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Title:
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Date:
2018-07-01

Citation:
Kearney, M. R., Deutscher, J., Kong, J. D. & Hoffmann, A. A. (2018). Summer egg diapause in a matchstick grasshopper synchronizes the life cycle and buffers thermal extremes. Integrative Zoology, 13 (4), pp.437-449. https://doi.org/10.1111/1749-4877.12314.

Persistent Link:
https://hdl.handle.net/11343/275100

ORIGINAL ARTICLE **doi: 10.1111/1749-4877.12314**

Running title: Diapause in a matchstick grasshopper

Summer egg diapause in a matchstick grasshopper synchronizes the life cycle and buffers thermal extremes

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Abstract

The phenological response is among the most important traits affecting a species' sensitivity to climate. In insects, strongly seasonal environments often select for a univoltine life cycle such that one seasonal extreme is avoided as an inactive stage. Through understanding the underlying mechanisms for univoltinism, and the consequences of its failure, we can better predict insect responses to climate change. Here we combine empirical data and simulation studies to investigate the consequences of an unusual diapause mechanism in a parthenogenetic matchstick grasshopper, *Warramaba virgo* (Key, 1963), from arid southern Australia. Our field body temperature measurements indicate that this species is a thermoconformer and our laboratory studies of the thermal response of feeding rate imply strong constraints on winter activity. However, the species exhibits no obligate winter diapause, and eggs can develop in 1 month under constant temperatures approximating the mean soil temperature at the time of oviposition (summer). We show that diurnal temperature cycles exceeding a peak of 36°C inhibit egg development in summer, and that this is sufficient to prevent autumnal hatching of eggs. Development is also strongly retarded below 24°C. Microclimate-driven simulation studies of egg development show that these thermal responses provide robust maintenance of a univoltine life cycle, thereby resulting in survival of heat stress as an egg (due to limited developmental state) and avoidance of cold stress as a nymph and adult (due to overwintering in the soil as an egg).

Key words: egg diapause, life cycle, matchstick grasshopper, phenology, thermal extremes, thermoregulation

INTRODUCTION

Organisms in seasonal environments frequently possess sophisticated developmental mechanisms that enable them either to avoid stressful times of year or to exploit resource-rich periods. In insects and other organisms, developmental responses such as quiescence (developmental slow-down) and diapause (developmental arrest) represent key "strategies of adaptation" (Levins 1968) to climate. These responses delay development so that stressful conditions are avoided and/or resource-rich periods are exploited. Developmental trajectories may be induced by environmental signals involving temperature, food levels, moisture and photoperiod, which are informative about future environments. However, climate change has the potential to alter these cues and the fitness consequences of the response. Inappropriate responses to environmental cues, which no longer match the onset of favorable conditions, can result in insects entering developmental traps that expose them to stressful conditions (Bale & Hayward 2010; Van Dyck *et al.* 2015) unless they make adaptive adjustments (Bradshaw & Holzapfel 2006; Van Asch *et al.* 2007).

Most studies of diapause as a response to climate variability focus on the induction of diapause by the onset of cold temperatures and short day photoperiods (Tauber *et al.* 1986). These studies are almost entirely restricted to Northern Hemisphere species, where high latitude insects typically overwinter by diapausing at the larval or pupal stages, with sufficient resources being accumulated and conserved during diapause to enable development to the adult stage in spring (Arrese & Soulages 2010; Hahn & Denlinger 2011). However, this strategy can be costly when mild winter conditions increase metabolic demand, with consequences for spring survival and subsequent reproductive output (Irwin & Lee 2000; Zhang *et al.* 2016).

Here we consider diapause in a Southern Hemisphere grasshopper species belonging to the family Morabidae (Matt *et al.* 2008), and assess whether diapause in this group involves a different strategy to that of northern hemisphere species. The species considered, *Warramaba virgo* (Key, 1963), is a strictly univoltine parthenogen; its eggs hatch around mid-October, with adults appearing by December and persisting as late as the end of April. Parthenogenesis is obligate, having arisen through hybridization between 2 undescribed sexual species from Western Australia (White *et al.* 1977; Kearney & Blacket 2008). Its phenology is, thus, a hybrid expression of the phenology of its parental species, and it has very limited potential for evolutionary change. The species, as presently described, occurs in 2 disjunct populations in semi-arid southern Australia. There are 2 distinct lineages present within *W. virgo*: the "Standard Phylad" and the "Boulder-Zanthus Phylad" (White 1980; White & Contreras 1981; Honeycutt & Wilkinson 1989; Kearney *et al.* 2006; Kearney & Blacket 2008). The Boulder-Zanthus Phylad likely originated more recently and occurs only in Western Australia, whereas the Standard Phylad has spread to the east. Both species have limited mitochondrial DNA diversity but have substantial nuclear diversity, likely due to repeated hybrid origins within the site of origin (Honeycutt & Wilkinson 1989; Kearney *et al.* 2006).

The late geneticist MJD White (Peacock & McCann 2010) studied the Standard Phylad for many years, and of the 227 entries on the species in his field diaries, spanning 1970 to 1982, 1st to 2nd instar nymphs were only found in mid-October and "small nymphs" (presumably also 1st to 2nd instar) in mid-December. The earliest that White recorded adults was 10 December and the latest was 30 April. Our observations are entirely consistent with this picture; we have collected the species throughout its range over 8 summers, as early as October and as late as March, and have only found 1st instar nymphs in October. White and colleagues found that eggs take approximately 30 days to develop at constant 32°C (N. Contreras, personal communication), which is typical of other summer-active matchstick grasshoppers (Blackith & Blackith 1968). As will be shown, this is approximately the mean soil temperature in January and February when the eggs are laid, and yet development is not complete until after winter. In the laboratory, the 5 nymphal instars take 63 days to reach adulthood when reared between 30 and 35°C (Blackith & Blackith 1968), and all stages feed on a range of shrubs and trees of the genus *Acacia* (White, 1966) as well as *Senna* and *Dodonaea* (M. Kearney, personal observation). The Boulder-Zanthus

Phylad is also strictly univoltine, but White and colleagues found that it had an obligate diapause broken by exposure to low temperature (i.e. eggs would not initiate development without prolonged exposure to temperatures of around 12°C; N. Contreras, personal communication). In contrast, the more widespread Standard Phylad has no obligate diapause.

How then does the Standard Phylad of *W. virgo* synchronize a univoltine life cycle? To resolve this issue, we characterized its thermal developmental response to constant and cycling temperature treatments. In addition, we made field observations of soil temperatures and of the species' behavioral capacity for thermoregulation, as well as laboratory measurements of its thermal response curve for feeding. We integrate this information into a mechanistic model of the consequences of its life cycle in terms of buffering thermal extremes to interpret the adaptive significance of its developmental response.

METHODS

Collection and maintenance of animals

Grasshopper stocks were collected in January 2005 from 4 locations in the eastern part of the species' range representing the Standard Phylad: Nowingi, Victoria, 34°35.635′S 144°13.391′E, Yatpool, NSW 34°24.364′S 142°11.194′E, Coonavitta Tank, NSW, 31°39.097′S 144°15.852′E, and Stirling Vale Creek, New South Wales 31°59.115′S 141°21.564′E. An additional 27 animals were obtained from the Nowingi site in January 2006 as adults or penultimate instar nymphs, for use in feeding rate measurements.

