1 Title: Novel applications of thermocyclers for phenotyping invertebrate thermal responses

2

3 Short Running Title: Characterising invertebrate thermal responses

4

5 Word Count: 6764

6

7 Jacinta D. Kong, Jason K. Axford, Ary A. Hoffmann & Michael R. Kearney

8 School of BioSciences, The University of Melbourne, Parkville, VIC 3010, Australia

9

10 Corresponding author: Jacinta D. Kong, jacintadkong@gmail.com

Summary

1. High-throughput genomic methods are increasingly used to investigate invertebrate thermal responses with greater dimensionality and resolution than previously achieved. However, corresponding methods for characterising invertebrate phenotypes are still lacking. To scale up the characterisation of invertebrate thermal responses, we propose a novel use of thermocyclers as temperature-controlled incubators.
2. Here, we tested the performance of thermocyclers as incubators and demonstrated the application of this method to efficiently characterise the thermal responses of model and non-model invertebrates.
3. We found the thermocyclers performed with high precision, accuracy and resolution under various and fluctuating ambient conditions. We were able to successfully characterise the temperature-dependent development of grasshopper eggs (*Warramaba virgo*), as well as the effects of fluctuating temperature cycles on the survival of mosquito eggs (*Aedes aegypti*) and developmental success of *Drosophila simulans* larvae, all with similar survival rates to conventional methods.
4. Thermocyclers are a general and transferrable means to scale up current methods of incubating small invertebrates. They permit rapid characterisation of high-dimensional physiological responses to natural thermal regimes. When combined with existing approaches in thermal and evolutionary biology, these methods will advance our understanding of, and ability to predict, biological adaptations and responses to environmental changes.

Key Words

Thermal response, invertebrate ecophysiology, mechanistic modelling, thermal biology

Introduction

Thermal adaptations and responses underpin many components of the ecology and evolution of organisms (Angilletta 2009). The adaptations of organisms to the thermal environment have important roles in defining the phenology and distribution of many species (Gaston 2003; Danks 2007; Bozinovic, Calosi & Spicer 2011). For example, adaptive changes to the thermal niche of the red-legged earth mite *Halotydeus destructor* facilitated the range expansion of this invasive pest into Australia (Hill, Chown & Hoffmann 2013). Identifying the relationships between adaptive traits and the thermal environment has become increasingly important to understand the responses of organisms to changing temperatures within the context of anthropogenic climate change (Chown *et al.* 2010; Bozinovic *et al.* 2011; Araújo *et al.* 2013). Accordingly, such relationships have been incorporated into process-explicit models of phenology or geographic distribution under current and future climates (e.g. Morin, Augspurger & Chuine 2007; Buckley 2010; Kolbe, Kearney & Shine 2010).

With growing interest in thermal responses within the context of environmental change, there is a need to develop efficient methods of assessing organismal responses to a range of temperatures. Such methods need to account for the fluctuations in temperature which occur in natural environments and which are crucial in establishing thermal limits (Terblanche *et al.* 2011). Efficient methods of characterising thermal response are particularly important in assessing the evolutionary potential of organisms to adapt to future temperature conditions (Chown *et al.* 2010; Bozinovic *et al.* 2011; Araújo *et al.* 2013; Overgaard, Kearney & Hoffmann 2014), where heritable variation may need to be established through comparisons of large numbers of families (e.g. Mitchell & Hoffmann 2010). Phenotypic data that can complement genomic data is imperative for identifying causal relationships between

genotypes, the environment and evolutionary process (Houle, Govindaraju & Omholt 2010; Fahlgren, Gehan & Baxter 2015; Großkinsky *et al.* 2015). Scaling-up current methods of characterising thermal phenotypes are essential in developing links between thermal phenotypes and genetic markers (Travisano & Shaw 2013), particularly now that Next Generation Sequencing (NGS) has potential to identify novel links between the genes underlying thermal stress response and the corresponding phenotypic traits and environmental cues (Porcelli *et al.* 2015).

Current methods of characterising phenotypic thermal responses using automated incubators or water baths have changed little over the years (e.g. Gibert & Huey 2001) and often rely on custom-built set-ups (e.g. Woods & Bonnecaze 2006). Such methods are often only able to process tens of individuals at once. Experimental studies can be further hindered by an inability of incubators to replicate fluctuating conditions in the laboratory, particularly seasonal or daily field temperature cycles. Fluctuating temperatures are more representative of natural conditions than constant temperatures, and may consequently change the dynamics of life history traits in manipulative experiments (Terblanche *et al.* 2011; Carrington *et al.* 2013; Colinet *et al.* 2015).

Thermocyclers, as commonly used to perform biochemical reactions such as Polymerase Chain Reactions (PCR), are able to generate constant and fluctuating temperature cycles with fine-scale resolution within a range of biologically relevant temperatures. Thermocyclers are also able to generate a gradient of temperatures to allow for parallel incubation of invertebrates within a single unit compared to using multiple incubator units. A single thermocycler unit is able to incubate tens of PCR tubes containing small invertebrates. To develop an efficient method of characterising invertebrate thermal response, we tested the use

of thermocyclers as temperature-controlled incubators. Here, we (i) measured the spatial and temporal variation in temperature across the thermocycler block under various ambient conditions to determine the accuracy and precision of the thermocycler in the context of characterising thermal responses, and (ii) illustrate this method in a case study approach to investigate egg development under constant temperatures in a grasshopper *Warramaba virgo* as well as the effect of daily cycling temperatures on egg survival of the mosquito *Aedes aegypti* and larval developmental success in *Drosophila simulans*. We show that thermocyclers provide an affordable and practical means to scale up the incubation of small invertebrates under replicated field conditions. Increasing the throughput of individuals, dimensionality and precision of phenotyping thermal response has potential to provide thermal biologists with the necessary tools to investigate thermal responses under changing environments.

Materials and Methods

Thermocycler description

We tested two Biometra thermocyclers (Göttingen, Germany), the 0.2 mL TProfessional TRIO 48 (hereafter TRIO 48) and the 0.2 mL TProfessional Gradient 96 (hereafter Gradient 96). The TRIO 48 has three independently-controlled heated blocks with individual lids. A maximum of 48 0.2 mL single PCR tubes in a six by eight arrangement can be placed in each heated block for a total of 144 PCR tubes per thermocycler unit. The Gradient 96 has a single heated block capable of generating a temperature gradient, with a maximum of 96 0.2 mL single PCR tubes in an eight by twelve arrangement. PCR plates or tube strips are also compatible for use with either thermocycler model. Both models of thermocyclers will run with the lid of the heated block left open. The thermocyclers can be programmed to run fluctuating temperature cycles or constant temperatures for an indefinite period of time.

