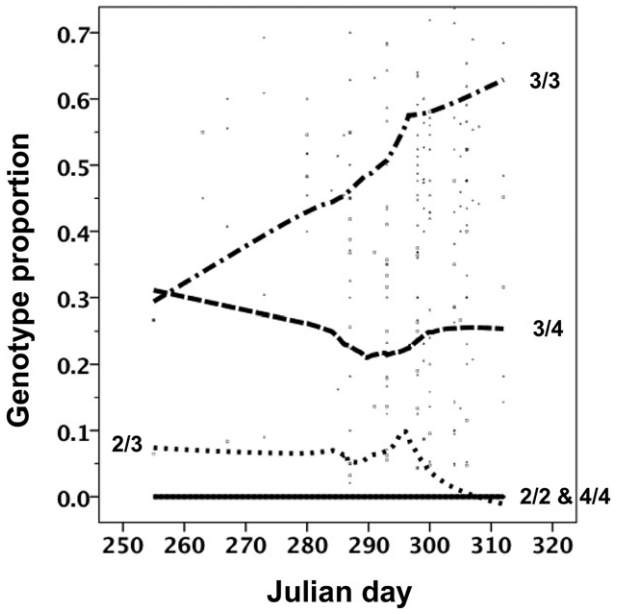

Figure 6. Proportion of *pgm* genotypes among all offspring individuals produced by 114 field females over the autumn seasons 2003–2005 (Julian day 252 = 1 September): 2/3 decreasing (finely hatched nonparametric regression line); 3/3 increasing (doubly hatched line); 3/4 slightly decreasing (coarsely-hatched line; genotypes 2/2 and 4/4 were rare: solid line).

Finally, confirming the findings reported in a study by Ward *et al*. (2004), the overall genotype distribution over the autumn season changed across all three years in that alleles 2 (i.e. genotypes 2/2 and 2/3) and 4 (genotypes 3/4 and 4/4) decreased, whereas the most common genotype 3 increased (Fig. 6; descriptive results without statistics). Again, from our *in vitro* enzyme activity study (Fig. 1), we had expected an increase in allele 4 towards the end of the season, although this did not occur.

DISCUSSION

Our extensive study revealed subtle but significant effects of *pgm* genotype on egg and egg-to-adult development times that were not congruent. Although, in our laboratory experiment, the egg development time of individuals possessing the most common *pgm* allele 3 (approximate 90% natural frequency) was fastest at all temperatures tested (Fig. 2, top), which we interpret as being best in terms of fitness given seasonal time constraints (Blanckenhorn, 1998; Teuschl *et al*., 2007), this effect disappeared when considering the whole juvenile period (Fig. 2, bottom), and, in our field experiment, egg-to-adult development times were fastest for individuals bearing allele 2 (approximate 2.5% natural frequency) and slowest for individuals bearing the most common allele 3 (Fig. 4). Based on our *in vitro* enzyme activity assay (Fig. 1), and assuming that *in vivo* enzyme activity reflects *in vitro* activity, we had expected that individuals possessing allele 3 would develop faster at the beginning of the season in September when it was warmer, whereas individuals possessing allele 4 (approximate 4.5% natural frequency) would develop faster later in the season when it was cooler. This was clearly not the case, and allele 4 also did not increase in our field population towards the end of the season (whereas allele 3 did; Fig. 6). Rather than our *in vitro* assay, our field results thus confirm the field study of Ward, Jann & Blanckenhorn (2004) based on 7000 flies over 2 years showing that the common allele 3 is most frequent at the beginning and the end of the year when it is coolest. We therefore conclude that, although PGM allozymes differ in activity (Fig. 1) and *pgm* genotype does have detectable differential effects on fitness-relevant life history traits (i.e. development time, and also pupal weight; Ward, 1998), these effects are difficult to reconcile within an adaptive life-history and sexual selection framework, as discussed in more detail below.

PGM-3 had the highest Q_{10} of all the allozymes investigated (Fig. 1). This may constitute a selective advantage that could explain the high frequency of the common *pgm-3* allele in nature, if the allozyme indeed functions effectively at a wide range of temperatures. The latter, however, cannot be resolved by the present study, nor is total enzyme activity as measured here necessarily the most relevant metric in this context (e.g. as opposed to temperature-specific enzyme kinetics; Eanes, 1999). At the same time, the common allele must be disadvantageous in some way for a polymorphism with alternative alleles to be maintained by presumed spatio-temporally variable selection. In this context, we note that the allele distribution in yellow dung flies appears to be rather stable because approximately 10 years lie between the study performed by Ward *et al*. (2004) and the present study. The slightly longer development time (< 1 day) associated with the *pgm*-3 allele found in the present study could be such a disadvantage, although the fitness consequences of such small variation will be limited. We concede that the present study is merely a start in analyzing the mechanism and functional significance of PGM allozyme variation in yellow dung flies because other yet unexplored life history trade-offs may well exist.