Grasshoppers were housed individually in enclosures made with 2 different-sized clear plastic drinking cups (320 and 425 mL, Genfac Plastics, Dandenong, Australia) pushed together at their open ends. The base of the top cup (the larger of the two) was cut out and mesh was glued on the outer side of the cup base to provide ventilation. The animals were fed by introducing fresh *Acacia sophorae* leaves every 1–3 days (White & Contreras 1979) and were sprayed with water daily. The animals were maintained at 32°C under a light : dark 14:8 photoperiod.

Incubation treatments

Females oviposited directly onto the floor of their enclosure and the eggs were transferred with a wetted fine brush to Petri dishes (55-mm diameter), half filled with moist sand and with a small sheet of filter paper on top. As matchstick grasshoppers do not produce a foam pod (Blackith & Blackith, 1968), the eggs could be separated on the filter paper after wetting. Eggs were maintained in constant darkness during development.

Eggs from the wild-caught individuals were incubated at constant 32°C and 58 hatchlings were successfully reared to the adult stage. Hatchlings from a given clutch were initially housed together and then transferred to single cages as they matured. The clutches produced by the F1 generation were incubated at 7 constant incubation treatments (9, 17.7, 23.6, 29.1, 31.7, 35.5 and 39.2°C) and 1 cycling regime (14–41°C, Suppl. Fig. S1). All clutches were split across 2 or more temperatures, always including 31.7°C and evenly allocating across all temperatures but with the particular set of temperatures varying among clutches.

We observed that the first molt, from the vermiform larva (protonymphal stage) into the first stadium, requires an increase in light intensity. To achieve this light contrast, eggs that had turned from yellow to brown [around embryonic stage 25 (Pfadt, 1966)] were transferred to 40-mL glass vials filled with moist sand, buried anterior end upwards, and held at 31.7°C under constant light.

Thermal dependence of feeding rate and lethal limit

These trials were carried out with 46 captive-reared F1 individuals as well as 27 wild-caught individuals collected in the same month. Grasshoppers were housed singly in enclosures, and tested at 15°C (*n* = 15), 17°C (*n* = 13), 20°C (*n* = 20), 25°C (*n* = 24), 30°C (*n* = 16), 35°C (*n* = 46), 40°C (*n* = 29) and 45°C (*n* = 27). Individuals were used repeatedly across temperature in these tests, starting at the lowest temperature. Wild-caught individuals were only measured at 35°C (*n* = 26), 40°C (*n* = 12) and 45°C (*n* = 27). No captive-bred individuals were measured at 45°C.

Each grasshopper was weighed and then fed 1 *A. sophorae* phyllode, with its basal end placed into a water-filled Eppendorf tube through a small hole cut into the tube's lid. All trials lasted approximately 24 h, except for the 45°C trial, which was terminated after 18 h due to high mortality. Each feeding trial was conducted with constant light provided by 35-W fluorescent tubes. Phyllodes were large enough (approximately 5–15 cm^2) so that some material remained unconsumed within 24 h, and were digitally scanned before and after the trial and assessed for area using the image analysis package ImageJ v1.4 (Schneider *et al.* 2012). At the completion of a trial, each partially consumed phyllode was dried in a flow of hot (approximately 50–60°C), dry air until desiccated, and then weighed (Mettler Toledo XS205DU, Port Melbourne, Australia) to the nearest 0.01 mg. The dry mass of food ingested by each grasshopper was estimated as the difference in phyllode area before and after the trial and the estimated specific dry mass of the given phyllode. We also measured the dry mass of frass produced at a subset of temperatures to determine its association with food consumption at 17, 20 and 35°C.

The 5-parameter thermal response function of Schoolfield *et al.* (1981) was applied to characterize the temperature response, using the nls.lm function of the R package “minpack.lm,” with the initial parameter estimates obtained using the approach described by Schoolfield *et al.* (1981). Following Kooijman (2010), we modified the equation of Schoolfield *et al.* (1981) to include the potential for an enzyme inactivation effect at the reference temperature.

A preliminary estimate of the lethal limit was obtained by heating 10 grasshoppers (different individuals to the feeding rate study) under a 100-W incandescent lamp until they voluntarily jumped away. Immediately upon jumping, we recorded the temperature between the mandible and the prosternum using a 40-gauge type T thermocouple thermometer. Post-experimental survival was then monitored for 24 h.

Thermoregulatory behavior in the field

We estimated the extent and precision of thermoregulatory behavior of *W. virgo* on 14 January 2006 at Nowingi, Victoria, where this species was abundant on *Acacia wilhelmiana* bushes (<2.5-m tall). We employed the protocols of Hertz *et al.* (1993), which include comparing the distribution of field measurements of body temperatures, T_{b}, to the expected or “null” distribution of body temperatures that would result if the animal behaved randomly with respect to its thermal environment (Heath 1964; Grant & Dunham 1988; Hertz 1992a,b). We estimated the latter distribution by randomly sampling operative temperatures, T_e, from the habitat used by the grasshoppers with a small copper tube of the same diameter and length as the grasshoppers (2 mm × 30 mm OD × length). The tube was painted with Krylon Jade Green paint, which has a solar reflectivity of 40%. Preliminary field measurements indicate that such a copper model provided a more accurate estimate of the temperature of a freshly-killed grasshopper (correlation coefficient $r = 0.83–0.88$, root mean square error $rmse = 1.2–1.5$°C) than did iButtons ($r = 0.74–0.75$, $rmse = 1.4−2.4$ °C).

Prior to sampling field T_b and T_e, we marked 57 randomly selected branch positions across 24 bushes. We then sequentially recorded the T_e at each location using a thermocouple probe inserted in the tube following a 30-s equilibration period. We concurrently sampled the same bushes for grasshoppers by shaking all branches on each bush into a calico hoop-bag and recorded the temperature in the cavity between the mandible and the prosternum with a 40-gauge type T thermocouple thermometer. All grasshopper temperature measures were made within 10 s of capture and care was taken to handle the animals at the base of the thorax, well away from the measurement point. Samples were taken continuously from 7:00 to 9:30 hours, and then from 10:20 to 13:25 hours.

Simulating egg and adult thermal responses

All simulations and analyses were performed in R (R Development Core Team, 2012).
We integrated thermal developmental and feeding responses with the NicheMapR microclimate model (Kearney *et al.* 2014; Kearney & Porter 2017) to simulate the life cycle in the field as a function of oviposition date and shade level (Supporting Information Appendix 1). We drove the microclimate model with gridded daily weather data from the Australian Water Availability Project (AWAP), as described in Kearney *et al.* (2014), and also included a daily gridded mean wind speed product (McVicar *et al.* 2008) with soil properties obtained from the Soil and Landscape Grid of Australia (Viscarra Rossel *et al.* 2015). Egg development was simulated at a depth of 3 cm, based on oviposition behavior of *W. virgo* in the laboratory. We tested the ability of the microclimate model to predict soil temperatures in the habitat of *W. virgo* against observed values measured with Thermochron iButtons at 3-cm depth at the Stirling Vale Creek site. One iButton was placed in the open and one in the deep shade of the food plant, *Acacia aneura*

(Suppl. Fig. S2). Shaded air temperature at 1 m was also recorded by an iButton placed in a large *A. aneura* tree.

Egg development rate was computed based on the 5-parameter Arrhenius equation developed in this study. Based on our laboratory measures, in some simulations we imposed a diapause at 30% development if eggs had experienced a temperature >35.5°C within a threshold period of time. We assumed that eggs were insensitive to the induction of diapause once they had passed 30% development, which was the approximate threshold at which developmental arrest was observed. Eggs were simulated to be laid at weekly intervals from the 1 January to 26 March.