111

112 *Thermocycler performance*

113 We evaluated the precision and accuracy of a TRIO 48 thermocycler under potential
114 conditions required for measuring thermal responses. Temperatures within individual PCR
115 tubes were recorded using thermocouple wire (TT-T-40-200, Omega Engineering,
116 Connecticut, U.S.A.) connected to a data logger (CR1000, Campbell Scientific, Utah,
117 U.S.A.). The thermocouple wires were secured through a hole in the closed lid of individual
118 PCR tubes. The thermocycler was programmed to run a temperature ramp between 10 °C and
119 39.5 °C in 0.5 °C increments lasting 10 min each. To examine the effect of ambient
120 temperature on the temperatures within the thermocycler, we placed the thermocycler unit
121 within a temperature-controlled room at either 15, 20 or 25 °C. To examine the effect of
122 exposure to ambient conditions on temperature in the thermocycler, we ran two heated blocks
123 simultaneously with either the lid of the thermocycler lowered but not sealed closed, or the
124 lid left completely open.

125

126 We further evaluated thermocycler performance by examining spatial variation of
127 temperatures in the thermocycler across a heated block and within a single PCR tube. We
128 tested for horizontal spatial variation of temperature across the heated block by comparing
129 temperatures within PCR tubes placed in each corner (n = 4) and in the centre of the block (n
130 = 3). To characterise vertical gradients of temperature within a single PCR tube, we placed
131 two thermocouple wires in each PCR tube: at the bottom of the tube where the closest contact
132 with the heated block is located, and 5 mm from the bottom of the tube. We tested for a
133 potential vertical thermal gradient within a PCR tube because contact with the heated block is
134 limited to the bottom 5 mm of the PCR tube, above which the PCR tube is not in direct
135 contact with a source of heat. This height also corresponds with the top of our experimental

grasshopper egg when placed in the tube, thus a gradient may influence the thermal microenvironment of the PCR tube.

Temperature-dependent Warramaba virgo egg development

To validate the suitability of the thermocycler as incubators for insect eggs, we used eggs from the non-model Orthopteran *Warramaba virgo*. We first examined whether *W. virgo* eggs could be incubated within PCR tubes in the thermocycler, and determined an appropriate method of incubating *W. virgo* eggs. Eggs were obtained from a field-collected colony of *W. virgo* maintained in the laboratory. Eggs were selected from different females to control for effects of maternal age and identity on egg development.

We examined whether the proportion of eggs developing successfully depended on the exposure of the egg to ambient conditions, the presence of substrate and the presence of liquid water in a factorial crossed design. Whether eggs were exposed to ambient conditions depended on whether the lid of the PCR tube was left open, closed with a hole in the lid to allow gas exchange, or closed without a hole in the lid. Eggs of *W. virgo* are laid in sandy soil in the field, thus the tubes either had nothing or sand as a substrate to mimic natural conditions. We further tested the hypothesis that *W. virgo* eggs do not require the presence of water during later stages of development. Eggs were either kept moist throughout the incubation period or only for the first 10 days, after which no additional water was provided.

Eggs (n = 144) were assigned to each incubation treatment and incubated in individual PCR tubes within the TRIO 48 thermocycler at 30 °C, with the thermocycler lid lowered as described previously. Eggs of *W. virgo* have been successfully incubated between 27 and 32 °C in preliminary studies (M. R. Kearney and J. D. Kong, unpublished data). A block design

was employed to minimise the effects of spatial variation in temperature across the thermocycler block. PCR tubes were randomly assigned places within the block design. We scored the proportion of eggs to successfully complete development as defined by when the eggs changed colour from yellow to brown, which indicates they are ready to hatch. Eggs that did not finish development at the end of the experimental period were considered to be unsuccessful.

To demonstrate the application of thermocyclers to characterise invertebrate thermal response, we examined the temperature-dependent egg development of *W. virgo* under a gradient of constant temperatures. We incubated eggs (n = 96) across a gradient of 12 temperatures between 23.3 °C and 38.5 °C in the Gradient 96 thermocycler with the lid of the thermocycler lowered. Eggs were individually incubated in PCR tubes which had a closed lid with a hole in the lid, sand as a substrate and were kept moist throughout incubation. We scored the proportion of eggs to initiate development as indicated by eggs visibly becoming turgid and swelling.

Aedes aegypti egg survival under fluctuating daily temperature cycles

We further validated the thermocycler for use with insect eggs by focussing on *Aedes aegypti* mosquitoes, which during the dry season rely on the ability of quiescent eggs to tolerate desiccation and thermal stress. To test egg survival, we incubated batches of eggs in the TRIO 48 thermocycler under fluctuating daily temperature cycles with a focus on upper thermal limits. Eggs were exposed to eleven programs representing a daily oscillation of 20 °C for one week. Each program differed by 1 °C with the least stressful program starting at a minimum of 15 °C and reaching a peak of 35 °C during midday, whilst the most stressful program ranged from 25 °C to 45 °C. We included a constant 26 °C program to act as a

control, which is the optimal temperature for survival and development of this *Ae. aegypti* stock. Two groups of six replicates were included to test for an effect of allowing gas exchange (PCR tube lids with a 1 mm hole) or not (no hole).

Eggs were collected from a mass-reared, wildtype *Ae. aegypti* laboratory colony onto filter paper over 24 hours and allowed to fully embryonate over three days thereafter. On the third day, dried eggs were dislodged into a glass vial (2.5 x 5 cm) using a paintbrush. From there they were divided into 0.2 mL PCR tubes through a funnel, in batches that ranged from 15 to 29 eggs. All eggs were confirmed resting in the bottom of each tube (below thermocycler heating element level) before treatment, where temperature control accuracy is greatest.

PCR tubes were placed randomly within each thermocycler block in a TRIO 48 thermocycler and all programs were initiated around the same time. Lids on the TRIO 48 were lowered but not closed. Once all programs were complete, egg survival was assessed by adding 200 µL of RO water with a concentration of 0.25 g L^{-1} of active dried yeast to each tube to stimulate hatching. First instar larvae were counted under a dissecting microscope and hatch rate per tube determined.

