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## RESEARCH ARTICLE

# Assessing the relative importance of environmental effects, carry-over effects and species differences in thermal stress resistance: a comparison of Drosophilids across field and laboratory generations

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### SUMMARY

**There is increasing interest in comparing species of related organisms for their susceptibility to thermal extremes in order to evaluate potential vulnerability to climate change. Comparisons are typically undertaken on individuals collected from the field with or without a period of acclimation. However, this approach does not allow the potential contributions of environmental and carry-over effects across generations to be separated from inherent species differences in susceptibility. To assess the importance of these different sources of variation, we here considered heat and cold resistance in Drosophilid species from tropical and temperate sites in the field and across two laboratory generations. Resistance in field-collected individuals tended to be lower when compared with F1 and F2 laboratory generations, and species differences in field flies were only weakly correlated to differences established under controlled rearing conditions, unlike in F1–F2 comparisons. This reflected large environmental effects on resistance associated with different sites and conditions experienced within sites. For the 8 h cold recovery assay there was no strong evidence of carry-over effects, whereas for the heat knockdown and 2 h cold recovery assays there was some evidence for such effects. However, for heat these were species specific in direction. Variance components for inherent species differences were substantial for resistance to heat and 8 h cold stress, but small for 2 h cold stress, though this may be a reflection of the species being considered in the comparisons. These findings highlight that inherent differences among species are difficult to characterise accurately without controlling for environmental sources of variation and carry-over effects. Moreover, they also emphasise the complex nature of carry-over effects that vary depending on the nature of stress traits and the species being evaluated.**

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### INTRODUCTION

Ectothermic species are often compared for their vulnerability to stressful climatic conditions. Typically species are scored *in situ*, or within a laboratory environment following a period of acclimation (e.g. Calosi et al., 2008; Janion et al., 2009). These approaches often do not allow for the separation of genetic and environmental effects that contribute to species differences. Separating such effects is crucial when determining whether species might be vulnerable to future climate change (e.g. Deutsch et al., 2008; Clusella Trullas et al., 2011). Otherwise, species might be classified as being relatively vulnerable to stressful conditions even when they have a moderate level of resistance – such as when individuals used for testing vulnerability happen to be raised in an environment where nutrition is poor. Controlling for environmental effects is also important when phylogenetic analyses are carried out to identify evolutionary lineages that are vulnerable to different types of climatic stresses (Huey et al., 2009; Strachan et al., 2011; Kellermann et al., 2012a). Without this partitioning, related taxa within lineages might appear to be similar because they share similar environmental conditions rather than because of any inherent similarity in their vulnerability.

In arthropods, there is abundant evidence that environmental conditions dramatically affect resistance to stressful climatic conditions. Perhaps the most well-known effects involve hardening and acclimation, where exposure to sub-lethal stress conditions increases the level of resistance to thermal stresses within a generation (e.g. Fischer and Karl, 2010; Allen et al., 2012; Colinet and Hoffmann, 2012), particularly when juvenile stages are reared under different conditions (e.g. Gibert et al., 2001; Rako and Hoffmann, 2006; Foray et al., 2013). In addition, carry-over effects (phenotypic effects lasting across generations, including those due to epigenetic mechanisms) can influence the stress resistance of arthropods, although these currently remain poorly defined and rarely tested (e.g. Jenkins and Hoffmann, 1994; Bacigalupe et al., 2007). The impacts of carry-over effects and acclimation on stress resistance within a field context have rarely been examined (Overgaard and Sørensen, 2008).

Here, we followed an experimental design aimed at assessing differences in species vulnerability based on a comparison of the resistance of species when tested directly from the field and when reared under controlled laboratory conditions for two generations. This design allowed us to test the relative importance of inherent

Table 1. Effects that potentially contribute to variation among species in the different generations

| Generation | Environmental (between sites) | Environmental (within sites) | Carry over (across one generation) | Genetic | Environmental (within generation) |
|---|---|---|---|---|---|
| Field/parental ($\mu_P$, $\sigma_P$) | +* | + | + | + | + |
| F1 ($\mu_{F1}$, $\sigma_{F1}$) | | | + | + | + |
| $F2_{19°C}$ ($\mu_{F2,19°C}$, $\sigma_{F2,19°C}$) | | | | + | + |
| $F2_{28°C}$ ($\mu_{F2,28°C}$, $\sigma_{F2,28°C}$) | | | | + | + |

μ, mean; σ, variance.

Variance components were estimated using ANOVA (supplementary material Table S2), while differences between generations or environments were calculated using *t*-tests.

+Indicates that this source of variation can contribute to species differences in the generation tested.

*This component was not included for tests involving 2 and 8 h cold stress because parental flies from the tropical or temperate sites could usually only be scored for one of these tests.

genetic effects, environmental effects and carry-over effects, and to therefore assess whether biases might be introduced when species differences are measured only on field individuals or after a generation of being reared in a controlled environment.

We applied this design to investigate variation in adult thermal resistance among Drosophilid species. We first considered species differences in adults sampled directly from the field, either from a tropical or a temperate site. This represents a situation where species differences in vulnerability are tested without acclimation and without the ability to control rearing conditions. Our interest was to compare site effects rather than species effects; specimens sampled included both widespread species as well as narrowly distributed climate specialists known to differ in cold resistance (Gibert et al., 2001; Kellermann et al., 2012a). We then reared the offspring of each species from the field in a controlled laboratory environment for two generations (F1, F2) to test for carry-over effects and inherent differences in resistance among the species. Flies at the F2 stage were reared at two different temperatures to assess developmental thermal acclimation (a form of phenotypic plasticity).

We show that the vulnerability of species assessed when they are sampled directly from the field is only weakly correlated to their vulnerability when assessed at the F2 generation. Carry-over effects in thermal resistance were detected for several species. Variance estimates for resistance were higher in comparisons of field-raised flies than in comparisons of laboratory-reared flies, reflecting the fact that environmental effects due to site differences and other sources of environmental variation inflated species differences.

## MATERIALS AND METHODS

### Design

The different components of the design are summarised in Table 1 and were used to examine (1) changes in mean resistance due to within- and across-generation effects, and (2) the impact of the different environmental sources of variation and genetic effects on species differences. We identified two sources of direct environmental variation, the variation among tropical and temperate sites experienced by species from each area, and the variation within the two environments. We then also considered carry-over effects across generations by characterising species differences after rearing flies under controlled laboratory conditions for a generation. Finally, species differences in the F2 generation should reflect mostly genetic effects (unless carry-over effects last across multiple generations), and in this generation we reared flies in two environments to directly assess their impact on resistance. Note that an assumption in this design is that genetic adaptation to laboratory conditions has limited impact on species differences. This has been tested previously (Kellermann et al., 2012b) and found to be a reasonable assumption, but it should be kept in mind particularly because some stress traits (and life history traits) can show evidence of laboratory adaptation (Sgrò and Partridge, 2001; Griffiths et al., 2005).

### Field flies and rearing

Flies were collected from a temperate (Nowra, NSW, Australia) and tropical (Cairns, Qld, Australia) site during April 2012 (Table 2). At Nowra, the average daily minimum temperature for the previous 2 weeks prior to collecting was 14°C and the average daily maximum was 24°C. At Cairns, the average daily minimum temperature for the previous 2 weeks was 20°C and the average maximum was 30°C. At their site of origin, flies from the *Drosophila* and *Scaptodrophila* genera were identified (*D. immigrans* Sturtevant, *D. pseudotakahashii* Mather, *D. setifemur* Malloch, *D. simulans* Sturtevant, *S. specensis* Bock, *S. lativittata* Malloch and *S. evanescens* van Klinken from the temperate environment; *D. bipectinata* Duda, *D. birchii* Dobzhansky and Mather, *D. bunnanda* Schiffer and McEvey, *D. hydei* Sturtevant, *D. melanogaster* Meigen, *D. pseudoananassae* Bock, *D. papuensis*-like, *D. rubida* Mather, *D. sulfurigaster* Duda and *S. novoguineensis* Duda from the tropical environment), sexed under $CO_2$ anaesthesia and allowed to recover for a minimum of 24 h on laboratory medium before testing for heat knockdown and cold recovery stress. Field flies used for testing were held as close to ambient temperature as possible; all testing was conducted at a room temperature (25°C). All flies were held and reared on laboratory 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), niapagin (1.6%) and acid mix (1.4% 10:1 proprionic acid:orthophosphoric acid). Concurrently, flies representing the same species were identified and sexed and sent back to the laboratory for rearing of F1 and F2 generations.