Finally, we computed hourly feeding rates based on predicted air temperature at 1-m height and determined run lengths of periods of no feeding with the rle function of R. We used this to estimate cold-induced starvation risk of individuals over winter.

RESULTS

Incubation

A total of 1455 eggs from 179 clutches were incubated (median clutch size 8), of which 98 hatched. Development was completed successfully only under the constant temperatures of 29.1°C (36.20 days, standard deviation [*sd*] = 2.39, $N = 5$) and 31.7°C (30.75 d, *sd* = 4.50, $n = 60$) (Fig. 1), being significantly faster at 31.7°C (ANOVA: $F_{1,63} = 7.079$, $P = 0.01$) (Fig. 2).

Eggs exposed to the cycling incubation temperatures failed to take up water, despite being in contact with saturated filter paper, and appeared not to develop. However, when they were transferred to 31.7°C they took up water and completed development 31% faster than those held at constant 31.7°C (17–26 days, mean ± SD = 21.3 ± 2.78 days, $N = 9$). The time spent at the cycling treatment (due to different laying dates) was not significantly correlated with the time taken to develop at 31.7°C post-cycling ($r = -0.49$, $P = 0.182$). Given the rate of development at 31.7°C, 30.6% of development appeared to have been completed before pausing.

The eggs at constant 35.5°C had failed to swell or show any signs of development after 52 days. To assess their viability, these eggs were transferred to 31.7°C. After 11 days, they had started to swell and half were transferred back to 35.5°C. The 3 eggs remaining at 31.7°C completed development in a further 23.3 days (values of 22, 24, 24 days), which was 29% faster than for the constant 31.7°C eggs, suggesting that 24.1% of development had already been completed at 35.5°C. Those transferred back to 35.5°C completed development in a further 21.0 days (range 20–23 days, $N = 7$), which was significantly faster than those remaining at 31.7°C ($t = 2.82$, df = 4.3, $P = 0.044$). Accounting for the 11 days at 31.7°C that these eggs experienced, this implies a developmental rate of 4% per day at 35.5°C. In addition, 1 egg held at 39.2°C for 29 days was transferred to 31.7°C and hatched after 39 days.

Although eggs did not hatch at 23.6°C within the time frame of the experiment, observable development did occur. This provided an opportunity to estimate the total time required for eggs to hatch at 23.6°C. Embryos from 5 eggs incubated at 23.6°C (ages 92, 110, 119, 125 and 153 days) were compared with 3 eggs incubated at 29.2°C (ages 16, 17 and 23 days) and 2 incubated at 31.7°C (ages 17 and 18 days). Embryo developmental stages and the associated percent development completed were determined from figure 10 in Pfadt (1966), fitting a 5th order polynomial to the relationship between development stage and percent completed development in that figure. The inferred rates and observations are given in Figure 2, along with the predicted rate from the estimated 5-parameter Arrhenius function of Schoolfield *et al.* (1981), with T_A = 4976.6 K, T_{AL} = 75 054.2 K and T_L = 299.4 K, k_{ref} (31.7°C) = 3.252% d^{-1}, and T_{AH} and T_H being based on the estimate from the feeding rates (see below).

Thermal response of feeding and lethal limit

No grasshoppers fed at 15°C and only 31% of grasshoppers fed at 17°C, with the mass-specific feeding rate (dry mass intake) rising to approximately 0.28 g d^{-1} g^{-1} between 30 and 40°C, and then declining at 45°C (Fig. 3). The source of grasshoppers (wild *vs* captive) had no effect on feeding rate at 35 and 40°C, nor was there a difference in the rate of feeding between these 2 temperatures (2-way ANOVA, all $P > 0.05$). The estimates of the 5-parameter Arrhenius function parameters of Schoolfield *et al.* (1981) across all temperatures were: T_A = 1851.3 K, T_{AL} = 47859.7 K, T_{AH} = 71588.9 K, T_L = 293.0 K, and T_H = 317.3 K, with k_{ref} (35.0°C) = 0.277 g d^{-1} g^{-1}.

Frass production was linearly related to dry mass intake (linear regression, $F_{1,41} = 289.1$, slope = 0.609, intercept = 0.0004, multiple $R^2 = 0.876$, $P < 0.0001$). Feeding efficiency was 38.4%, and did not differ with temperature (ANOVA with mass as a covariate, $F_{1,36} = 0.182$, $P = 0.672$).

In the 45°C feeding treatment, 10 of the 27 animals died within 18 h, and 9 of the remaining 17 died over the following 3 days. The body temperatures at which grasshoppers voluntarily jumped away from a strong heat source (100-W incandescent lamp) ranged from 37.9 to 50.5°C (mean 44.0°C). Three individuals died within 30 min and a further 4 died within 24 h.

Thermoregulatory behavior

Sixty-two grasshoppers were sampled across 565 branch-shakes over 5.5 h. Across this interval, 114 operative temperatures were measured, representing 2 samples of each of the 57 randomly allocated sites. The T_b and T_e measurements tracked the locally-measured shaded air temperature but were at times as much as 5.1°C and 7.1°C higher (Fig. 4). This variation was largely within the range predicted by the microclimate model for heights from 10 to 120 cm above ground, although the AWAP-predicted air temperatures were approximately 1.5°C higher than what we observed (Fig. 4). There were no significant differences between T_b and T_e overall, nor when considering the morning and midday periods separately (*t*-tests, all $P > 0.15$), and the distributions of these variables was similar. The overall mean deviation of T_e from the peak feeding rate range of 30–40°C, the d_e statistic of Hertz *et al.* (1993), was 5.2°C while the deviation of T_b from this range, the d_b statistic, was 6.2°C. The effectiveness of thermoregulation statistic $E = 1 - (d_b/d_e)$ was −0.2. At midday, these statistics were $d_b = 0.1$°C and $d_e = 0.2$°C, with $E = 0.46$. However, there were no significant differences between the operative and observed temperatures for the morning sample, the midday sample or the whole period (*t*-tests, $P > 0.16$).

Simulation results

Shaded air temperatures measured at the Stirling Vale Creek study site over April to June 2006 are shown in Supplementary Figure S3a. Predicted air temperature extracted for the site from the AWAP dataset (Jones *et al.*, 2009) were found to be biased downward 1.25°C and initial predictions of soil temperature were biased in the same direction. After correction for this bias, the observed and predicted air temperatures showed a close correspondence (Suppl. Fig. S3b) (correlation coefficient *r* = 0.92, root mean square error *rmse* = 2.6°C). Soil temperature predictions were made from 0 to 90% shade in 10% increments. The soil temperature measured in the sun at 3-cm depth corresponded most closely with the predictions under 30% shade (*r* = 0.97, *rmse* = 2.0°C) and the shaded soil temperature measurements were closest to the 70% shade simulation (*r* = 0.95, *rmse* = 1.8°C) (Fig. 3c–f).