Drosophila simulans egg to pupae survival under fluctuating daily temperature cycles

We determined the suitability of the thermocycler to characterise thermal responses in the model organism *Drosophila simulans* under three daily fluctuating temperature cycles. A TRIO 48 thermocycler was used to run each program concurrently for 3 days and included temperature cycles in the ranges of 15 – 23 °C, 20 − 28 °C and 25 – 33 °C. *Drosophila simulans* was sourced from a laboratory line derived from Brisbane, Australia which had been reared on a sucrose-dead yeast-agar medium at a constant 19 °C since 2013. To

211 determine a suitable method for incubating *D. simulans* in a TRIO 48 thermocycler we added
212 a 1 mm hole to the lid of the PCR tube in half of the replicates (48 total) to allow gas
213 exchange, and assessed the effect of high or low levels of food by adding either 200 µL
214 (high) or 100 µL (low) of media to each tube using a pipette. Single eggs less than 1 hour old
215 were then added using a straight teasing needle to the top of the fly media in each tube. We
216 assigned tubes randomly to each thermocycler block within 10 minutes of the egg being
217 placed. Once the programs were complete the tubes were removed and scored over time for
218 survival until all individuals had either pupated or died.
219
220 *Statistical analysis*
221 Variation in temperature within the thermocycler under various ambient conditions was
222 assessed with Analysis of Covariance (ANCOVA) with the programmed temperature as the
223 covariate and the mean recorded temperature as the response variable. The effect of
224 incubation method on the proportion of eggs to successfully complete development was
225 assessed by a Generalised Logistic Regression following a binomial distribution (binomial
226 GLM). The effect of temperature on the proportion of *W. virgo* eggs to initiate development
227 was assessed by a binomial GLM. The effect of temperature, treated as a factor for each
228 thermocycler program, upon the hatch rate of quiescent *Ae. aegypti* eggs was assessed in an
229 Analysis of Variance (ANOVA) after data were checked to test for normality and
230 homoscedasticity. Incubation method (tubes with or without a hole) was included as a factor
231 in the ANOVA model. Where significant effects were detected, posthoc Tukey Honest
232 Significance Differences (Tukey HSD) were used to compare treatments. The constant 26 °C
233 treatment was set as a reference group. The proportion of *D. simulans* surviving to pupation
234 was assessed in a binomial GLM to test for the effects of temperature and incubation method

on survival to the pupal stage. Statistical analysis was done in R v.3.2.2 (R Core Team 2015) run in RStudio v.0.99.484 (RStudio Team 2015).

Results

*Thermocycler performance*The thermocyclers performed with high precision across all ambient conditions (Fig. 1). We present the accuracy of a thermocycler as differences between the recorded temperatures and the programmed temperature of the thermocycler. The overall accuracy of the thermocycler remained within 4.78 °C of the programmed temperature consistently across all ambient conditions, while temperatures at the bottom of a PCR tube remained within 1.67 °C of the programmed temperature across all ambient conditions (Fig. 1). For all cases, the accuracy of the thermocyclers at each programmed temperature decreased relative to the ambient temperature (Fig. 1, ANCOVA, $F_{1, 100715} = 7.97$, $P < 0.05$). Temperatures within the thermocycler varied vertically between the top and the bottom of a PCR tube (Fig. 1, ANCOVA, $F_{1, 100715} = 71134.0$, $P < 0.001$), and horizontally between the centre and edges of the thermocycler block (ANCOVA, $F_{1, 100715} = 6079.3$, $P < 0.001$). These thermal gradients were consistent across all ambient temperatures. The accuracy of the thermocyclers was greatest at the bottom of the tube and in the centre of the thermocycler block, with the mean difference (± standard error) between the programmed and recorded temperatures ranging between −0.82 °C (± 0.028 °C) and 1.52 °C (± 0.023 °C) across all possible ambient conditions.

We found that large fluctuations in ambient temperature decreased the precision of the thermocycler when the lid of the thermocycler was left open, and that the lid of the thermocycler buffeted against this noise (Fig. 1). When the lid of the thermocycler was left open, any differences between the centre and the edge of the thermocycler block were

masked by the noise of the ambient temperature. When the thermocycler lid was lowered, the differences in temperature between the centre and the edge of the block at each expected temperature was proportional to ambient temperature, and this effect was more pronounced for temperatures at the top of the tube (Fig. 1).

Temperature-dependent Warramaba virgo egg development

The proportion of *W. virgo* eggs that successfully developed within the thermocycler varied among the incubation methods (Fig. 2). Development success ranged between 0% for PCR tubes with the lid open and without a substrate and 75% for PCR tubes with a hole in the lid, sand in the tube and eggs kept moist throughout incubation, which was the most successful method of incubating *W. virgo* eggs (Fig. 2). There was no difference in development success between PCR tubes with closed lids with or without a hole in the lid, for PCR tubes with sand and water throughout incubation (Binomial GLM, $Z = 0.45$, $P = 0.65$).When we incubated eggs along a gradient of temperatures using the most successful method of incubation, we found a significant effect of temperature on the proportion of eggs which initiated development (Fig. 3, Binomial GLM, $Z = -4.83$, $P < 0.01$). Most *W. virgo* eggs incubated across a gradient of temperatures below 37 °C initiated development as indicated by visible swelling. The proportion of eggs which initiated development fell sharply at temperatures above 37 °C where no eggs initiated development despite remaining visibly viable (Fig. 3).

Aedes aegypti egg survival under fluctuating daily temperature cycles

The mean hatch rate of eggs (± standard error) held at a constant 26 °C was 0.974 (± 0.015), which remained high for those exposed to daily fluctuating temperature cycles of 15 – 35 °C to 20 – 40 °C ranging from 0.813 to 0.902 (with SEs within the range 0.016 – 0.027). The mean hatch rate of eggs in the 21 – 41 °C and 22 – 42 °C treatments was 0.508 (± 0.056) and

285 0.012 (± 0.006), respectively (Fig. 4). No survival was observed for daily cycles greater than
286 23 – 43 °C. In an analysis of the overall data, egg survival was significantly affected by
287 temperature (ANOVA, $F_{11,120} = 469.69$, $P < 0.001$) as well as incubation method ($F_{12,120} =$
288 4.42, $P < 0.001$). All treatments except 17 – 37 °C and 20 – 40 °C differed significantly from
289 the control (constant 26 °C) in a posthoc analysis (Tukey HSD, $P < 0.01$). A posthoc analysis
290 of the incubation method revealed a single significant effect in the 21 – 41 °C treatment
291 (Tukey HSD, $P < 0.001$) where intermediate survival was observed.
292
293 *Drosophila simulans egg to pupae survival under fluctuating daily temperature cycles*
294 The proportion of *D. simulans* that survived to pupation within the thermocycler differed
295 among the three daily temperature regimes and incubation methods (Fig. 5). No survival was
296 observed in PCR tubes without a hole, regardless of temperature and level of food. This
297 treatment was thus removed from further analyses. Survival to pupation did not depend on
298 the level of food provided in tubes with a hole and so data were pooled. Survival to pupation
299 depended on temperature, with the proportion surviving in the ranges of 15 – 23 °C, 20 – 28
300 °C and 25 – 33 °C being 0.38, 0.75 and 0.66 respectively (Fig. 5). Survival to pupation in the
301 20 − 28 °C treatment was highest and so was used as the reference group in a GLM model.
302 Survival at 15 – 23 °C differed significantly from the 20 – 28 °C treatment (Binomial GLM,
303 $Z = -2.545$, $P < 0.05$) whereas survival at 25 – 33 °C did not (Binomial GLM, $Z = -0.633$, P
304 > 0.05).
305
306 **Discussion**
307 The overall aim of this study was to test the novel use of thermocyclers as temperature-
308 controlled incubators. Thermocyclers allow control of both constant and fluctuating
309 temperature programs between 3 °C and 99 °C to temperature resolutions as fine as 0.1 °C,

thus accommodating many biologically realistic temperature regimes relevant to hot and temperate conditions. The thermocyclers are capable of heating and cooling rates of up to 5 °C s^{-1} and were able to rapidly achieve and maintain the desired temperature within the short time intervals between temperature changes. Overall, the thermocyclers performed as a temperature-controlled incubator with high precision, accuracy and resolution under various ambient conditions (Fig. 1).

