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Separating multiple sources of variation on heat resistance in *Drosophila hydei*

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ABSTRACT

While numerous insect studies have demonstrated the effects environmental conditions, genetic variation and other factors have on thermal resistance, often showing patterns consistent with adaptive plasticity and local adaptation, few experiments have considered the effects of multiple factors simultaneously. Here however, we have investigated the impact of sex, rearing conditions, hardening, population, and laboratory rearing period on adult heat resistance in stocks of *Drosophila hydei*, a cosmopolitan species that occurs across a range of climatic zones. We show that population and putative laboratory adaptation effects are larger than those associated with rearing temperature and hardening, although there was also a notable interaction between hardening and sex, in that females showed a cost of hardening that was not present in males. In separate experiments, we found that environmental effects across a generation were small and similar in magnitude to those within a generation. These findings suggest multiple sources of variation on heat resistance and place potential genetic versus environmental sources in context.

1. Introduction

With the advent of human induced climate change and extreme temperatures, there has been increasing interest in factors influencing heat resistance in insects (Chown et al., 2015; Garcia-Robledo et al., 2016; Hoffmann et al., 2013). Heat resistance is known to be affected both by environmental conditions and by genetic factors (Schilthuizen and Kellermann, 2014; Sgro et al., 2016). In addition, there is the potential for across-generation effects to influence resistance levels (Schiffer et al., 2013). All of these factors could impact on the ability of insects to counter extreme temperatures, and to persist under abnormally hot conditions. For instance, hardening can increase resistance to heat in several Drosophila species and other insects (Chidawanyika and Terblanche, 2011; Nyamukondiwa and Terblanche, 2010), although this is not universally the case (Mitchell et al., 2011).

Although the impact of these factors on heat resistance has been examined individually, there are few studies that consider their combined effects. While populations of Drosophila species are known to vary in their level of heat resistance, the relative level of resistance across populations needs to be assessed with respect to the environmental conditions (Ayrinhac et al., 2004; Hoffmann et al., 2005). Similarly, hardening and rearing effects on heat resistance when placed together can provide an indication of the extent to which these factors interact to influence resistance levels, which has been considered in Drosophila (Mitchell et al., 2011) as well as in Ceratitis fruit flies (Nyamukondiwa and Terblanche, 2010) and in kelp flies (Marais et al., 2009). Ideally the relative contribution of all factors needs to be considered when ascertaining their potential impact, to provide insight into the overall extent to which plastic responses might affect thermal tolerance and the conditions under which the effects of natural selection on population divergence can be detected.

In this paper we assess the relative contribution of environmental and genetic factors on heat resistance in *Drosophila hydei* Sturtevant, a cosmopolitan species that is often found around rotting fruit and decaying vegetables (Atkinson and Shorrocks, 1977). This comparatively large drosophilid can be easily reared in the laboratory and collected from a range of climates spanning tropical and temperate regions. Our aim is to investigate two aspects of plasticity: hardening and rearing conditions; as well as two sources of genetic variation: population variation and laboratory rearing time. Laboratory rearing time is often interpreted in terms of laboratory adaptation, but is usually ignored as a source of variance in species comparisons even though there is evidence that levels of thermal resistance are influenced by the number of generations

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that insects are reared in the laboratory (Griffiths et al., 2005; Hoffmann et al., 2001). This means that inherent levels of resistance might be overestimated or underestimated depending on the species and the particular situation.

As well as considering these genetic and environmental effects, we also address the issue of across-generation effects. This source of variation in insect genera often involves maternal effects (Mousseau and Dingle, 1991) but can also consist of transgenerational effects that act through both parents across one or more generations. However, the overall influence of maternal effects and other forms of transgenerational effects on thermal resistance in insects remains unclear (Sgro et al., 2016). Previous Drosophila data suggest that there may be some benefits for thermal resistance but these could be small (Schiffer et al., 2013). Moreover, transgenerational effects can also produce costs on other fitness components both in Drosophila (Magiafoglou and Hoffmann, 2003) and other insects (Eggert et al., 2015).

2. Methods

2.1. Flies and rearing

The first part of this study compared rearing conditions, collection time, population origin, and hardening effects of different populations of *D. hydei*, and consisted of two separate experiments, the first being conducted in February 2015, and the second in October 2015. Five populations were used in the February experiment and six in the October experiment. The aim of these experiments was to compare the heat resistance of different populations, taking into consideration both geographic differences in stock origin, and the time stocks had been in the laboratory. Note that we refer to the time a stock spent in the laboratory as "laboratory adaptation". However, we acknowledge that when a population is compared to another population collected at an earlier time but from the same field site, differences between the stocks may represent not only laboratory adaptation (and laboratory genetic drift) but also any changes in genetic composition of flies in the field between the collection times.