Simulations of development at 0% shade with the high temperature diapause mechanism imposed resulted in the first 11 of the 13 cohorts hatching on 23 October, with the remaining 2 hatching on 31 October and 11 November (Fig. 5a). When the diapause was not imposed, egg hatching was staggered either side of winter, with the first 7 cohorts hatching between 9 February and 26 April, and the remaining 6 cohorts hatching between 16 September and 11 November (Fig. 5b). Under 30% shade, diapause simulations resulted in the first 10 cohorts hatching on 18 November, and the remainder on 20, 25 and 30 November (Fig. 5c). Without diapause at 30% shade, the first 6 cohorts hatched between 4 February and 20 April, with the remaining 7 between 1 October and 29 November (Fig. 5d). Under 70% shade, diapause simulations resulted in hatch dates spread evenly between 21 November and 30 December, with the 3 latest cohorts failing to complete development by the end of the year (Fig. 5e). The corresponding no-diapause simulation (i.e. development continues if exposed to 35.5°C or higher) had pre-winter hatch dates for the first 3 cohorts between 14 February and 16 March, the remaining cohorts hatching between 18 October and 30 December, again with the 3 latest cohorts not developing within the year (Fig. 5f). Under the highest shade simulation (90%: results not shown), only the first 4 cohorts laid in January completed development within the year under either diapause scenario, hatching as early as 21 November (diapause) and 13 October (non-diapause).

The simulated feeding rate frequently dropped to zero during the winter period (Fig. 5). All cohorts hatching before winter would have experienced up to 11.8 consecutive days without feeding, while those hatching after winter were always able to feed each day post-hatching.

DISCUSSION

High-temperature induced diapause

At constant temperature, the developmental range of Standard Phylad *W. virgo* has a lower limit somewhere between 24 and 29°C and an upper limit between 33 and 35.5°C (Figs 1–2). This is remarkably narrow when compared to observed and predicted temperatures at oviposition depth (3 cm), which may vary from approximately 0 to 55°C (Fig. 6 and Suppl. Fig. S3). The hatching success in our study was very low (6.7%), but this was most likely due to egg rupture during transfer to the Petri dishes after laying (i.e. a result of poor handling techniques). Subsequent and ongoing work has dramatically improved this hatch rate and produced the same developmental responses (Kong *et al.* 2016). The narrow developmental temperature range of approximately 10°C of *W. virgo* contrasts with the wider range of temperatures reported for other grasshoppers (Acrididae) in both temperate and semi-arid regions, typically between 15 and 20°C (Birch 1942; Hunter-Jones 1970; Wingerden *et al.* 1991; Ando 1993; Gehrken & Doumbia 1996; Hao & Kang 2004; Fielding & Defoliart 2010). For example Gregg (1983) reported egg development in *Chortoicetes terminfera* between 16.2 and 39.0°C. Similarly narrow limits to *Warramaba* have been reported for the grasshopper *Caledia captiva* but primarily due to eggs entering diapause at lower experimental temperatures (Groeters & Shaw 1992).

Under a more realistic diurnal incubation regime cycling between 14 and 41°C, we found that eggs would not develop. Instead freshly-laid eggs exposed to temperatures >35.5°C for some period during the day (10 h or shorter, given our temperature cycle, Suppl. Fig. S1) suspended development unless transferred to lower temperatures. The time to complete development post-high-temperature exposure was then shortened by around 25–30%, regardless of how long eggs had been kept at high temperature. Thus, it appears that eggs partly developed to a fixed point during high-temperature exposure before entering diapause.

Most studies of diapause have focused on winter diapause in species from temperate areas, whereas documented cases of summer diapause, or of winter diapause in species from non-temperate regions, are less common (Tauber *et al.* 1986; Danks 1987). However, this likely reflects a bias in the literature, with summer diapause occurring as a widespread yet underappreciated trait in many species (Masaki 1980). Summer diapause represents a key trait for withstanding high temperature stress or as a mechanism to synchronize the timing of reproduction, and is most commonly reported in pupae or imago stages (Katsoyannos *et al.* 1997;

Nibouche 1998; Liu *et al.* 2006). Examples of summer diapause in orthopterans are limited (Wardhaugh 1986; Dingle & Mousseau 1994; Tanaka 1994; Fielding 2008; Maiga *et al.* 2010), particularly at the egg stage, whereas well-documented examples of summer egg diapause are found in Australia in other invertebrates such as earth mites and the lucerne flea, *Sminthurus viridis* (Wallace 1968; Wardhaugh 1986; Umina & Hoffmann 2003). Winter diapause has been extensively studied in model Tettigoniidae and Acrididae systems as a mechanism for phenological control (Wardhaugh 1986; Dingle & Mousseau 1994; Tanaka 1994; Fielding 2008). High temperature-induced diapause at the egg stage has been described in other orthopterans (Andrewartha 1943; Masaki 1962; Maiga *et al.* 2010). For example, Higaki and Ando (2002) reported that *Eobiana engelhardti subtropica* eggs at 30°C did not develop but immediately resumed development after transfer to lower temperatures. Most examples of univoltine phenologies are maintained by an obligate diapause. For example, the small plague locust, *Austroicetes cruciata*, also passes the hot, dry southern Australian summers in an obligate embryonic diapause, which is terminated by winter temperatures, thereby maintaining its univoltine phenology (Andrewartha 1943, 1944). However, because many insects pass the summer in diapause, and the transition between diapause termination and post-diapause quiescence is often not distinguished, the importance of summer diapause may be underappreciated (Groeters 1994; Hunter *et al.* 2001; Hodek 2002).

Under constant temperature incubation, the eggs of matchstick grasshoppers (including Standard Phylad *W. virgo*) typically absorb their initial weight in water over 7 days (Blackith & Blackith, 1968). However, the eggs exposed to temperatures >35.5°C did not take up water despite being exposed to a near-saturated substrate. Water uptake is necessary for the completion of development in grasshopper eggs, and the potential for water uptake to occur is associated with the termination of egg diapause in other grasshopper species (Slifer 1958; Moriarty 1970). The nature of diapause in Standard Phylad *W. virgo* is highly unusual in being maintained by frequent exposure to high temperature as opposed to the more typical case of being broken by prolonged exposure to low temperature. Nonetheless, water uptake occurs rapidly in *W. virgo* once eggs are removed from the high temperature environment, and development then resumes as is the case in many other grasshopper species. It is possible that high temperatures induce a response in the embryo leading to active blocking of water uptake: for example, through the production of waxes by cells beneath the hydropyle (Slifer 1958). It is not clear from our data how long the effect lasts, but presumably more than a day to prevent occasional, cold wet days in summer prematurely breaking diapause.

Thermoregulation and thermal tolerance of non-embryonic *Warramaba virgo*

Our studies of the thermal sensitivity of feeding in *W. virgo* reveal a broader thermal response curve than seen in egg development, with feeding rate rising rapidly above 17°C and reaching a plateau between 30 and 40°C (Fig. 3). Temperatures above 40°C appear to be stressful given that sustained (hours) exposure to 45°C in the feeding trials and shorter-term (minutes) exposure to temperatures between 40°C and 50°C caused mortality. Nonetheless, ambient temperatures in the habitat of *W. virgo* during their active phase from October to April can go well below 17°C and well above 40°C (Suppl. Fig. S3).

We found no evidence that *W. virgo* grasshoppers behaviorally thermoregulate to avoid exposure to high or low temperatures. Instead, they conform to the expected body temperature distribution of an animal behaving randomly with respect to temperature (Figs 4 and 5). The E statistic of Hertz *et al.* (1993) for quantifying the extent of thermoregulation was −0.2 when considering the whole time period, and higher ($E = 0.45$) when considering the midday period where air temperatures were approaching 40°C, but body temperature was never statistically different from the randomly sampled ones. The degree of thermoconformity in *W. virgo* is not surprising given their small size (and the small size of the leaves on which they perch), and the relatively high wind speeds they are exposed to on bushes, resulting in strong convective coupling (Gates 1980; Willmer & Unwin 1981). The main spatial variation available for thermoregulation in this species is the vertical gradient in air temperature, but this will vary strongly with the height of the food plant (centimeters to meters).