In the laboratory, 10 iso-female lines per species were set up and cultured at 19°C on a 12 h:12 h light:dark photoperiod to produce the F1 generation. The F2 generation was produced by rearing offspring of these females at 19°C or 28°C, as rearing temperature is known to influence cold recovery (Gibert and Huey, 2001; Rako and Hoffmann, 2006). As in the case of the field flies, F1 and F2 adults were sexed under $CO_2$ anaesthesia and allowed to recover on food medium for a minimum of 24 h before testing for resistance.

### Heat knockdown

Ten field flies of each sex and of each species were individually placed into numbered glass specimen vials (50 mm height × 12 mm diameter, SAMCO, San Fernando, CA, USA). The vials were then randomised and placed on a custom-built Perspex frame before

Table 2. Species tested from temperate and tropical sites in Australia with information on distribution and range

| | Species | Distribution | Australian southern limit | Australian northern limit (including Torres Strait Islands) |
|---|---|---|---|---|
| Temperate | *D. immigrans* | Cosmopolitan, temperate, urbanised, does not tolerate hot and dry conditions | Hobart, Tas 42°53′S | Cairns, Qld 16°52′S |
| | *D. pseudotakahashii* | Australia, rainforest | Nowra, NSW 34°51′S | Cairns, Qld 16°52′S |
| | *D. setifemur* | Australia, often frequents urban bushland | Ferntree Gully, Vic 37°53′S | Mt Bellenden Ker, Qld 17°16′S |
| | *D. simulans* | Cosmopolitan, urban | Hobart, Tas 42°53′S | Heathlands, Qld 11°37′S |
| | *S. evanescens* | Australia, rainforest | Nowra, NSW 34°51′S | Enoggera Creek, Qld 27°25′S |
| | *S. lativittata* | Australia, widespread, urban | Launceston, Tas 41°26′S | Atherton, Qld 17°16′S |
| | *S. specensis* | Australia, rainforest | Mt Saddleback, NSW 34°41′S | Cooktown, Qld 15°50′S |
| Tropical | *D. bipectinata* | South East Asia, Melanesia, Australia, urban | Townsville, Qld 19°22′S | Thursday Island 10°34′S |
| | *D. birchii* | Melanesia, Australia, rainforest | Byfield, Qld 22°49′S | Bamaga, Qld 10°53′S |
| | *D. bunnanda* | Australia, rainforest | Townsville, Qld 19°22′S | Heathlands, Qld 11°37′S |
| | *D. hydei* | Cosmopolitan, urban | Fairfield, Vic 37°47′S | Lake Eacham, Qld 17°17′S |
| | *D. melanogaster* | Cosmopolitan, urban | Hobart, Tas 42°53′S | Thursday Island 10°34′S |
| | *D. pseudoananassae* | South East Asia, New Guinea, Australia, rainforest/urban | Rockhampton, Qld 23°20′S | Moa Island 10°12′S |
| | *D. papuensis*-like | Australia, rainforest | Townsville, Qld 19°22′S | Cooktown, Qld 15°28′S |
| | *D. rubida* | New Guinea, Australia, rainforest | Townsville, Qld 19°18′S | Iron Range, Qld 12°43′S |
| | *D. sulfurigaster* | South East Asia, Melanesia, Australia, rainforest | Sydney, NSW 33°52′S | Moa Island 10°12′S |
| | *S. novoguineensis* | New Guinea and Australia, rainforest | Laceys Creek, Qld 27°11′S | Heathlands, Qld 11°14′S |

being immersed in a 28°C custom-built water bath. Temperature was controlled using a Ratek SP599 thermoregulator with a REX-P24 controller (Ratek, Boronia, Vic, Australia). The temperature was held at 28°C for 15 min then increased incrementally by 0.2°C $min^{-1}$ until reaching 38°C, after which the temperature stayed constant for the remainder of the experiment. This level of increase represents the maximum rate of increase in temperature likely to be encountered in the field (Terblanche et al., 2011). Flies were scored for time until heat knockdown; heat knockdown was defined as the point at which the flies were rendered unconscious, and identified when the flies were no longer able to hold themselves upright and did not respond to a light stimulus (a beam of light from a 12 LED hand torch). At the point of knockdown, the time was recorded to the nearest second, and then the corresponding specimen vial was removed from the water bath.

Ten F1 and F2 males and females for each species and rearing temperature were also assayed for heat knockdown following the procedure used for the field flies. Laboratory-reared flies were tested after 4–7 days of eclosion. We collected flies across this age range to allow sufficient numbers of flies to emerge for all the species.

### Cold recovery

For the assay on field flies, 10 flies of each sex and for each species were individually placed into numbered glass specimen vials (50 mm height × 12 mm diameter). The vials were then randomised and placed in ice where parentals were held at 0°C for 2 h (for tropical species) or 8 h (for temperate species). Note that we used different exposure times because most of the tropical field flies did not recover after 8 h, and most of the temperate flies were not knocked down after 2 h. After being cold stressed, the flies were removed from the ice and lined up for observation in a 25°C environment. Flies were scored to the second for time to recovery, which was defined as the ability to maintain a standing position.

The same procedure was used to score cold recovery after 2 or 8 h of stress at 0°C for the F1 and F2 generation (both variants of cold stress were scored for all temperate and tropical species). As for heat resistance, 10 males and females were tested from each species and flies were 4–7 days post-eclosion.

### Analysis

We tested the relative impact of the different environments, acclimation conditions and carry-over effects on mean resistance using ANOVA, and we also used *t*-tests to carry out independent contrasts in comparing means between generations and across rearing environments. All analyses were undertaken on untransformed data given that data were mostly normally distributed. We also estimated variance components to assess the magnitude of environmental, carry-over and genetic effects. For a single species, we defined $\mu_P$ as the mean of parental flies measured in the field, $\mu_{F1}$ as the mean of F1 flies measured in the laboratory after rearing at 19°C, $\mu_{F2,19°C}$ as the mean for the F2 generation after rearing at 19°C, and $\mu_{F2,28°C}$ as the mean for the F2 generation reared at 28°C (Table 1). We were interested in the contrast $\mu_P$–$\mu_{F2}$ measuring a combination of field environmental effects (including carry-over effects), the contrast $\mu_{F1}$–$\mu_{F2}$ measuring carry-over effects, and the contrast $\mu_{F2,19°C}$–$\mu_{F2,28°C}$ measuring the effects of developmental acclimation. Patterns were visualised by plotting species means for these generations against one another and comparing means with lines of unity (equal resistance). These also provided an indication of any differences between the tropical and temperate groups. *P*-values were corrected for multiple comparisons using the Bonferroni approach, correcting for the number of species within a stress test and generation.

To assess effects of the environmental conditions on the nature of species differences, we computed correlations among species means across the generations. Differences among species that were assumed to be genetic came from species reared under the same conditions and in the absence of field-related environmental and carry-over effects (i.e. the F2 generation, reared at 19 or 28°C). These were plotted against means obtained under the field conditions (which included the tropical *versus* temperate rearing conditions as well as other environmental and age-related effects) and those obtained in the F1 generation reared in the laboratory (which captured carry-over effects).