Flies collected in the field were sexed and these females produced F_1 offspring. Massbred populations were established from the offspring of 21 field females for each population. The F1 generation was cultured at 25 °C at a 12 h:12 h light:dark photoperiod, and these conditions were also used in subsequent generations. All flies were held and reared on a medium composed of dextrose (7.5% w/v), cornmeal (7.3% w/v), inactive yeast (3.5% w/v), soy flour (2% w/v), agar (0.6% w/v), nipagin (1.6% w/v), and acid mix (1.4% 10:1 proprionic acid:orthophosphoric acid). Flies were cultured in 600 ml bottles. Prior to testing, all flies were sexed using $C0_2$ anaesthesia and allowed to recover on laboratory medium for a minimum of 24 h.

The February experiment compared: (i) three populations from Kulnura, New South Wales, collected in March 2013, March 2014 and October 2014; (ii) one population from Innisfail, Queensland, collected in March 2014; and (iii) one population from Melbourne, Victoria collected in October 2014. The longest established population had been in the laboratory for 17 generations, compared to 7 generations for the March 2014 populations, and 2 generations for the October 2014 populations. Populations were defined by both their collection State and collection date ('2013 March NSW', '2014 March NSW', '2014 October NSW', '2014 March QLD', '2014 October VIC' for the order of populations described in (i) to (iii) above).

In the October population comparison, six populations were used. These were the three Kulnura populations and an additional population from the same site collected in April 2015 ('2015 April NSW') which at this stage meant that populations from the same site had been cultured for 23, 13, 7 and 2 generations in the laboratory. In addition, the 2014 October VIC population was remeasured, along with a new Melbourne population collected in April 2015 ('2015 April VIC') which was 2 generations removed from the field.

In the second part of this study, we investigated across-generation effects using the 2014 March NSW and 2014 March QLD populations. The experiments were run separately for the populations and sexes and were specifically designed to investigate across-generation versus within-generation effects (see Fig. 1).

2.2. Experimental design

For the population comparisons, populations were reared at 19 °C or 28 °C for two generations (and designated '1919' or '2828' respectively). Flies which emerged from these treatments were held at 25 °C prior to testing. Flies from different bottles were kept separately so that culture bottle, and block effects could be investigated. To ensure that all treatments could be compared simultaneously, treatments were set up in a staggered manner to compensate for the marked differences in developmental time when culturing at 19 °C versus 28 °C. Flies from each of the rearing treatment- population comparisons were either tested without hardening, or hardened prior to testing in a 37 °C water bath for one hour followed by 6 h of recovery at 25 °C.

To measure heat resistance, flies were placed individually in vials in a randomised order, and 10 assays were undertaken. The glass specimen vials (50 mm height x 12 mm diameter) were placed on a Perspex frame before being immersed into a 28 °C water bath. Temperature was controlled using a Ratek SP599 thermoregulator with a REX-P24 controller. The '0.2' ramping protocol (Schiffer et al., 2013) was used, which represents the maximum temperature increase likely to be experienced in the field (Terblanche et al., 2011). This involved having the water at 28 °C for the first 15 min of the experiment followed by an incremental increase of 0.2 °C per minute until reaching 38 °C at the 65-min mark, after which time the temperature was held at this level for the remainder of the experiment. Heat knockdown was defined from the starting time at which they were placed in a water bath to the point at which a fly was rendered unconscious, and unable to hold itself upright or be seen to be moving its mouthparts, and when it did not respond to a light stimulus (a beam of light from a 12 LED hand torch). Heat knockdown time was recorded to the nearest second and then converted to minutes for data analysis. Flies were tested between the ages of 9 and 12 days old to ensure they were sexually mature upon testing (Markow, 1985).

For the across-generation experiment, flies were reared in bottles at 19 °C, 25 °C or 28 °C to form the parental generation (Fig. 1). The 19 °C and 28 °C flies were then raised at either 19 °C or28 °C in the next generation to form four treatments. All flies were aged and held at 25 °C from emergence and tested for heat resistance. A treatment raised for two generations at 25 °C was also included as a reference/control. The treatments were compared again after an extra one or two generations of rearing at 25 °C (Fig. 1).

2.3. Analysis

Because data were normally distributed, ANOVAs were undertaken (in R) with post hoc analysis using a Tukey HSD test to compare groups. A block (assay) effect was included in the ANOVAs. Flies from bottles were initially treated separately, but because bottle effects were never significant these were excluded from the final models in both sets of experiments. Higher order interactions were pooled into the error term where these were not signif-