Adaptive significance of the egg thermal response

While field experiments are valuable for testing the adaptive significance of the unique thermal response of *W. virgo* eggs, simulation studies can help to develop and test hypotheses and to explore the consequences of novel environments. Useful simulation of egg developmental responses to temperature depends critically on an ability to predict realistic thermal environments. The NicheMapR microclimate model has previously provided accurate predictions of soil temperature (within 10%) across the soil profile when driven by gridded daily meteorological data available for Australia (Kearney *et al.* 2014). The same system predicted 3-cm shaded and unshaded soil temperatures at one of our study sites with an error of around 2°C (Suppl. Fig. S3).

Our simulations of the development of eggs laid in full sun from January to March, when including the high-temperature diapause response, resulted in a tight range of hatch dates; the majority hatched on a single day, 23 October, and the whole hatch period extended only to 11 November. This is consistent with the field observations (see Introduction). When development was simulated without the high-temperature diapause, synchronization was lost and the early clutches hatched before the winter (as early as mid-February), with the later clutches hatching earlier in the spring (mid-September) (Fig. 5b). As a consequence, the early clutches would have been exposed to temperatures (up to 54°C) at the highly developed embryo stage well in excess of the putative lethal limit of 45°C, and would have experienced a poor thermal environment for feeding over the winter, potentially running down their reserves (Irwin & Lee 2000). Detailed studies of lethal temperatures of embryos at different stages of development, and of the consequences of limited feeding potential over winter, are needed to elucidate fitness implications of pre-winter hatching. Our analyses suggest that the high-temperature diapause mechanism, together with the low-temperature quiescence, act to buffer *W. virgo* against both high and low temperature stress.

An important gap in our understanding of the developmental ecology of *W. virgo* is oviposition-site selection. Matchstick grasshoppers are only known to lay their eggs in the ground (Blackith & Blackith 1968). We have observed this in *W. virgo*, with 1 clutch of 6 eggs being dug out of unshaded sandy soil at the Nowingi site on 18 September 2010, approximately 300 mm from a low *Acacia wilhelminana* bush (Suppl. Fig. S3). These eggs hatched successfully in the laboratory. Whether this species also lays its eggs in deep shade is unknown. Our simulations of the effect of shade on development show that partial (30%) shade would have little effect on the phenology, delaying spring hatch slightly (Fig. 5c), but that high shade (70%) would result in eggs failing to hatch before the end of December.

Another area requiring investigation is the effect of soil moisture on phenology. Morabine grasshopper eggs typically require around 7 days of high soil moisture to complete water uptake. If soil water potential is too low over the summer for water uptake to be complete, then the eggs may be prevented from hatching even without the high temperature-induced diapause. If that were frequently the case, the high temperature-induced diapause mechanism could act as a secondary mechanism to account for occasional high rainfall summers. To address this issue, we need further information on water uptake speed and the threshold water potential for uptake.

In conclusion, the novel high temperature-induced diapause mechanism that we discovered in *W. virgo*, in combination with suppressed developmental rates below 25°C, is likely the cause of its univoltine life cycle. From our simulations and field measures of soil temperatures, we propose that this acts to prevent embryos reaching a developmental state susceptible to heat stress in the summer, and prevents them hatching when it is too cold to feed. In contrast, this grasshopper appears to have little capacity to buffer high temperature extremes after hatching. The egg stage may, therefore, be well buffered from the effects of climate change, and the post-embryonic stage may be the most vulnerable to the direct effects of warming.

ACKNOWLEDGMENTS

We thank Nelida Contreras and the late Graeme Webb for advice on collection and culturing of *Warramaba virgo*, Danuta Loesch-Mdzewska for insights into MJD White's field work, and Stephen Simpson for suggesting the protocol for measuring the feeding rate. This research was supported by Australian Research Council grants DP0450050 to MRK and DP160100279 to MRK and AAH.

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Figure 1 Egg viability at constant incubations (open circles) and at the cycling regime (filled point at 27.7°C) and 35.5 and 39.2°C incubation after subsequent incubation at constant 31.7°C (filled circles). Numbers above each point indicate sample size/number hatched.

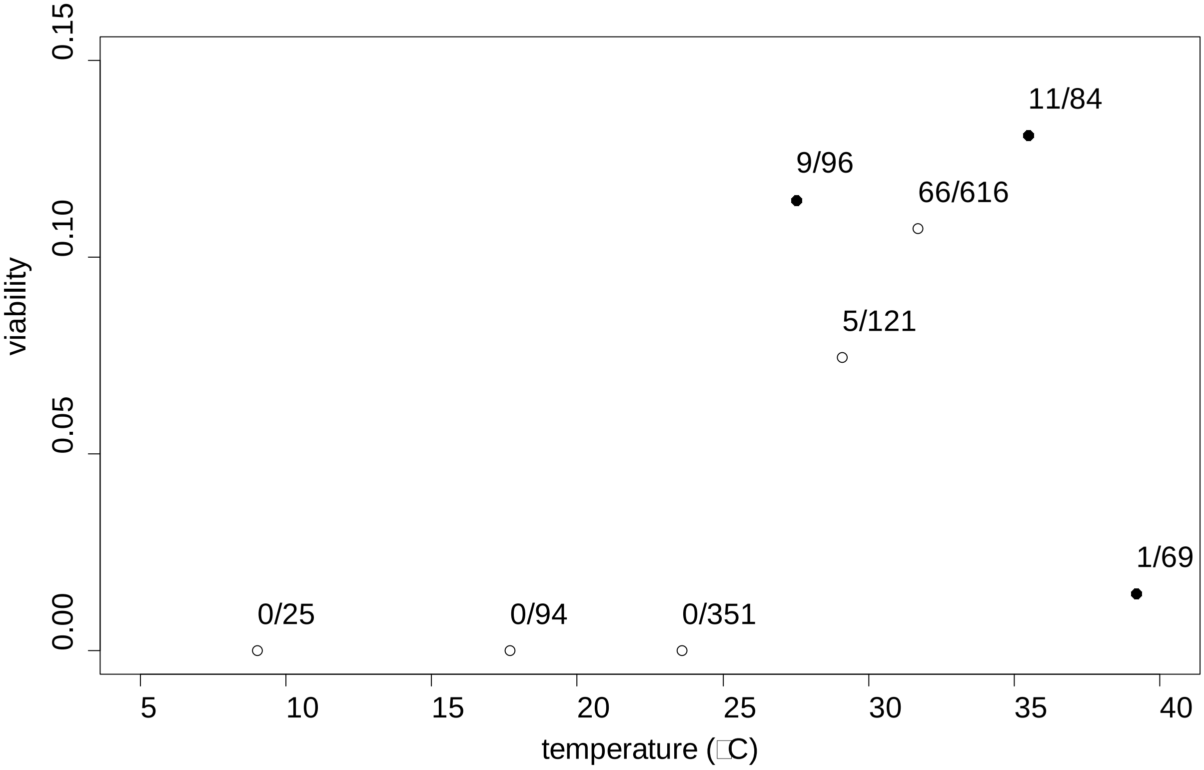
Figure 2 Egg development rate observed at constant temperatures (open circles) and inferred indirectly (closed symbols). The inferred rates for 23.6, 29.2 and 31.7°C were from dissections of eggs that had partially completed development. The inferred rate at 35.5°C was from eggs that had been partially incubated at this quiescence-inducing temperature, and then returned to 31.7°C. The fitted 5-parameter Arrhenius curve used the feeding response curve parameters for the upper thermal limit (T_{AH} and T_H).