Discrepancies between the programmed temperatures and the recorded temperatures within the thermocycler have three sources: (i) the ambient temperature, (ii) exposure to the ambient temperature and (iii) the location of the temperature reading within the thermocycler. Thermocyclers had reduced accuracy at programmed temperatures lower and greater than the ambient temperature. The reduction in accuracy was greater for programmed temperatures lower than ambient temperatures than for programmed temperatures greater than ambient temperature. These trends were consistent across all ambient conditions and are potentially a major technical limitation of the use of thermocyclers as incubators. The ambient temperature treatments were within the recommended working conditions set by the manufacturer, and were selected to represent a range of laboratory conditions.

We found temperatures within the thermocycler were sensitive to fluctuations in the ambient temperature, which in our study fluctuated within 6 °C of the set temperature. In the present study, noise from ambient temperatures could be almost entirely removed by having the lid of the thermocycler lowered. This would be at the expense of the potential for continuous monitoring; an open lid allows for continuous monitoring of the samples within the thermocycler, either visually or with a photo or video monitoring set up, which may be possible in more stable ambient conditions.

There were thermal gradients within a PCR tube and across the thermocycler block (Fig. 1). The magnitude of the vertical thermal gradient at each programmed temperature was proportional to the ambient temperature offset, although this difference was within 2 °C. The difference between PCR tubes at the centre and edge of the thermocycler block was minimal at programmed temperatures lower than ambient conditions, and this difference remained within 1 °C at programmed temperatures greater than ambient conditions. Thermal gradients within the thermocycler can be reduced by turning on the heating function of the thermocycler lid, which is able to maintain a constant temperature between 30 °C and 99 °C. This heating function would be suitable for use with constant incubation temperatures within the thermocycler lid heating temperature range, but would not be suitable for use with fluctuating temperature cycles where the heated thermocycler lid would bias the effective incubation temperature. The absence of a sample within the PCR tube exacerbates the vertical thermal gradient due to the low thermal conductivity of air and represents an upper bound; a liquid sample in the tube would provide the lower bound on this gradient, as would be the case in the usual application of thermocyclers for PCR.

The biological significance of these discrepancies in temperature for the incubation of organisms depends on the intended study system. We do not envision these temperature differences to have much biological impact, and certainly no more than observed in standard laboratory incubation methods. The development of insect eggs did not vary in relation to the spatial arrangement of PCR tubes across the thermocycler block when a blocking design was used to minimise any effect of spatial variation in incubation temperatures as described above. In quantifying this temperature variation, it is also possible to correct for discrepancies in temperature for improved accuracy if required.

A key aim of the present study was to demonstrate the feasibility of the use of thermocyclers for incubating invertebrates for characterising thermal response. Using a non-model Orthopteran *W. virgo* as a case study, we first showed that its eggs could successfully complete development within a PCR tube in the thermocycler. The most successful method for incubating *W. virgo* were closed PCR tubes with a hole in the lid, with a sand substrate and water available through the incubation period. The development success of 75% using this method was comparable to 61% when *W. virgo* eggs (n = 140) were incubated at 30 °C within an temperature-controlled incubator (J. D. Kong, unpublished data) under the standard protocol for incubating these grasshoppers eggs in petri dishes with moist sand (Blackith & Blackith 1969). Second, using this method we were able to characterise the egg development of *W. virgo* incubated across a fine gradient of constant temperatures, identifying 37 °C as an upper thermal limit of egg development for *W. virgo*. This approach provides a powerful and precise means to rapidly assay thresholds for development as well as thermal response curves for development rate for species with eggs small enough to fit in PCR tubes.

This incubation method for *W. virgo* may be directly transferrable to other insect species which lay their eggs in soil. However, incubation methods will depend on the requirements of the intended species. For example, the importance of water for egg development in the case of *W. virgo* was exemplified by the variable development success across the different incubation methods. The incubation methods with the greatest development success were those which promoted a moist microenvironment within the tube with minimal evaporation (a closed tube lid, presence of sand and provision of water throughout the incubation period), compared to incubation methods which permitted evaporation of water (open tube lids, no sand and no water during later stages of incubation).

385

386 The evaporation of water within a PCR tube was driven by the constant presence of the
387 vertical gradient of temperature. The water vapour condensed on the closed lid of PCR tubes
388 and remained at the top of the tube. This condensation could potentially change the rate of
389 gas exchange through the hole in the tube lid if the hole was covered by water. The presence
390 of sand in the PCR tube also allowed a greater amount of water to be placed in those tubes
391 than tubes without sand. The sand potentially further reduced the rate of evaporation from the
392 bottom of the tube, and therefore increased the availability of liquid water for egg
393 development compared to tubes without sand. The hydric microenvironment observed within
394 the PCR tubes has implications for species whose egg development are sensitive to hydric
395 microenvironments or have low desiccation resistance, and is a potential limitation of this
396 incubation method for those species. Alternative methods of incubation to those described
397 here would be appropriate for such species or others that lay eggs above ground.
398

399 We also demonstrated the ability to successfully incubate and characterise the thermal
400 response of *Ae. aegypti*, the primary vector of dengue fever, at the quiescent egg phase in a
401 thermocycler. Our results showed that quiescent eggs could survive for one week in a PCR
402 tube, with or without gas exchange, at a constant 26 °C. Quiescent *Ae. aegypti* eggs were also
403 stressed under a range of daily fluctuating temperature cycles which identified an upper
404 thermal limit of 41 to 42 °C, similar to that noted by Focks *et al.* (1993). Previous
405 experiments on the thermal response of immature stages of *Aedes* mosquitoes have typically
406 held them under constant conditions in incubators or water baths where a large number of
407 individuals can be exposed to particular conditions (e.g. Richardson *et al.* 2011; Alto and
408 Bettinardi 2013) while fluctuating conditions have more rarely been considered both through
409 the use of incubators (e.g. Westby & Juliano 2015; Carrington *et al.* 2013) and (in the case of