Finally, we considered variance estimates reflecting the effect of different components of the environment on species differences as outlined in Table 1. We calculated the effects of the

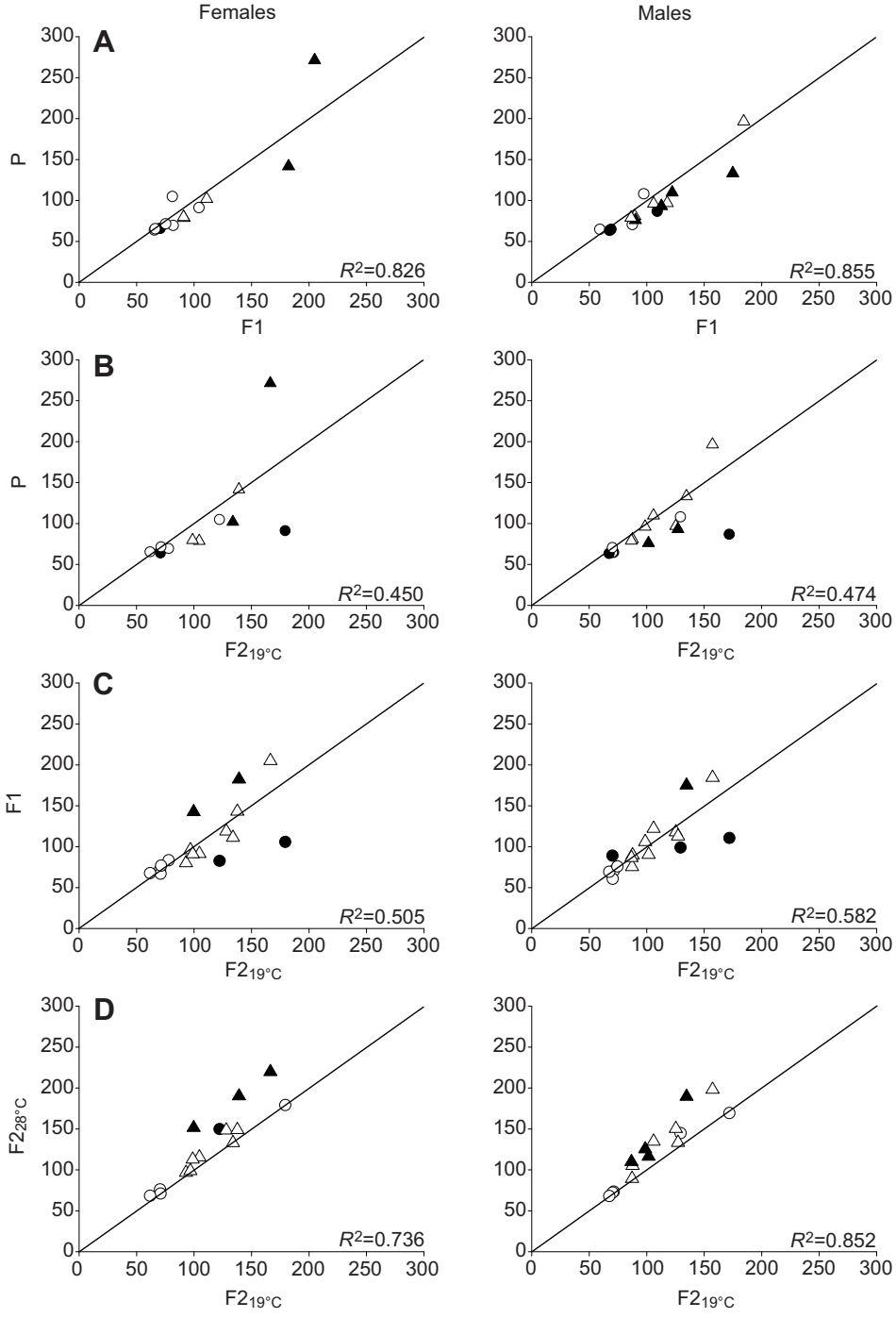


Fig. 1. Scattergrams depicting changes across generations for heat knockdown times of males and females. Species means (presented here in minutes) for comparisons of: the field parents (P) and both the F1 (A) and F2 (B) generations; the F1 and F2 generation; (C); and the F2 generations measured at 19 and 28°C (D). These correspond to the contrasts in Table 3. The solid lines reflect expectations based on identical values across the generations, and $R^2$ values are based on regression lines where the F2$_{19°C}$ treatment or F1 treatment is the independent variable. Triangles correspond to species collected from the tropical site; circles represent species collected from the temperate site. Solid symbols identify significant differences for individual species. *P*-values were corrected for multiple comparisons using the Bonferroni approach, correcting for the number of species within a stress test and generation. Note that species numbers vary in the graphs because we did not always capture enough individuals (particularly females) in the field generation.

environment, carry-over effects and genetic factors on species differences by estimating variance components among species in the different generations using ANOVA testing for species differences within different generations and environments. Species were only used in estimates of variance components when data were available for a particular species across all three generations. While most species could be included when obtaining estimates for the heat resistance assays, only temperate species were included for the 8 h cold exposure because species collected from the tropical site failed to survive this stress when tested at the parental field stage. Similarly, for the 2 h cold stress only tropical site species were included because temperate site species recovered almost immediately or were not knocked down under this stress. Variance components were not calculated for females for the 2 h cold stress as there were insufficient field females collected for some species. We then calculated the variance component due to overall environmental effects as the difference in variance components between field and F1 flies, and the component due to carry-over effects as the difference between the F1 and the F2 generation. The component due to genetic effects was extracted as the variance component in species differences in the F2 flies reared at 19°C (F2$_{19°C}$). The error variance component in the F2$_{19°C}$ comparison was used to estimate within-species and environmental variation, the variation left after the effect of species had been removed and when the species had been reared in the same environment.

Table 3. Comparisons across species in mean differences between generations (and 19/28°C environments for F2)

| | Females | | Males | |
|---|---|---|---|---|
| | Temperate | Tropical | Temperate | Tropical |
| Heat knockdown | | | | |
| Environmental (P–F1) | –199.9±588.4 (5) | –57.0±719.1 (5) | –760.6±588.2 (5) | –806.9±315.3 (9)* |
| Carry-over ($F2_{19°C}$–F1) | 1072.8±564.4 (7) | –382.4±559.4 (10) | 961.4±563.3 (7) | –301.6±307.9 (10) |
| Acclimation ($F2_{28°C}$–$F2_{19°C}$) | 539.6±623.6 (7) | 1306.4±559.4 (10)* | 91.1±627.1 (6) | 1457.5±307.9 (10)*** |
| Cold recovery (2 h stress) | | | | |
| Environmental (P–F1) | – | – | – | 56.3±154.5 (10) |
| Carry-over ($F2_{19°C}$–F1) | –190.1±134.2 (8) | –177.5±153.8 (10) | –128.2±78.9 (8) | –236.5±155.8 (10) |
| Acclimation ($F2_{28°C}$–$F2_{19°C}$) | 191.0±149.1 (6) | 707.9±153.8 (10)*** | 156.7±87.6 (6) | 464.0±155.5 (10)** |
| Cold recovery (8 h stress) | | | | |
| Environmental (P–F1) | – | – | 126.7±209.1 (4) | – |
| Carry-over ($F2_{19°C}$–F1) | –24.0±198.5 (7) | 513.4±186.6 (8)* | –284.4±214.0 (7) | 270.7±225.8 (8) |
| Acclimation ($F2_{28°C}$–$F2_{19°C}$) | 841.8±244.1 (6)*** | 299.4±310.7 (2) | 1126.6±255.1 (7)*** | 423.8±392.8 (2) |

Standard errors of contrasts are presented for mean values, and species numbers are given in parentheses. Note that species numbers vary between contrasts because insufficient numbers of individuals were available for testing some generations or conditions were too stressful (particularly for cold recovery after 8 h stress) or not stressful enough to generate knockdown (particularly for temperate field flies recovering after 2 h cold stress).
Significance levels for the contrasts are based on *t*-tests: *$P$<0.05, **$P$<0.1, ***$P$<0.001.