Figure 3 Mean feeding rate achieved at constant temperatures for captive-reared (open circles) and wild-caught (filled circles) individuals along with the 5-parameter Arrhenius curve fitted by pooling captive-reared and wild-caught individuals.

Figure 4 Time course of various temperature measurements at the Nowingi site on 14 January 2006: grasshopper body temperatures (crosses), operative temperatures (grey circles), field-measured air temperature (black solid line), and predicted air temperature at 10 and 120 cm (upper and lower solid blue lines, respectively). Also indicated are the thresholds between which feeding rate is maximized (dashed horizontal lines).

Figure 5 Simulated development rates (black lines) for 13 *Warramaba virgo* cohorts laid weekly from 1 January to 26 March 2006 at the Stirling Vale Creek site, under 0% shade (a,b), 30% shade (c,d) and 70% shade (e,f). The left-hand panels show simulations with a high temperature diapause imposed, and the right panels show the consequence of no diapause mechanism. In (a) the arrows indicate hatch events, with 11 of these happening synchronously on 23 October. In the absence of diapause, only a subset of cohorts successfully developed within the period considered in the simulations. Simulated soil temperature at 3 cm is shown in orange and simulated feeding rate assuming grasshoppers are at 1 m air temperature is shown in green. Also indicated is the diapause-inducing temperature threshold, 35.5°C (dashed black horizontal line) and the putative upper lethal temperature limit of 45°C (dashed red horizontal line).

Figure 1



0.15
0.10
0.05
0.00
viability
11/84
9/96
66/616
5/121
1/69
0/25
0/94
0/351
5
10
15
20
25
30
35
40
temperature (□C)

Figure 2

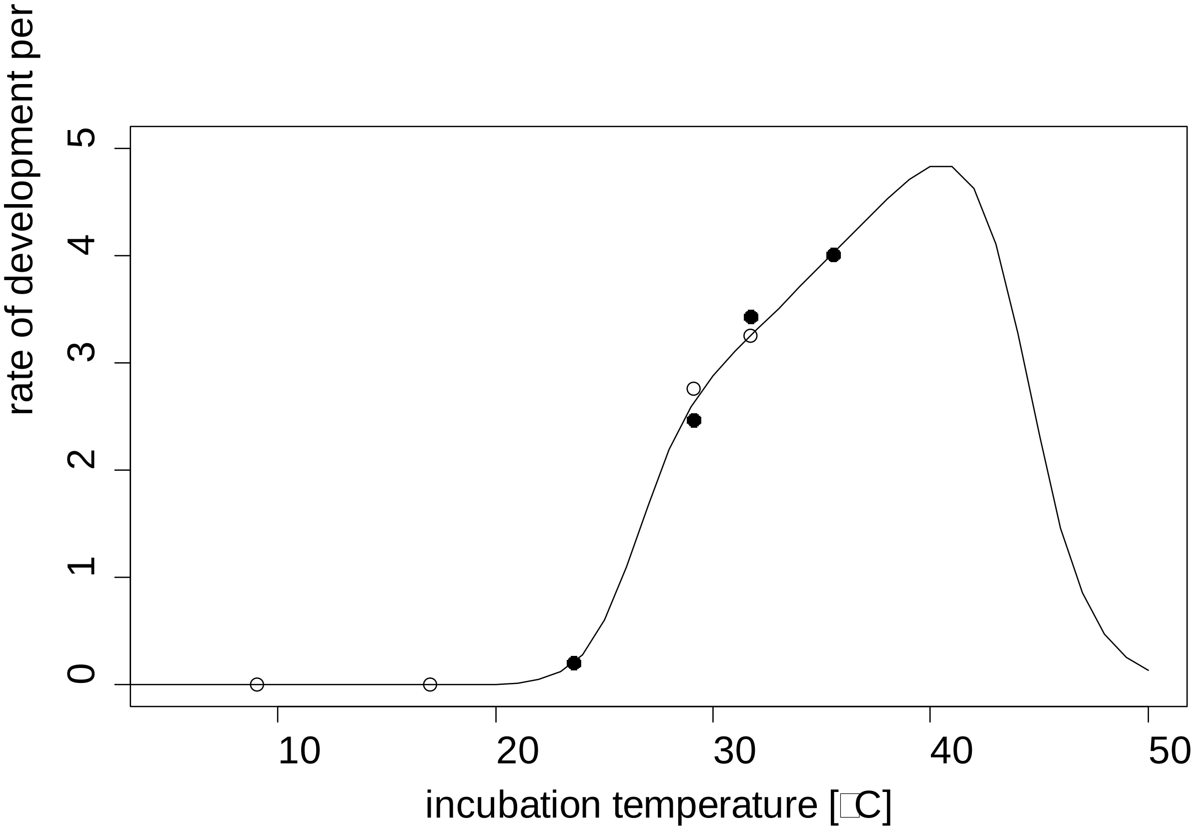

rate of development per
0
1
2
3
4
5
10
20
30
40
50
incubation temperature [□C]