410 diapausing eggs) by holding eggs outside (Ritchie *et al*. 2015). While these treatments allow
411 large numbers of eggs or larvae to be considered under a particular set of conditions, the
412 approach described here would have the advantage of allowing for a greater range of
413 treatments (particularly in a gradient set-up) with more stringent temperature control than
414 might be expected in an incubator although a water bath provides for accurate maintenance of
415 constant conditions.
416
417 We successfully incubated *D. simulans* from egg to larvae in a TRIO 48 thermocycler. Over
418 the three temperature ranges tested we showed it was possible to generate a thermal response.
419 For the fruit flies to survive any treatment in a PCR tube, the lid must have a hole to allow
420 gas exchange. No effect of the level of food was found, most probably because food was in
421 excess even at the lowest level used. In our experiment, food level may have been
422 confounded with temperature due to the small vertical temperature gradients in PCR tubes.
423 However, these gradients should be reduced in liquid medium rather than air. The issue could
424 be resolved by providing identical volumes of media but with different nutritional densities. It
425 is also feasible to incubate adult *Drosophila* spp. within PCR tubes (e.g. Gibert & Huey
426 2001). *Drosophila* species have been used very widely in experiments on the effects of
427 constant and fluctuating conditions on thermal resistance involving water baths, incubators
428 and (in the case of adults) knockdown tubes (Hoffmann, Sorensen & Loeschcke 2003) and
429 there has been a strong interest in the use of different thermal ramping rates on resistance
430 (Terblanche *et al.* 2011) which have normally used programmable incubators but could easily
431 be simulated with the approach described here.
432
433 There are a number of advantages of using thermocyclers compared with incubators. First,
434 thermocyclers increase the throughput of individuals compared with incubators. We were

able to examine up to 12 incubation temperatures and 96 individual *W. virgo* eggs in parallel within one compact thermocycler unit, which would otherwise require 12 full sized incubator units. Thermocyclers are also more compact and occupy a smaller space in the laboratory than most temperature-controlled incubators.

Second, the temperature range and flexibility of programming of a thermocycler is greater than most commercially available diurnal incubators. The fine temperature resolution of the thermocyclers allows precise characterisation of thermal limits, such as critical or lethal temperatures. The ability of the thermocycler to run both constant and fluctuating temperatures eliminates the need for multiple incubators capable of different temperature cycles. Thermocyclers used here are able to conduct fluctuating temperature cycles of up to 30 set temperature points and 99 cycle repetitions. The thermocyclers are thus able to replicate hourly-scale diurnal temperature cycles, or daily seasonal cycles for an extended period of time automatically. However, thermocyclers are typically not able to generate temperatures below 3 °C and thus might not be suitable to replicate field temperatures of cooler climates.

Third, although it is possible to assemble a purpose-built incubator with customised components (for examples see Huey *et al.* 1992, Gibert & Huey 2001 and Woods & Bonnecaze 2006), the thermocyclers did not require any additional modifications after purchase for the experiments described in this study, and were used with standard laboratory products, e.g. PCR tubes. There are several types of thermocyclers of varying technical specifications likely to be suitable for incubating small invertebrates, including thermocycler models which are capable of holding larger PCR tubes than those used in this study, allowing the incubation of larger species or life stages.

In summary, our study demonstrates that thermocyclers are a simple and transferrable method to scale up the incubation of small invertebrates with high precision. We have demonstrated two types of thermal response, development and survival, under constant and fluctuating temperatures in three different model and non-model invertebrate species. We have yet to try to assess other common thermal responses of invertebrates, for example heat tolerance, but we envision thermocyclers would be suitable for such evaluations.

Our methods are applicable for broader ecological and evolutionary studies on the thermal response of invertebrates (Belén Arias, Poupin & Lardies 2011; Chen *et al.* 2015; Donohue *et al.* 2015). For example, the thermal traits of egg development as described in this study can be combined with newly developed microclimate models to parameterise a mechanistic niche model of egg development (Horton 2012; Kearney *et al.* 2014). Such models can predict adaptive developmental responses of invertebrate eggs to climate or, when combined with georeferenced climate data, can be used to investigate the processes underlying the life cycle or distribution of species under changing climates (Kearney *et al.* 2009; Richardson *et al.* 2011). Efficient methods of characterising thermal traits can better enable us to draw connections from the outputs of high-throughput genotypic exercises to environment-specific phenotypic consequences, which can help to understand adaptive responses of species to environmental change (Laughlin & Messier 2015).

Data Accessibility

We will not be archiving data because all data used in this manuscript are presented herein.

Acknowledgements

This research was supported by an ARC LIEF grant (LEI150100083) and an ARC Discovery Project (DP160100279) awarded to M.R.K. and A.A.H., as well as an NHMRC program grant (APP1037003) to A.A.H., while J.D.K was supported by a Holsworth Wildlife Research Endowment – Equity Trustees Charitable Foundation.

References

Alto, B.W. & Bettinardi, D. (2013) Temperature and dengue virus infection in mosquitoes: Independent effects on the immature and adult stages. *American Journal of Tropical Medicine and Hygiene,* **88,** 497-505.

Angilletta, M.J. (2009) *Thermal adaptation: A theoretical and empirical synthesis*. Oxford University Press, Oxford, U. K.

Araújo, M.B., Ferri-Yáñez, F., Bozinovic, F., Marquet, P.A., Valladares, F. & Chown, S.L. (2013) Heat freezes niche evolution. *Ecology Letters,* **16,** 1206-1219.

Belén Arias, M., Poupin, M.J. & Lardies, M.A. (2011) Plasticity of life-cycle, physiological thermal traits and Hsp70 gene expression in an insect along the ontogeny: Effect of temperature variability. *Journal of Thermal Biology,* **36,** 355-362.

Blackith, R.E. & Blackith, R.M. (1969) Observations on the biology of some Morabine grasshoppers. *Australian Journal of Zoology,* **17,** 1-12.

Bozinovic, F., Bastías, D.A., Boher, F., Clavijo-Baquet, S., Estay, S.A. & Angilletta Jr, M.J. (2011) The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiological and Biochemical Zoology,* **84,** 543-552.

Bozinovic, F., Calosi, P. & Spicer, J.I. (2011) Physiological correlates of geographic range in animals. *Annual Review of Ecology, Evolution, and Systematics,* **42,** 155-179.

Buckley, L.B. (2010) The range implications of lizard traits in changing environments. *Global Ecology and Biogeography,* **19,** 452-464.

Carrington, L.B., Armijos, M.V., Lambrechts, L., Barker, C.M. & Scott, T.W. (2013) Effects of fluctuating daily temperatures at critical thermal extremes on *Aedes aegypti* life-history traits. *Plos One,* **8,** e58824.