## RESULTS

### Heat knockdown

F1 flies tended to be similar or more resistant to heat than the parentals (Fig. 1A). A significant overall increase in resistance was evident for the tropical males although all contrasts were negative (Table 3). Females of *D. melanogaster* represented a notable exception to the overall pattern because for this group the F1 flies were less resistant than the parentals (supplementary material Table S1). Significant increases in male resistance in the F1 generation were detected for *D. hydei* and *S. novoguineensis* from the tropical collection site and *D. immigrans*, *D. setifemur* and *S. lativittata* from the temperate site. Differences between species were consistent across the parental and F1 generations, leading to high $R^2$ values ($R^2$>0.8, Fig. 1A) in both sexes. The $R^2$ value between sexes (not shown) was also high ($R^2$>0.9). Species from the tropical site were more resistant than species from the temperate site (ANOVA, $P$<0.001 in both parents and F1 when sexes were combined).

For comparisons of the parental and F2 (19°C) generations, $R^2$ values were substantially reduced ($R^2$<0.5, Fig. 1B), but correlations between generations were still significant (females, $r$=0.671, $P$=0.017; males, $r$=0.689, $P$=0.005). Where significant differences between generations were evident, species showed greater heat resistance in the F2 generation (19°C) (*D. rubida*, *D. sulfurigaster* and *S. novoguineensis* from the tropics and *D. immigrans*, *D. pseudotakahashii* and *S. lativittata* from the temperate site), except for *D. melanogaster*, where the parental generation had higher resistance.

In the F1 *versus* F2 (19°C) comparisons, $R^2$ values were only around 0.5, and several species differed significantly between these generations (Fig. 1C), with some tropical species (*D. hydei* and *D. pseudoananassae*) showing significantly higher heat resistance in the F1 flies, whilst some temperate species (*D. simulans* and *S. lativittata*) recorded higher resistance in the F2 generation (19°C) (supplementary material Table S1). However, overall there were no significant differences between these environments as evidenced by the contrast means (Table 3).

For the F2 generation, the flies reared at 28°C had higher heat resistance, as might be expected (Fig. 1D). The $R^2$ values obtained from a comparison of species means between temperature treatments were relatively high ($R^2$>0.7). The benefits of high temperature rearing were greater for the tropical site species compared with the temperate site species (with particularly large changes for *D. melanogaster*, *D. pseudoananassae* and *D. hydei* – see supplementary material Table S1). Acclimation effects were significant for both sexes in the case of the tropical site species (Table 3) and these species showed significantly larger changes in resistance than temperate site species (ANOVA, $F_{1,16}$=401.8, $P$<0.001 when sexes combined).

### Cold recovery

For the 2 h treatment, a comparison between parental and F1 flies was undertaken only for the tropical males because of the low number of field females available for this test; nevertheless, tropical female results are plotted for comparison (Fig. 2A). There were no consistent changes in resistance following laboratory culture (Table 3). Two species (*D. bipectinata* and *D. bunnanda*) showed higher resistance after laboratory culture, whereas *D. hydei* showed reduced resistance (supplementary material Table S1). For the males, $R^2$ values across generations were low ($R^2$<0.2), and there was only a low correlation between species means when compared at the parental and F1 stages ($r$=0.434, $P$=0.210, Fig. 2A). This was also evident in the P *versus* $F2_{19°C}$ comparison ($r$=0.130, $P$=0.721, Fig. 2B). The $R^2$ values were higher across the sexes within the same generation ($R^2$>0.6); the sexes were not significantly correlated in the parental generation ($r$=0.790, $P$=0.112), but highly correlated in the F1 ($r$=0.851, $P$<0.001) and F2 ($r$=0.851, $P$<0.001 for $F2_{19°C}$ and $r$=0.951, $P$<0.001 for $F2_{28°C}$) generations.

For the F1 *versus* $F2_{19°C}$ comparison, $R^2$ values were low (Fig. 2C), although correlations remained significant for both males ($r$=0.642, $P$=0.006) and females ($r$=0.492, $P$=0.045). F1 flies tended to have relatively long recovery times (lower resistance levels), resulting in positive contrasts (Table 3), although these were not significant. For the $F2_{19°C}$ *versus* $F2_{28°C}$ comparison, the 28°C treatment tended to reduce resistance relative to the 19°C rearing treatment, pointing to plastic responses (Fig. 2D), and this change was significant in the tropical species (both sexes), which comprised the majority of the species tested. Species showing individually

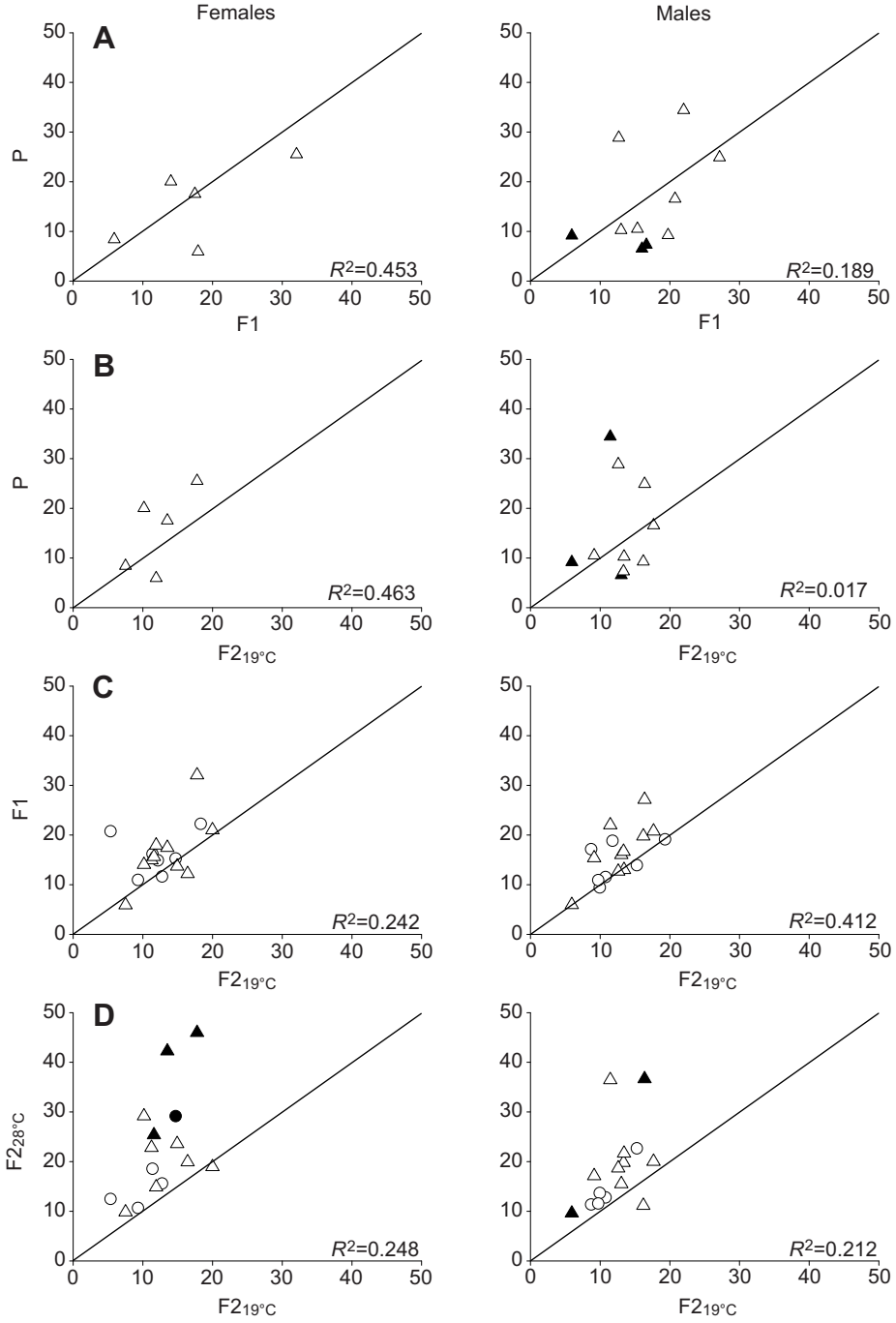


Fig. 2. Scattergrams depicting changes across generations for cold recovery time (2 h stress) of males and females. See Fig. 1 legend. Note that temperate species from the field (circles) were not included in these comparisons because they were not knocked down by the 2 h stress.