Figure 3

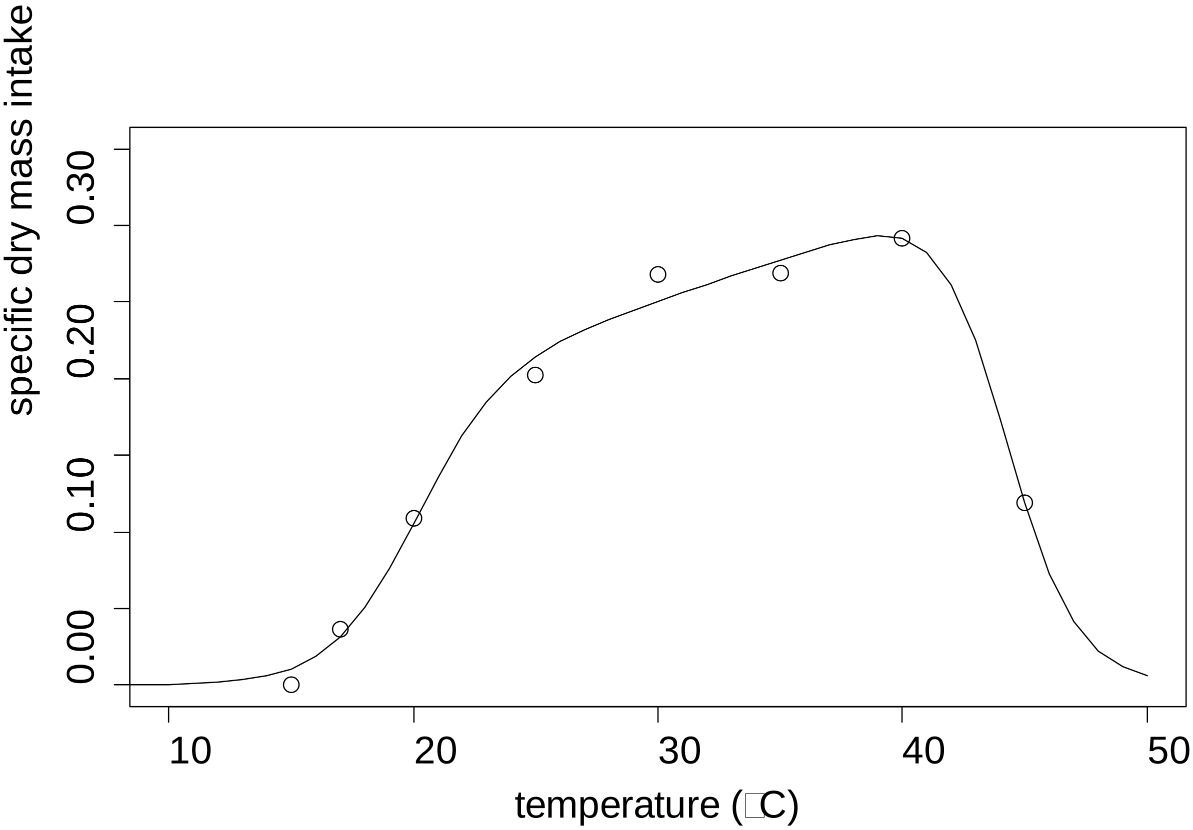

specific dry mass intake
0.00
0.10
0.20
0.30
10
20
30
40
50
temperature (□C)

Figure 4

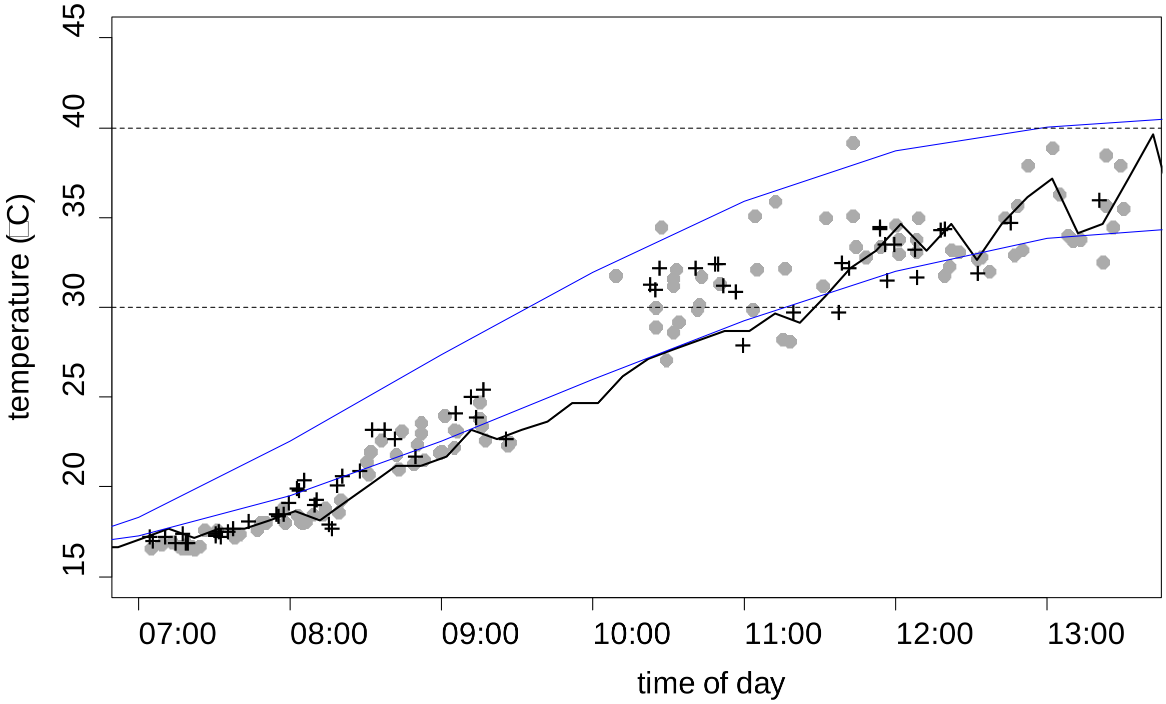
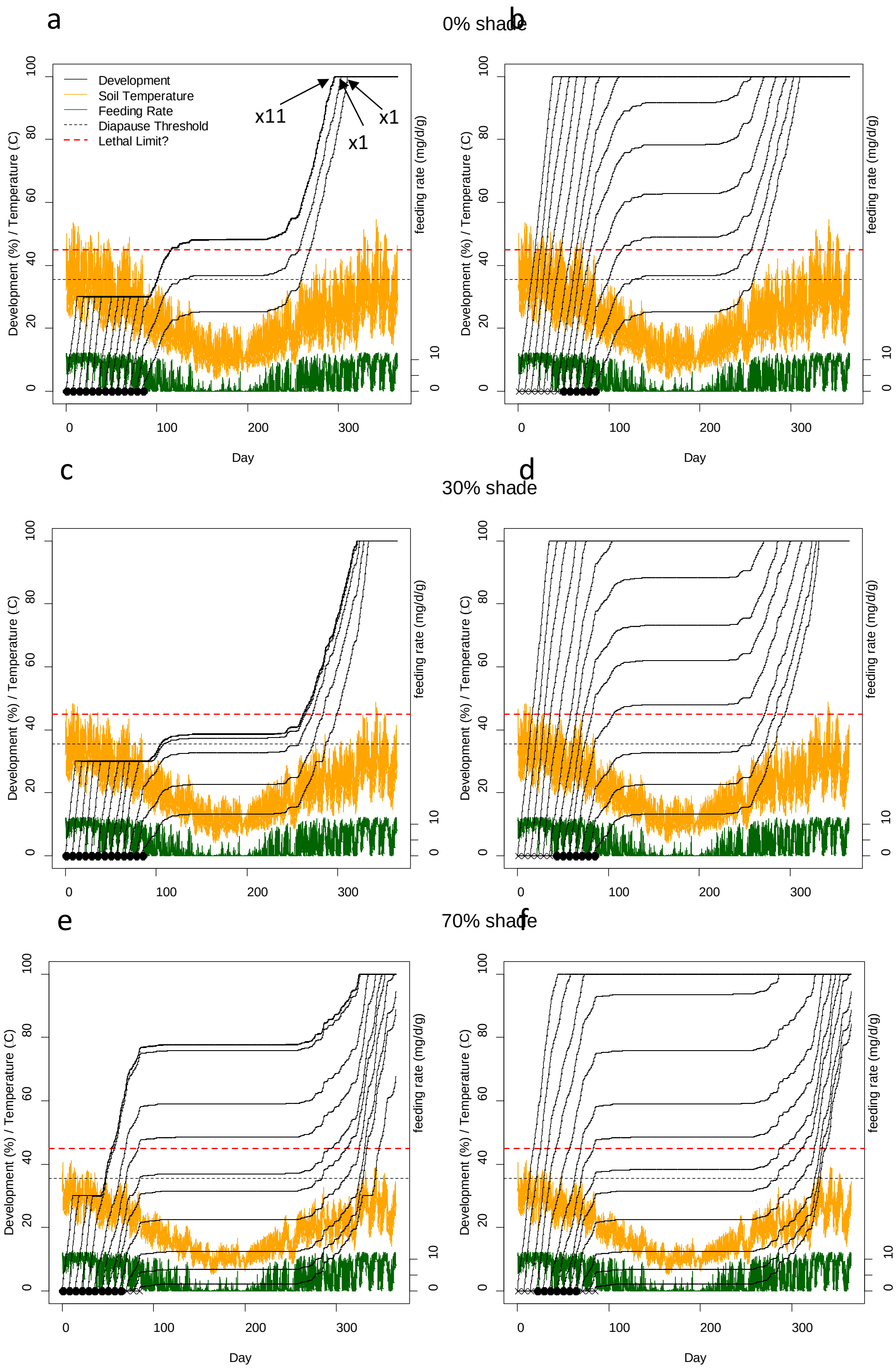
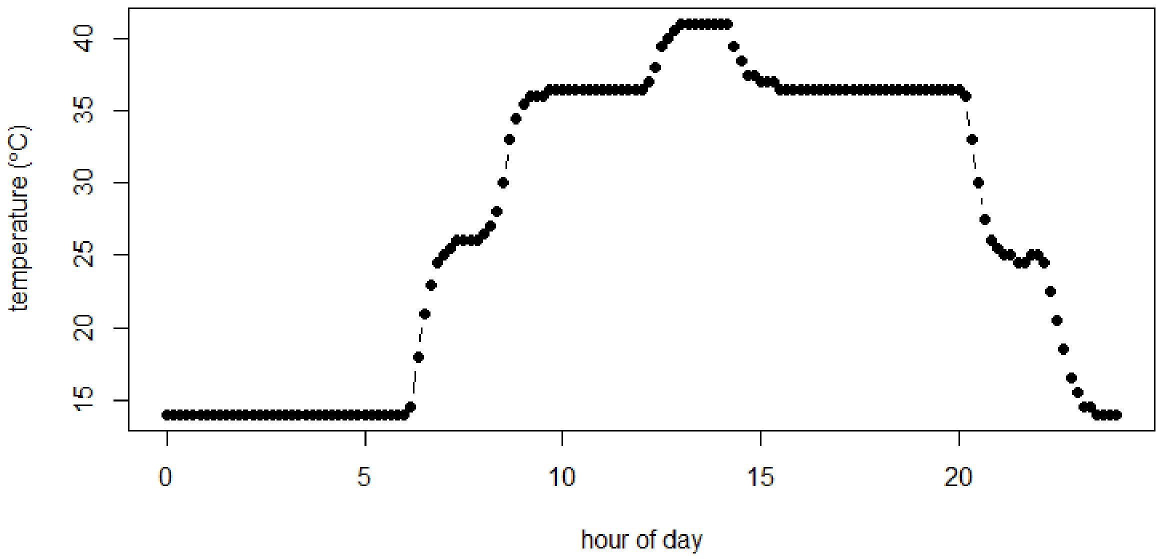