514 Chen, H., Jackson, P.L., Ott, P.K. & Spittlehouse, D.L. (2015) A spatiotemporal pattern
515 analysis of potential mountain pine beetle emergence in British Columbia, Canada.
516 *Forest Ecology and Management,* **337,** 11-19.
517 Chown, S.L., Hoffmann, A.A., Kristensen, T.N, Angilletta Jr, M.J., Stenseth, N.C & Pertoldi,
518 C. (2010) Adapting to climate change: A perspective from evolutionary physiology.
519 *Climate Research,* **43,** 3-15.
520 Colinet, H., Sinclair, B.J., Vernon, P. & Renault, D. (2015) Insects in fluctuating thermal
521 environments. *Annual Review of Entomology,* **60,** 123-140.
522 Danks, H.V. (2007) The elements of seasonal adaptations in insects. *The Canadian*
523 *Entomologist,* **139,** 1-44.
524 Donohue, K., Burghardt, L.T., Runcie, D., Bradford, K.J. & Schmitt, J. (2015) Applying
525 developmental threshold models to evolutionary ecology. *Trends in Ecology &*
526 *Evolution,* **30,** 66-77.
527 Fahlgren, N., Gehan, M.A. & Baxter, I. (2015) Lights, camera, action: High-throughput plant
528 phenotyping is ready for a close-up. *Current Opinion in Plant Biology,* **24,** 93-99.
529 Focks, D.A., Haile, D.G., Daniels, E. & Mount, G.A. (1993) Dynamic life table model for
530 *Aedes aegypti* (Diptera: Culicidae): Analysis of the literature and model development.
531 *Journal of Medical Entomology,* **30,** 1003-1017.
532 Gaston, K.J. (2003) *The structure and dynamics of geographic ranges*. Oxford University
533 Press, New York, N. Y.
534 Gibert, P. & Huey, R.B. (2001) Chill-coma temperature in *Drosophila*: Effects of
535 developmental temperature, latitude, and phylogeny. *Physiological and Biochemical*
536 *Zoology,* **74,** 429-434.

Großkinsky, D.K., Svensgaard, J., Christensen, S. & Roitsch, T. (2015) Plant phenomics and the need for physiological phenotyping across scales to narrow the genotype-to-phenotype knowledge gap. *Journal of Experimental Botany,* **66,** 5429-5440.

Hill, M.P., Chown, S.L. & Hoffmann, A.A. (2013) A predicted niche shift corresponds with increased thermal resistance in an invasive mite, *Halotydeus destructor*. *Global Ecology and Biogeography,* **22,** 942-951.

Hoffmann, A.A., Sorensen, J.G. & Loeschcke, V. (2003) Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology,* **28,** 175-216.

Horton, B. (2012) Models for estimation of hourly soil temperature at 5 cm depth and for degree-day accumulation from minimum and maximum soil temperature. *Soil Research,* **50,** 447-454.

Houle, D., Govindaraju, D.R. & Omholt, S. (2010) Phenomics: The next challenge. *Nature Reviews Genetics,* **11,** 855-866.

Huey, R.B., Crill, W.D., Kingsolver, J.G. & Weber, K.E. (1992) A method for rapid measurement of heat or cold resistance of small insects. *Functional Ecology,* **6,** 489-494.

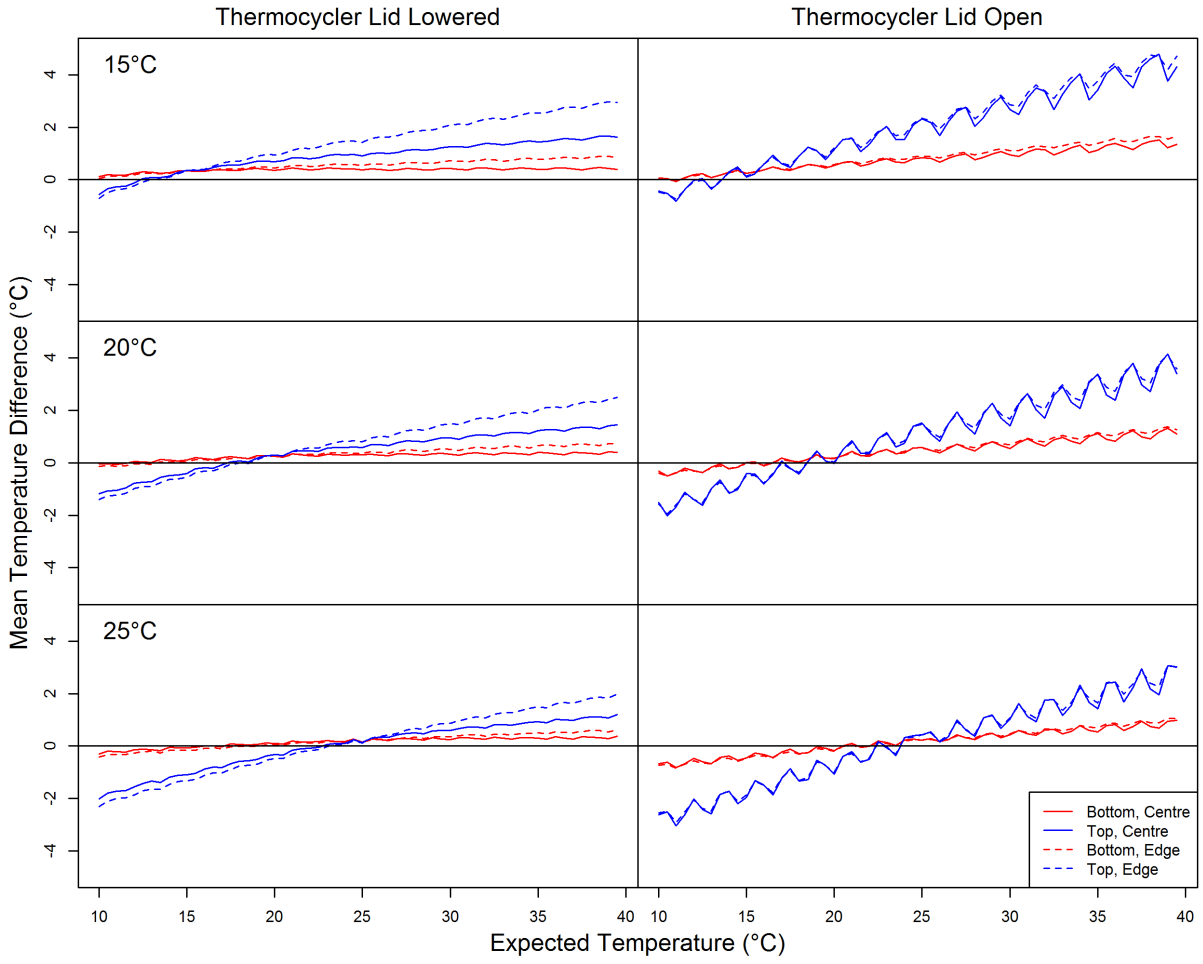
Kearney, M., Porter, W.P., Williams, C., Ritchie, S. & Hoffmann, A.A. (2009) Integrating biophysical models and evolutionary theory to predict climatic impacts on species' ranges: The dengue mosquito *Aedes aegypti* in Australia. *Functional Ecology,* **23,** 528-538.