significant changes included *D. hydei*, *D. rubida*, *D. pseudoanannasae*, *D. setifemur* and *S. novoguineensis* (supplementary material Table S1).

For the 8 h treatment, parental data were only analysed for the temperate site males, given that female numbers for both sites were low and species from the tropical site mostly did not recover from this treatment. The temperate field males showed inconsistent changes in resistance when compared with the F1 generation (Fig. 3A) with no significant change overall (Table 3). However changes in individual species were significant in some cases, with an increase (*D. setifemur* and *S. lativittata*) or a decrease (*D. hydei*) in F1 resistance when compared with the parentals (supplementary material Table S1). The parental and F1 values for species means were not significantly correlated in males ($r$=0.544, $P$=0.130) and the correlation for females was also relatively weak ($r$=0.696, $P$=0.055) with low $R^2$ values (Fig. 3A). $R^2$ values in comparisons of species means between sexes from the same generation were higher ($R^2$>0.68) and correlated in the parental ($r$=0.825, $P$=0.012) and F1 ($r$=0.940, $P$<0.001) generations.

A carry-over effect was detected in tropical females (Table 3), with the F2 flies being less resistant than the F1 flies, although none of the other groups showed this pattern (Fig. 3C). Moreover, none of the individual species comparisons were significant with the exception of *D. melanogaster*. Acclimation effects due to rearing temperature were detected and significant for both sexes in the temperate site species, with the colder rearing temperature leading to a higher level of resistance (Table 3). Temperate site species

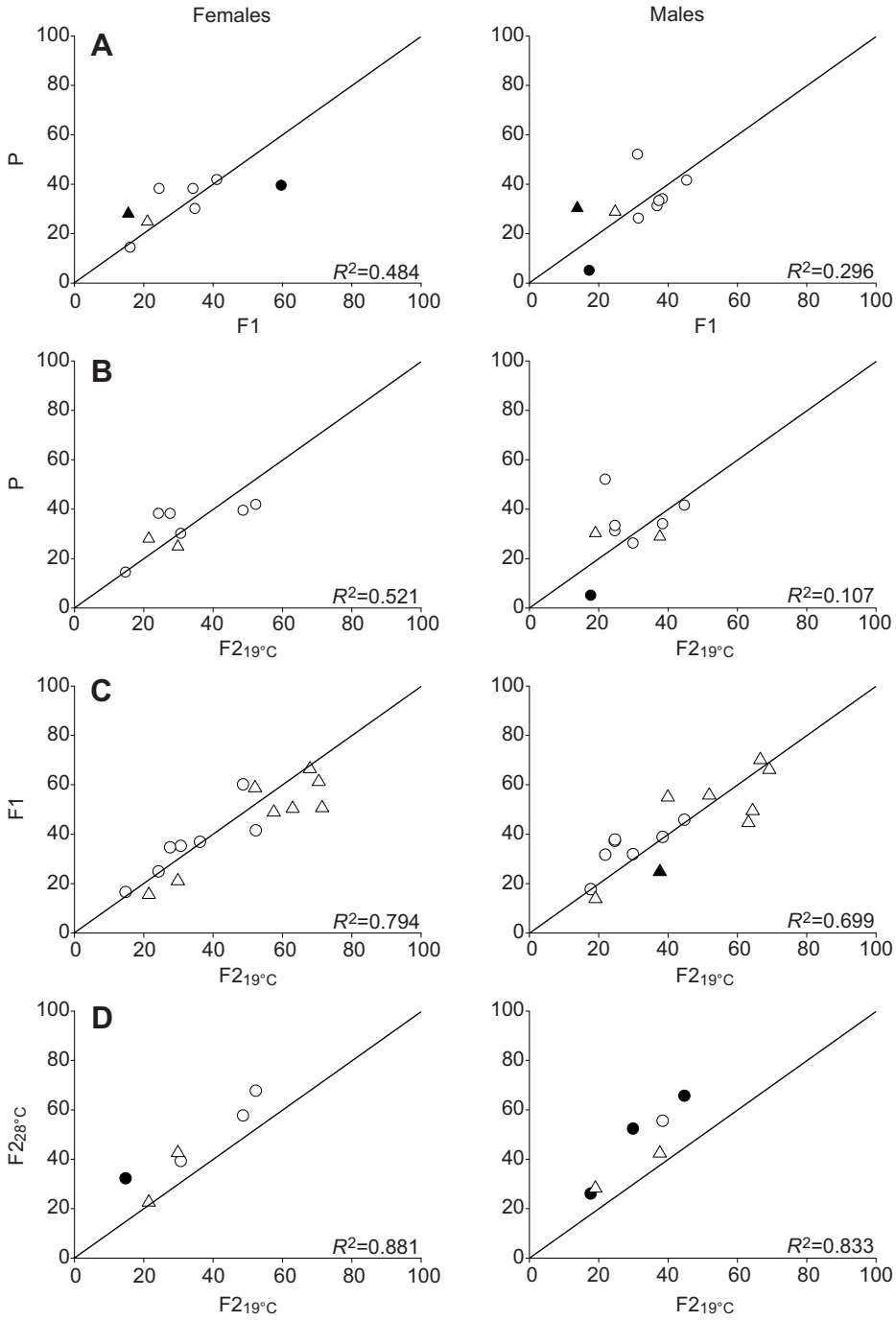


Fig. 3. Scattergrams depicting changes across generations for cold recovery time (8 h stress) of males and females. See Fig. 1 legend. Note that few tropical site species (triangles) were included in the comparisons with field flies because they often failed to recover from this stress.

showing a significant increase in resistance after being reared at 19°C were *D. simulans*, *D. setifemur*, *S. specensis* and *S. lativittata* (supplementary material Table S1).

### Variance components

We calculated variance components for the different comparisons to assess the relative contribution of genetic, carry-over and environmental effects across/within field sites on resistance levels. Variance components were extracted from ANOVA testing for species differences in different generations and environments, which are shown in supplementary material Table S2. For heat resistance, inherent differences among species contributed substantially to variability in both sexes (particularly in males), along with environmental effects across and within sites, while carry-over effects were smaller (Table 4). For 2 h cold resistance, environmental effects tended to be the most important, along with variability within species remaining after laboratory culture. Finally, for 8 h cold resistance, genetic and environmental effects had a similar level of importance in both sexes. Note that for heat resistance, species from both sites were tested, whereas for 8 h cold the comparison involved mostly temperate site species as opposed to tropical species for the 2 h treatment; this is likely to have influenced the magnitude of the environmental and species effects detected.