Another unexpected result that we obtained was the development time increase toward the end of the season of offspring produced in the field when developing under standard conditions (constant 18 °C) in the laboratory (Fig. 3), comprising a robust outcome based on more than 2500 scored individuals that was

consistent over 3 years and independent of *pgm* genotype. We had expected the opposite because yellow dung flies have to reach the pupal stage to overwinter successfully and are therefore increasingly time-constrained as the winter approaches, with as little as 1–2 days development time difference conferring a crucial selective advantage (Blanckenhorn, 1997, 1998; Teuschl *et al*., 2007). Consistent with the latter hypothesis, larva-to-adult development times were shorter for those individuals hatching from eggs incubated at cooler temperatures in our laboratory experiment (12 °C versus 17 °C or higher; Fig. 2, bottom). Nevertheless, a longer development time towards the end of the season is likely maladaptive in general because early frost would kill any larvae that have not yet reached the pupal stage (Blanckenhorn, 1998).

What could be the reasons for our largely incongruent results? In deriving our concrete expectations, we assumed that the *in vivo* and *in vitro* activities of PGM allozymes are correlated. We further assumed that higher *in vivo* activity would lead to faster development (Freriksen *et al*., 1994), which, with everything else being equal, does confer an advantage under most ecological circumstances and particularly under time constraints towards the end of the season (Blanckenhorn, 1997, 1998; Teuschl *et al*., 2007). Both assumptions may of course be wrong or at least simple-minded, although congruence between *in vitro* activity and *in vivo* performance has been previously reported for particular enzymes (Watt, 1977, 1983) and life-history traits have been found to relate to the level of allozyme heterozygosity in a number of species (Mitton & Grant, 1984; Eanes, 1999; Saastamoinen & Hanski, 2008). In recent years, physiological ecologists have (re-)discovered the intricacies of physiological assays and the complexity of responses of organisms to thermal manipulations, demanding ever more multifarious designs (Rank *et al*., 2007; Overgaard, Kristensen & Sørensen, 2012), such that congruence between life stages, treatments, or environments must not necessarily be assumed and needs to be specifically tested, as carried out in the present study. Being involved in the mobilization and storage of glycogen (Bailey, 1975; Sacktor, 1975), it is also possible that *pgm* has little effect on juvenile traits and fitness, whereas it might be more relevant for adult dispersal and survival relating to flight capacity, as is the case for PGI in *Colias* butterflies (Watt, 1977, 1983, 1985). Unfortunately, survivorship of yellow dung flies in the field is very difficult to follow (Burkhard, Ward & Blanckenhorn, 2002). Future research needs to determine whether *in vivo* PGM activity indeed follows *in vitro* activity, and whether different *pgm* genotypes show more consistent temperature-dependent performance differences at the adult life stage (e.g. in flight activity).

The present study was originally motivated by the intriguing findings that yellow dung fly females apparently exert cryptic female choice based on the *pgm* locus (Ward, 1998, 2000; Ward *et al*., 2002, 2004; Bussière *et al*., 2010). Although it is unclear how females would detect the *pgm* genotype of their mating partner or his sperm, nor what information they derive from the *pgm* genotype or any trait potentially correlated with it, we set out to test not cryptic female choice *per se* but, instead, the plausibility of the general hypothesis that such choice could confer development or growth advantages should it indeed occur. Ward *et al*. (2002) found nonrandom genotypic distributions of eggs according to microhabitat but did not track the mothers that laid these eggs. In the present study, we investigated whether females place eggs of different *pgm* genotypes in different microhabitats on the dung pat because they presumably develop faster there, and we further investigated the developmental consequences of variation in *pgm* genotype of the offspring. We found no significant differences in the *pgm* composition of the eggs laid on the (cooler) north and (warmer) south slopes of a dung pat, when these eggs stemmed from the same female that had a choice (paired test in 2003 and 2004), or when they stemmed from different females that had no choice (unpaired test in 2005). Because alleles other than *pgm-3* are naturally rare, we spared no effort in setting up almost 200 females over 3 years to acquire the sample sizes necessary to uncover an effect if it exists. We obtained no effects despite the fact that females increasingly preferred the warmer, south-exposed oviposition sites as the weather became cooler towards the end of the season (Fig. 5), which is clearly an adaptive response. Yellow dung flies have been previously found to exert analogous small-scale oviposition site preferences with regard to egg desiccation and drowning probability (Ward *et al*., 1999).

In conclusion, the present study shows that, in yellow dung flies, *pgm* genotype does have subtle effects on development time, a central life-history fitness component, although these effects are small and inconsistent for egg and total development time, and also do not agree with results from our *in vitro* enzyme activity assays. Such inconsistencies among treatments and between laboratory and field results may be more common than assumed and testify to the complex plasticity underlying thermal responses (Rank *et al*., 2007; Overgaard *et al*., 2012). It is conceivable that more congruent *pgm* effects could be obtained for other larval or adult fitness components. Most centrally, the present study revealed no evidence supporting the plausibility of yellow dung fly females exerting *pgm*-based cryptic microhabitat choice conferring growth or development advantages

for their offspring (Ward, 1998, 2000; Ward *et al*., 2002, 2004; Bussière *et al*., 2010), supporting the results of a recent field test of cryptic female choice using molecular paternity analysis (Demont, Martin & Bussière, 2012). In the end, any experimental investigation of the PGM allozyme polymorphism in yellow dung flies will be hampered by the high frequency of the dominant *pgm-3* allele in nature and the inherent difficulties associated with conclusively demonstrating cryptic female choice (Birkhead, 1998; Simmons, 2001).

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