Kearney, M.R., Shamakhy, A., Tingley, R., Karoly, D.J., Hoffmann, A.A., Briggs, P.R. & Porter, W.P. (2014) Microclimate modelling at macro scales: a test of a general microclimate model integrated with gridded continental-scale soil and weather data. *Methods in Ecology and Evolution,* **5,** 273-286.

562 Kolbe, J.J., Kearney, M. & Shine, R. (2010) Modeling the consequences of thermal trait
563 variation for the cane toad invasion of Australia. *Ecological Applications,* **20,** 2273-
564 2285.
565 Laughlin, D.C. & Messier, J. (2015) Fitness of multidimensional phenotypes in dynamic
566 adaptive landscapes. *Trends in Ecology & Evolution,* **30,** 487-496.
567 Mitchell, K.A. & Hoffmann, A.A. (2010) Thermal ramping rate influences evolutionary
568 potential and species differences for upper thermal limits in *Drosophila*. *Functional*
569 *Ecology,* **24,** 694-700.
570 Morin, X., Augspurger, C. & Chuine, I. (2007) Process-based modeling of species'
571 distributions: What limits temperate tree species' range boundaries? *Ecology,* **88,**
572 2280-2291.
573 Overgaard, J., Kearney, M.R. & Hoffmann, A.A. (2014) Sensitivity to thermal extremes in
574 Australian *Drosophila* implies similar impacts of climate change on the distribution of
575 widespread and tropical species. *Global Change Biology,* **20,** 1738-1750.
576 Porcelli, D., Butlin, R.K., Gaston, K.J., Joly, D. & Snook, R.R. (2015) The environmental
577 genomics of metazoan thermal adaptation. *Heredity,* **114,** 502-514.
578 R Development Core Team (2015) *R: A Language and Environment for Statistical*
579 *Computing*. R Foundation for Statistical Computing, Vienna. URL https://www.R-
580 project.org [accessed 12 December 2015]
581 RStudio Team (2015). *RStudio: Integrated Development for R*. RStudio, Inc., Boston, MA
582 URL http://www.rstudio.com/ [accessed 12 December 2015]
583 Richardson, K., Hoffmann, A.A., Johnson, P., Ritchie, S. & Kearney, M.R. (2011) Thermal
584 sensitivity of *Aedes aegypti* from Australia: Empirical data and prediction of effects
585 on distribution. *Journal of Medical Entomology,* **48,** 914-923.

586 Ritchie, S.A., Townsend, M., Paton, C.J., Callahan, A.G. & Hoffmann, A.A. (2015)
587 Application of wMelPop *Wolbachia* Strain to Crash Local Populations of Aedes
588 aegypti. *Plos Neglected Tropical Diseases,* **9,** e0003930.
589 Terblanche, J.S., Hoffmann, A.A., Mitchell, K.A., Rako, L., le Roux, P.C. & Chown, S.L.
590 (2011) Ecologically relevant measures of tolerance to potentially lethal temperatures.
591 *Journal of Experimental Biology,* **214,** 3713-3725.
592 Travisano, M. & Shaw, R.G. (2013) Lost in the map. *Evolution,* **67,** 305-314.
593 Westby, K.M. & Juliano, S.A. (2015) Simulated seasonal photoperiods and fluctuating
594 temperatures have limited effects on blood feeding and life history in *Aedes*
595 *triseriatus* (Diptera: Culicidae). *Journal of Medical Entomology,* **52,** 896-906.
596 Woods, H.A. & Bonnecaze, R.T. (2006) Insect eggs at a transition between diffusion and
597 reaction limitation: Temperature, oxygen, and water. *Journal of Theoretical Biology*,
598 **243**, 483-492.

599



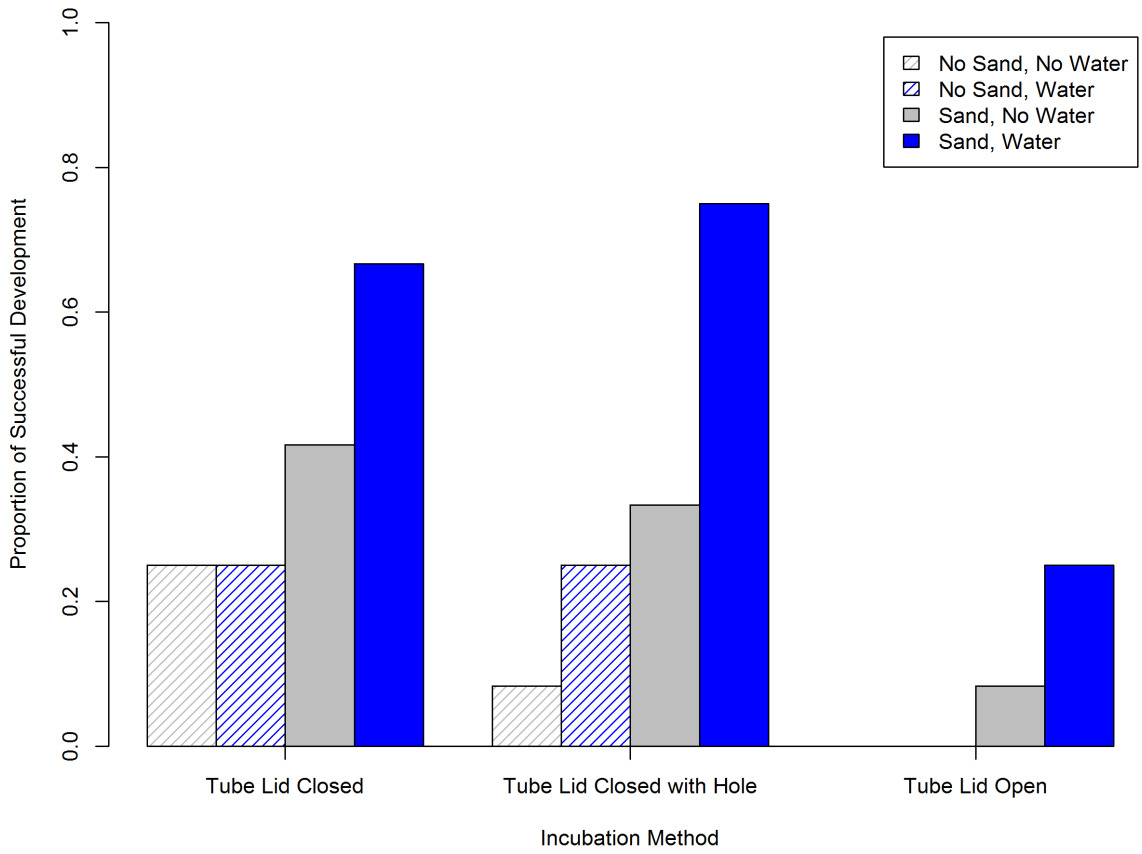
600

601 **Figure 1**

602 Mean accuracy of temperature (°C) within a TRIO 48 thermocycler under various ambient

603 conditions. The lid of the thermocycler was either open or lowered but not sealed closed.