## DISCUSSION

The results suggest that the environmental factors and to a lesser extent carry-over effects can have a substantial impact on species

Table 4. Variance components as estimated using ANOVA

| Source of variation | Estimation | Heat | | Cold 2 h | Cold 8 h | |
|---|---|---|---|---|---|---|
| | | Females | Males | Males | Females | Males |
| Environment | P–F1 | 39 | 5 | 48 | 3 | 16 |
| Carry-over | F1–$F2_{19°C}$ | 16 | 16 | 16 | 15 | 8 |
| Genetic* (d.f.) | $F2_{19°C}$ | 39 (11) | 64 (13) | 7 (9) | 48(7) | 41 (8) |
| Within species and environments** (d.f.) | Error $F2_{19°C}$ | 6 (91) | 15 (127) | 30 (72) | 34 (69) | 35 (72) |

Estimates of variance components (%) were calculated using ANOVA (supplementary material Table S2) on species differences attributable to various sources of variation based on comparisons in different generations and environments (see Table 1 for source contributions – these indicate which figures were subtracted to obtain components). The contribution from variation within species and environments is also estimated.

*This term reflects species differences in the $F2_{19°C}$ comparison: the d.f. reflect the number of species tested in the $F2_{19°C}$ generation that were also tested in the earlier generations.

**This term reflects the error variance component in the $F2_{19°C}$ comparison: variation left after the effect of species has been removed and the species have been reared in the same environment.

variation for heat and cold resistance. In the case of cold resistance (particularly for the male data), these effects resulted in a low level of similarity between resistance levels of the species when measured on flies obtained directly from the field and flies reared under controlled laboratory conditions. For other traits (heat, female cold resistance) there was a moderate level of similarity, with $R^2$ values around 0.5. In contrast, when species were compared after being reared in different laboratory environments or when sexes of the same species were compared, $R^2$ values were often around 0.8 or higher even when few species were available for comparison. Some of the environmental variation is undoubtedly connected to the plastic effects associated with the different sites from where the parental generation of the species was collected. However, for cold resistance there was a low correlation between species means from the field and laboratory generations even when species came from the same collection site. The lower correlations across generations involving the field generation point to the impact of environmental conditions on species variation in resistance.

It is therefore important when making comparisons between taxa to consider the environment in which taxa are reared. This is particularly the case when evolutionary inferences are being made about species differences, as when establishing relationships within a phylogenetic framework (Strachan et al., 2011; Kellermann et al., 2012a). When most of the variation among species is due to environmental effects rather than heritable factors (Table 3) and acclimation/carry-over effects are not considered, it might be incorrectly concluded that a clade collected from one environment has a relatively higher level of resistance than another clade from a different environment, and that a phylogenetic signature for the resistance trait is present.

When species cannot be reared in the laboratory, it may be possible to hold them for a period in a uniform environment to 'remove' some of the environmental effects. This was done by Slabber and colleagues (Slabber et al., 2007) when comparing resistance levels among species of springtails along with the effects of acclimation. If carry-over effects or rearing effects have little impact on a trait, a period of acclimation may be sufficient to produce meaningful comparisons of resistance among species; however, when making species comparisons, some prior knowledge would be required before making such an assumption. For instance, heat resistance in field flies was often less than that in laboratory-reared F1 flies regardless of the collection location, and this may reflect the fact that the field flies developed under poor conditions.

Recently, there has been a renewed interest in carry-over effects of traits in general (Bonduriansky and Day, 2009; Yanagi and Tuda, 2010; Bonduriansky et al., 2012) and also for stress resistance (Donelson et al., 2012). We have searched for these effects in F1 *versus* F2 comparisons and found evidence for them for heat resistance; in several cases tropical site species exhibited F1 flies that were more resistant than the F2 flies, with both these generations being reared at a lower average temperature than experienced by the field generation. Substantial carry-over effects were also observed for heat resistance in several temperate species, although in these instances the F2 generation was more resistant than the F1 generation.

Carry-over effects have previously been noted for heat resistance in *D. simulans* tested directly from the field (Jenkins and Hoffmann, 1994) and also for other stress and life history traits in *Drosophila* species tested under controlled laboratory conditions (Crill et al., 1996; Hercus and Hoffmann, 2000; Magiafoglou and Hoffmann, 2003; Rako and Hoffmann, 2006). Carry-over effects may be adaptive, although this will depend on whether offspring encounter similar conditions to those experienced by the parental generation (in the case of positive effects) or dissimilar conditions (in the case of negative effects). This in turn will depend on the generation length of the species and seasonal temperature fluctuations. Positive carry-over effects might be adaptive in many *Drosophila* species from warm conditions because successive generations are likely to experience similar conditions. However, our results also point to a high level of variability in these effects across species, and it is perhaps worth noting that three of the species showing positive effects are widespread species.

In contrast to the results for heat resistance, carry-over effects were generally small for cold resistance, although for the 8 h cold treatment, tropical *D. melanogaster* F1 males were more resistant than the F2 flies (supplementary material Table S1). Carry-over effects that reduce progeny fitness after parental cold stress exposure have previously been documented in widespread *Drosophila* (Watson and Hoffmann, 1996). However, there are also reports of positive carry-over effects after thermal acclimation in *D. melanogaster* (Rako and Hoffmann, 2006) and in this species cold hardening can also increase progeny heat resistance (Sejerkilde et al., 2003).

In summary, these results highlight the challenges involved in meaningfully characterising thermal resistance variation across species for the purpose of using data in comparative analyses. Moreover, they indicate the ways in which field conditions can influence adult stress resistance both within and across generations. These sources of variation need to be considered in determining the vulnerability of species to climatic extremes, and highlight that caution is required in making inferences about species differences when environmental control across generations is not possible.

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## AUTHOR CONTRIBUTIONS

A.A.H. conceived the idea. M.S. designed the experimental protocols and specialised equipment required to execute the investigation. M.S. collected field specimens, carried out taxonomic identifications and conducted all field experiments. S.H. conducted all laboratory experiments. S.H. calculated variance components and generated scattergrams. M.S., A.A.H. and S.H. were all involved in the analysis and writing of the manuscript.

## COMPETING INTERESTS

No competing interests declared.

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**Table S1.** Contrasts for individual species, standard error of estimates, and associated P values from t tests. P values were corrected for multiple comparisons using Bonferroni and correcting for the number of species within a stress test and generation.