45
40
35
30
25
20
15
temperature (□C)
07:00
08:00
09:00
10:00
11:00
12:00
13:00
time of day

Figure 5


a
b
0% shade
c
d
30% shade
e
f
70% shade
Development
Soil Temperature
Feeding Rate
Diapause Threshold
Lethal Limit?
x11
x1
x1
Development (%) / Temperature (C)
feeding rate (mg/d/g)
Day
0
20
40
60
80
100
10
0
100
200
300

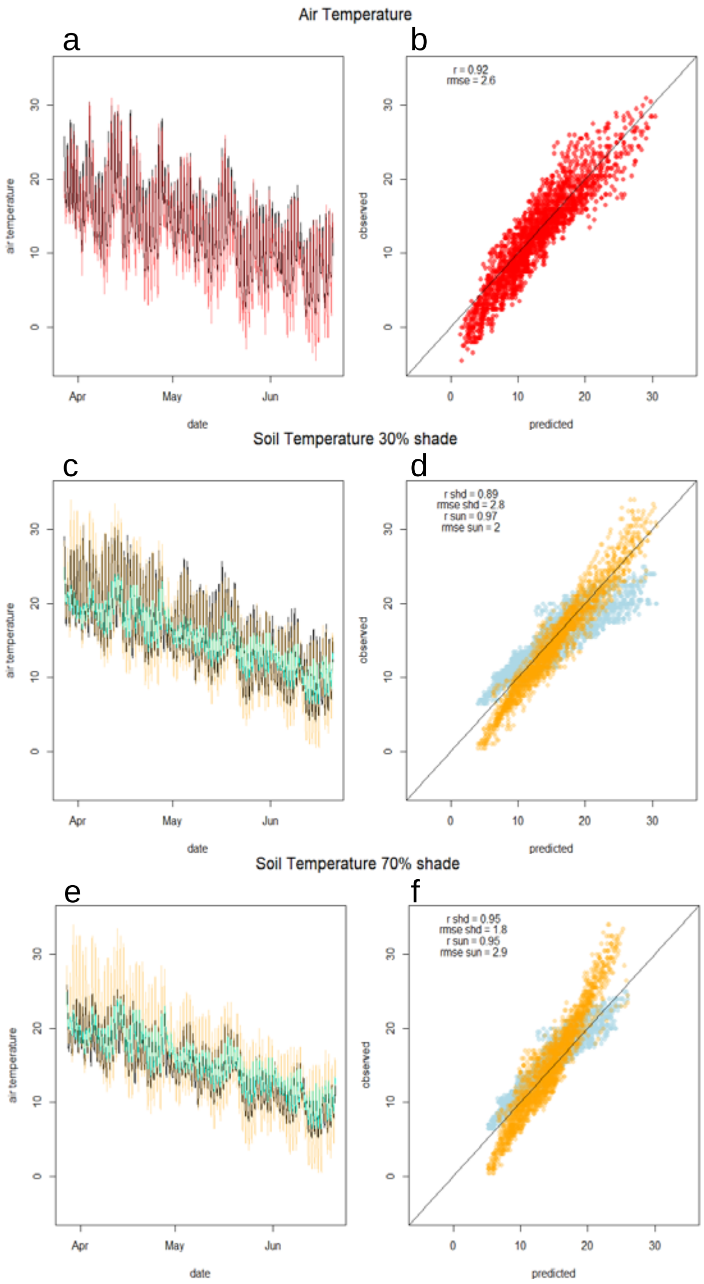
SUPPLEMENTARY MATERIALS

Supplementary Figure S1 The diurnal temperature cycle in an incubator used to measure larval development under 14–41°C, as measured by a Thermochron iButton data logger r(Maxim Integrated, San Jose, USA). The incubator (*Steridium* E500, Brendale, Qld, Australia) was programmed to step across 4 set-points: 14, 26, 36.5 and 41°C.



Supplementary Figure S2 Stirling Vale Creek site where soil and air temperature loggers were installed in 2006. *Warramaba virgo* lives and feeds on the mulga (*Acacia aneura*) trees in the foreground.

Supplementary Figure S3 Predicted shaded air temperature (a and b), and predicted soil temperature in sun (c and d) and shade (e and f) compared to observed temperature. Black lines show predictions in the left-hand panels, with air temperature observations in red, sunny soil temperature observations in orange and shaded soil temperature observations in cyan. The correlation coefficients *r* and root mean square deviations *rmse* are indicated on the scatter plots of observed and predicted values (right hand column), with the 1:1 lines indicated in black.



Supplementary Figure S4 Habitat of *Warramaba virgo* at the Nowingi site showing an *Acacia wilhelmiana* bush (food plant) and with the black arrow indicating the site where a clutch of eggs was found on 18 September 2010.