604 Ambient temperatures were 15, 20 or 25 °C.



605

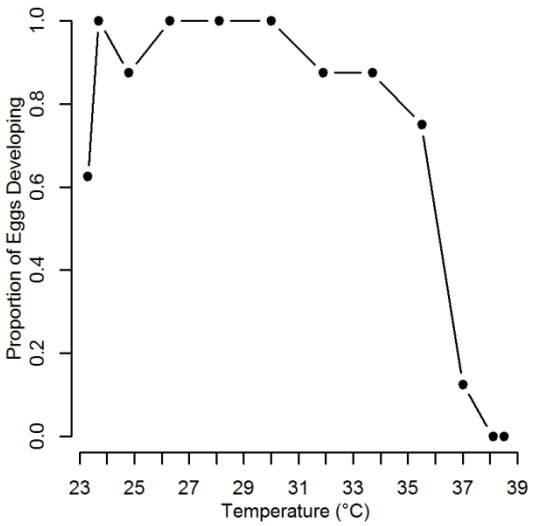
606 **Figure 2**

607 Proportion of *Warramamba virgo* eggs (n = 144) that successfully developed under

608 difference incubation methods. Development success was defined by eggs changing colour

609 from yellow to brown, which indicates they are ready to hatch. Eggs were incubated within

610 PCR tubes in a TRIO 48 thermocycler at 30 °C.



611

612 **Figure 3**

613 Proportion of *Warramamba virgo* eggs (n = 96) that initiated development at each incubation

614 temperature (°C). Initiation of development was defined as the proportion of eggs that visibly

615 swelled and became turgid. Eggs were incubated within PCR tubes in a Gradient 96

616 thermocycler.

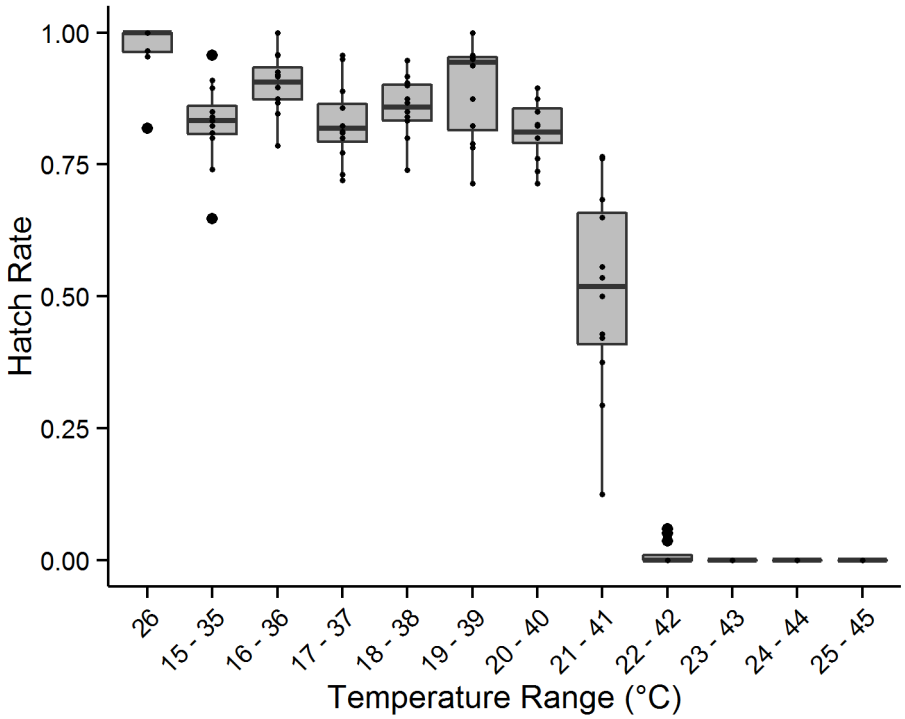


Figure 4

Box plot showing the hatch rate of *Aedes aegypti* eggs (n = 12) after being incubated for 1 week at the temperatures shown. Eggs were incubated at 26 °C (control) or fluctuating daily temperature cycles in the ranges shown for the remaining treatments. Small circles indicate hatch rate while large circles represent outliers.

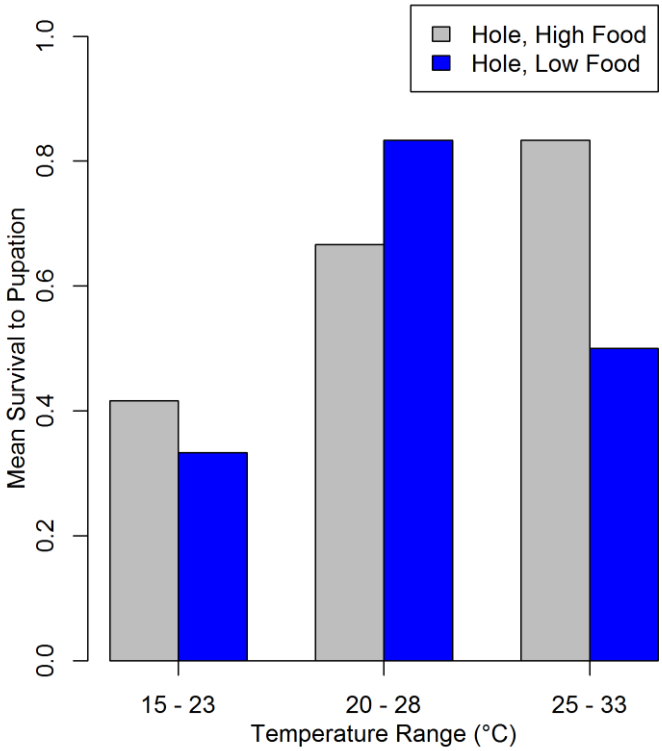


Figure 5

Mean survival of *Drosophila simulans* to pupation (n = 144) after 3 days of development as an egg and larvae at the three temperature ranges shown and two incubation methods, within PCR tubes in a TRIO 48 thermocycler. Tubes were then kept at 25 °C for the remainder of development. Tubes with no holes are not displayed due to zero survival.