| | | | P–F1 | | | P–F2 | | | $F2_{19°C}–F_1$ | | | $F2_{28°C}–F2_{19°C}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Sex** | **Origin** | **Estimate** | **SE** | ***P*** | **Estimate** | **SE** | ***P*** | **Estimate** | **SE** | ***P*** | **Estimate** | **SE** | ***P*** |
| **Heat Knockdown** | | | | | | | | | | | | | | |
| *D. bipectinata* | F | Trop | | | | | | | 560 | 653 | 7.187 | 1213 | 653 | 1.339 |
| *D. bipectinata* | M | Trop | -744 | 753 | 4.614 | 223 | 753 | 11.528 | -967 | 753 | 3.728 | 1722 | 753 | 0.478 |
| *D. birchii* | F | Trop | | | | | | | 781 | 293 | 0.320 | 213 | 293 | 8.613 |
| *D. birchii* | M | Trop | | | | | | | 718 | 358 | 1.139 | 119 | 358 | 12.667 |
| *D. bunnanda* | F | Trop | | | | | | | 53 | 629 | 16.803 | 116 | 629 | 15.401 |
| *D. bunnanda* | M | Trop | -543 | 336 | 1.611 | -427 | 336 | 3.188 | -116 | 336 | 13.174 | 1040 | 336 | 0.065 |
| *D. hydei* | F | Trop | -2433 | 687 | **0.011** | 156 | 687 | 8.220 | -2589 | 687 | **0.011** | 3058 | 687 | **0.020** |
| *D. hydei* | M | Trop | -2504 | 641 | **0.006** | -91 | 641 | 13.320 | -2413 | 641 | **0.011** | 3288 | 641 | **0.019** |
| *D. melanogaster* | F | Trop | 3987 | 813 | **0.011** | 6295 | 813 | **0.011** | -2308 | 813 | 0.133 | 3195 | 813 | **0.007** |
| *D. melanogaster* | M | Trop | 717 | 978 | 6.556 | 2347 | 978 | 0.326 | -1631 | 978 | 1.874 | 2447 | 978 | 0.289 |
| *D. papuensis*-like | F | Trop | | | | | | | -320 | 442 | 8.554 | 670 | 442 | 2.547 |
| *D. papuensis*-like | M | Trop | -1260 | 663 | 0.914 | -1711 | 663 | 0.212 | 450 | 663 | 9.025 | 1502 | 663 | 0.502 |
| *D. pseudoananassae* | F | Trop | | | | | | | -2564 | 461 | **0.020** | 3107 | 461 | **0.020** |
| *D. pseudoananassae* | M | Trop | -600 | 498 | 3.301 | -163 | 498 | 11.189 | -438 | 498 | 6.932 | 1598 | 498 | **0.048** |
| *D. rubida* | F | Trop | -765 | 312 | 0.193 | -1560 | 312 | **0.011** | 795 | 312 | 0.274 | 646 | 312 | 0.823 |
| *D. rubida* | M | Trop | -858 | 248 | **0.020** | -1542 | 248 | **0.017** | 684 | 248 | 0.162 | 874 | 248 | **0.020** |
| *D. sulfurigaster* | F | Trop | -671 | 324 | 0.454 | -1144 | 324 | **0.011** | 473 | 324 | 2.747 | 834 | 324 | 0.256 |
| *D. sulfurigaster* | M | Trop | -452 | 272 | 1.473 | -467 | 272 | 1.422 | 15 | 272 | 17.235 | 1383 | 272 | **0.019** |
| *S. novoguinensis* | F | Trop | -543 | 569 | 3.462 | -1911 | 569 | **0.018** | 1369 | 569 | 0.383 | -55 | 569 | 16.623 |
| *S. novoguinensis* | M | Trop | -1181 | 322 | **0.008** | -2052 | 322 | **0.017** | 871 | 371 | 0.421 | 355 | 371 | 5.855 |
| *D. immigrans* | F | Temp | -369 | 102 | **0.009** | -368 | 102 | **0.009** | -1 | 102 | 17.903 | 248 | 102 | 0.356 |
| *D. immigrans* | M | Temp | -345 | 103 | **0.027** | -487 | 103 | **0.017** | 142 | 103 | 3.224 | 29 | 103 | 13.318 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. pseudotakahashii* | F | Temp | -199 | 105 | 0.670 | -519 | 105 | **0.001** | 320 | 105 | 0.081 | -79 | 105 | 8.264 |
| *D. pseudotakahashii* | M | Temp | 240 | 279 | 5.538 | -430 | 279 | 1.974 | 670 | 279 | 0.389 | 18 | 279 | 16.131 |
| *D. setifemur* | F | Temp | -137 | 272 | 6.179 | 127 | 265 | 6.357 | -264 | 265 | 5.895 | 307 | 294 | 5.452 |
| *D. setifemur* | M | Temp | -354 | 100 | **0.018** | -333 | 98 | **0.029** | -21 | 98 | 14.972 | -55 | 146 | 12.056 |
| *D. simulans* | F | Temp | 1332 | 473 | 0.079 | -1136 | 473 | 0.217 | 2468 | 473 | **0.002** | 1563 | 473 | **0.040** |
| *D. simulans* | M | Temp | 547 | 487 | 3.767 | -1372 | 487 | 0.120 | 1919 | 487 | **0.007** | 836 | 501 | 1.766 |
| *S. specensis* | F | Temp | | | | | | | -234 | 316 | 8.397 | -601 | 316 | 1.262 |
| *S. specensis* | M | Temp | | | | | | | -1037 | 294 | **0.038** | -76 | 317 | 13.840 |
| *S. lativittata* | F | Temp | -871 | 452 | 0.620 | -5374 | 452 | **0.001** | 4503 | 452 | **0.002** | -102 | 452 | 14.819 |
| *S. lativittata* | M | Temp | -1432 | 432 | **0.029** | -5196 | 432 | **0.011** | 3764 | 432 | **0.020** | -243 | 432 | 9.821 |
| *S. evanescens* | F | Temp | | | | | | | -234 | 185 | 4.001 | -84 | 167 | 11.219 |
| *S. evanescens* | M | Temp | | | | | | | 17 | 280 | 17.165 | | | |
| **Cold Recovery (2 h stress)** | | | | | | | | | | | | | | |
| *D. bipectinata* | F | Trop | | | | | | | 75 | 221 | 13.295 | 520 | 227 | 0.488 |
| *D. bipectinata* | M | Trop | 570 | 113 | **0.011** | 392 | 113 | **0.014** | -178 | 113 | 2.228 | 148 | 113 | 3.149 |
| *D. birchii* | F | Trop | | | | | | | -223 | 253 | 7.092 | 696 | 241 | 0.194 |
| *D. birchii* | M | Trop | 292 | 194 | 1.474 | -81 | 194 | 6.798 | -373 | 224 | 1.991 | 479 | 215 | 0.579 |
| *D. bunnanda* | F | Trop | | | | | | | 255 | 215 | 4.435 | 210 | 221 | 5.606 |
| *D. bunnanda* | M | Trop | 562 | 150 | **0.006** | 364 | 150 | 0.203 | -197 | 150 | 3.541 | 386 | 150 | 0.230 |
| *D. hydei* | F | Trop | -150 | 82 | 0.372 | -54 | 82 | 2.583 | 97 | 82 | 4.403 | 140 | 82 | 1.520 |
| *D. hydei* | M | Trop | -192 | 60 | **0.030** | -192 | 60 | **0.030** | -1 | 59 | 17.878 | 221 | 59 | **0.010** |
| *D. melanogaster* | F | Trop | 719 | 293 | 0.096 | 358 | 293 | 1.152 | -362 | 293 | 4.063 | 177 | 293 | 8.784 |
| *D. melanogaster* | M | Trop | 630 | 379 | 1.056 | 416 | 379 | 2.796 | -213 | 379 | 10.390 | -302 | 379 | 6.890 |
| *D. papuensis*-like | F | Trop | | | | | | | -61 | 212 | 13.982 | -63 | 212 | 12.310 |
| *D. papuensis*-like | M | Trop | 252 | 144 | 0.890 | 67 | 144 | 6.467 | -185 | 144 | 3.721 | 141 | 144 | 5.328 |
| *D. pseudoananassae* | F | Trop | | | | | | | -242 | 169 | 2.956 | 827 | 169 | **0.018** |
| *D. pseudoananassae* | M | Trop | 164 | 175 | 3.548 | 190 | 175 | 2.858 | 26 | 175 | 15.925 | 494 | 175 | 0.125 |
| *D. rubida* | F | Trop | 392 | 476 | 2.077 | -464 | 476 | 1.681 | -856 | 476 | 1.447 | 1691 | 476 | **0.018** |
| *D. rubida* | M | Trop | 135 | 334 | 6.898 | -510 | 334 | 1.361 | -645 | 325 | 0.999 | 1216 | 325 | **0.011** |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. sulfurigaster* | F | Trop | -361 | 477 | 2.271 | -593 | 477 | 1.111 | -232 | 477 | 11.344 | 1141 | 477 | 0.355 |
| *D. sulfurigaster* | M | Trop | -972 | 424 | 0.279 | -975 | 424 | 0.274 | -3 | 424 | 17.888 | 364 | 424 | 6.333 |
| *S. novoguinensis* | F | Trop | -5 | 380 | 4.946 | -242 | 380 | 2.642 | -237 | 370 | 9.472 | 1725 | 370 | **0.018** |
| *S. novoguinensis* | M | Trop | -747 | 454 | 1.076 | -1379 | 454 | **0.041** | -633 | 510 | 3.989 | 1501 | 510 | 0.083 |
| *D. immigrans* | F | Temp | | | | | | | -909 | 390 | 0.502 | 412 | 401 | 5.022 |
| *D. immigrans* | M | Temp | | | | | | | -490 | 254 | 1.159 | 145 | 254 | 9.181 |
| *D. pseudotakahashii* | F | Temp | | | | | | | -273 | 215 | 3.883 | 416 | 215 | 1.029 |
| *D. pseudotakahashii* | M | Temp | | | | | | | -29 | 85 | 13.199 | 106 | 85 | 3.624 |
| *D. setifemur* | F | Temp | | | | | | | -18 | 142 | 16.234 | 853 | 176 | 0.018 |
| *D. setifemur* | M | Temp | | | | | | | 96 | 127 | 8.219 | 431 | 169 | 0.298 |
| *D. simulans* | F | Temp | | | | | | | 83 | 187 | 11.907 | 155 | 187 | 6.600 |
| *D. simulans* | M | Temp | | | | | | | 47 | 128 | 12.942 | 209 | 128 | 1.858 |
| *S. specensis* | F | Temp | | | | | | | -221 | 648 | 13.279 | | | |
| *S. specensis* | M | Temp | | | | | | | 28 | 381 | 16.974 | | | |
| *S. lativittata* | F | Temp | | | | | | | -83 | 98 | 7.243 | 68 | 98 | 7.923 |
| *S. lativittata* | M | Temp | | | | | | | -55 | 73 | 8.249 | 95 | 73 | 3.278 |
| *S. evanescens* | F | Temp | | | | | | | -149 | 458 | 13.568 | | | |
| *S. evanescens* | M | Temp | | | | | | | -407 | 238 | 2.673 | | | |
| **Cold Recovery (8 h stress)** | | | | | | | | | | | | | | |
| *D. bipectinata* | F | Trop | | | | | | | 555 | 417 | 3.272 | | | |
| *D. bipectinata* | M | Trop | | | | | | | -211 | 546 | 11.256 | | | |
| *D. birchii* | F | Trop | | | | | | | -397 | 1366 | 12.474 | | | |
| *D. birchii* | M | Trop | | | | | | | -901 | 453 | 1.309 | | | |
| *D. bunnanda* | F | Trop | | | | | | | 753 | 463 | 1.974 | | | |
| *D. bunnanda* | M | Trop | | | | | | | 894 | 418 | 0.832 | | | |
| *D. hydei* | F | Trop | -750 | 204 | **0.005** | -396 | 204 | 0.361 | 354 | 204 | 1.462 | 68 | 204 | 6.673 |
| *D. hydei* | M | Trop | -988 | 253 | **0.002** | -674 | 253 | 0.081 | 314 | 253 | 3.555 | 551 | 253 | 0.362 |
| *D. melanogaster* | F | Trop | -228 | 341 | 3.052 | 299 | 341 | 2.315 | 527 | 341 | 2.094 | 767 | 341 | 0.276 |
| *D. melanogaster* | M | Trop | -247 | 209 | 1.471 | 527 | 209 | 0.113 | 773 | 209 | **0.011** | 286 | 209 | 1.787 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. papuensis*-like | F | Trop | | | | | | | 88 | 580 | 14.102 | | | | |
| *D. papuensis*-like | M | Trop | | | | | | | 183 | 463 | 11.214 | | | | |
| *D. pseudoananassae* | F | Trop | | | | | | | 1248 | 459 | 0.243 | | | | |
| *D. pseudoananassae* | M | Trop | | | | | | | 1114 | 426 | 0.291 | | | | |
| *D. sulfurigaster* | F | Trop | | | | | | | 520 | 598 | 6.397 | | | | |
| *D. sulfurigaster* | M | Trop | | | | | | | -232 | 459 | 9.920 | | | | |
| *D. immigrans* | F | Temp | | | | | | | -10 | 376 | 15.670 | 812 | 376 | 0.361 | |
| *D. immigrans* | M | Temp | | | | | | | -559 | 624 | 6.056 | 1783 | 624 | 0.081 | |
| *D. pseudotakahashii* | F | Temp | -22 | 608 | 5.828 | 655 | 608 | 1.735 | 677 | 608 | 4.378 | 895 | 670 | 1.715 | |
| *D. pseudotakahashii* | M | Temp | 286 | 550 | 3.640 | 288 | 535 | 4.157 | 2 | 550 | 15.947 | 999 | 550 | 0.779 | |
| *D. setifemur* | F | Temp | 1238 | 416 | **0.035** | 577 | 396 | 0.935 | -661 | 407 | 1.845 | 516 | 590 | 3.507 | |
| *D. setifemur* | M | Temp | 254 | 285 | 2.285 | 216 | 271 | 3.030 | -38 | 279 | 14.275 | 1230 | 404 | **0.049** | |
| *D. simulans* | F | Temp | 304 | 288 | 1.786 | 61 | 288 | 5.003 | -243 | 288 | 6.454 | 487 | 288 | 0.895 | |
| *D. simulans* | M | Temp | 341 | 239 | 0.970 | 247 | 239 | 2.148 | -94 | 239 | 11.157 | 1324 | 239 | **0.011** | |
| *S. specensis* | F | Temp | | | | | | | -395 | 199 | 0.944 | 612 | 199 | **0.049** | |
| *S. specensis* | M | Temp | | | | | | | -731 | 265 | 0.179 | 367 | 265 | 1.795 | |
| *S. lativittata* | F | Temp | 124 | 244 | 3.680 | 45 | 244 | 5.124 | -79 | 244 | 11.960 | 1020 | 244 | **0.002** | |
| *S. lativittata* | M | Temp | 758 | 152 | **0.007** | 784 | 152 | **0.008** | 27 | 152 | 13.808 | 474 | 152 | **0.036** | |
| *S. evanescens* | F | Temp | | | | | | | -11 | 248 | 15.469 | | | | |
| *S. evanescens* | M | Temp | | | | | | | -762 | 283 | 0.296 | 493 | 193 | 0.242 | |

**Table A2**. ANOVAs testing for species differences between generations

| | | females | | | | males | | | |
|---|---|---|---|---|---|---|---|---|---|
| **heat knockdown** | | | | | | | | | |
| generation | source | df | MS | F | P | df | MS | F | P |
| P | species | 13 | 44357947 | 45 | **<0.001** | 9 | 142334706 | 169 | **<0.001** |
| $F_1$ | species | 13 | 47602929 | 30 | **<0.001** | 9 | 83925995 | 92 | **<0.001** |
| $F_{2(19)}$ | species | 13 | 38327877 | 43 | **<0.001** | 9 | 60366212 | 64 | **<0.001** |
| | | | | | | | | | |
| **cold recovery (2 h stress)** | | | | | | | | | |
| generation | source | df | MS | F | P | | | | |
| P | species | 9 | 4485602 | 6 | **<0.001** | | | | |
| $F_1$ | species | 9 | 1234896 | 5 | **<0.001** | | | | |
| $F_{2(19)}$ | species | 9 | 414429 | 3 | **0.002** | | | | |
| | | | | | | | | | |
| **cold recovery (8 h stress)** | | | | | | | | | |
| generation | source | df | MS | F | P | df | MS | F | P |
| P | species | 7 | 3106509 | 8 | **<0.001** | 8 | 5700858 | 7 | **<0.001** |
| $F_1$ | species | 7 | 7595642 | 18 | **<0.001** | 8 | 3580003 | 16 | **<0.001** |
| $F_{2(19)}$ | species | 7 | 6218662 | 16 | **<0.001** | 8 | 3289811 | 13 | **<0.001** |