Estimating the differences in critical thermal maximum and metabolic rate of *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) across life stages

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Temperature is a crucial driver of insect activity and physiological processes throughout their life-history, and heat stress may impact life stages (larvae, pupae and adult) in different ways. Using thermolimit respirometry, we assessed the critical thermal maxima (CT_{max} - temperature at which an organism loses neuromuscular control), CO_2 emission rate ($\acute{V}CO_2$) and Q10 (a measure of $\acute{V}CO_2$ temperature sensitivity) of three different life stages of *Helicoverpa punctigera* (Wallengren) by increasing their temperature exposure from 25°C to 55°C at a rate of 0.25°C min^{-1}. We found that the CT_{max} of larvae (49.1°C±0.3°C) was higher than pupae (47.4°C ±0.2°C) and adults (46.9°C±0.2°C). The mean mass-specific CO_2 emission rate (ml $\acute{V}CO_2$ h^{-1}) of larvae (0.26±0.03 ml $\acute{V}CO_2$ h^{-1}) was also higher than adults (0.24±0.04 ml $\acute{V}CO_2$ h^{-1}) and pupae (0.06±0.02 ml $\acute{V}CO_2$ h^{-1}). The Q_{10}:25–35°C for adults (2.01±0.22) was significantly higher compared to larvae (1.40±0.06) and Q_{10}:35–45°C for adults (3.42±0.24) was significantly higher compared to larvae (1.95±0.08) and pupae (1.42±0.98) respectively. We have established the upper thermal tolerance of *H. punctigera*, which will lead to a better understanding of the thermal physiology of this species both in its native range, and as a pest species in agricultural systems.

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2 *punctigera* (Wallengren) (Lepidoptera: Noctuidae) across life stages

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15 **Abstract**

16 Temperature is a crucial driver of insect activity and physiological processes throughout their life-

17 history, and heat stress may impact life stages (larvae, pupae and adult) in different ways. Using

18 thermolimit respirometry, we assessed the critical thermal maxima (CT_{max} - temperature at which

19 an organism loses neuromuscular control), CO_2 emission rate ($\acute{V}CO_2$) and Q10 (a measure of

20 $\acute{V}CO_2$ temperature sensitivity) of three different life stages of *Helicoverpa punctigera*

21 (Wallengren) by increasing their temperature exposure from 25°C to 55°C at a rate of 0.25°C min^{-1}.

22 We found that the CT_{max} of larvae (49.1°C±0.3°C) was higher than pupae (47.4°C ±0.2°C) and

23 adults (46.9°C±0.2°C). The mean mass-specific CO_2 emission rate (ml $\acute{V}CO_2$ h^{-1}) of larvae

24 (0.26±0.03 ml $\acute{V}CO_2$ h^{-1}) was also higher than adults (0.24±0.04 ml $\acute{V}CO_2$ h^{-1}) and pupae

25 (0.06±0.02 ml $\acute{V}CO_2$ h^{-1}). The Q_{10}:25–35°C for adults (2.01±0.22) was significantly higher

26 compared to larvae (1.40±0.06) and Q_{10}:35–45°C for adults (3.42±0.24) was significantly higher

27 compared to larvae (1.95±0.08) and pupae (1.42±0.98) respectively. We have established the upper

28 thermal tolerance of *H. punctigera*, which will lead to a better understanding of the thermal

29 physiology of this species both in its native range, and as a pest species in agricultural systems.

30 **Key words**: *Helicoverpa punctigera*, life stage, critical thermal maxima, metabolic rate,

31 thermolomit respirometry

32 **Introduction**

33 Insects have a specific range of temperatures within which they can survive, grow and reproduce

34 (Andrew et al., 2011; Huang et al., 2015; DeVries et al., 2016), and have their distribution strongly

35 defined by a thermal envelope (Gullan & Cranston, 2014; Li et al., 2019). Assessing the impact of

36 extreme temperature exposures on insect distribution is vital to understanding insect ecology and

37 the current and future effects of climate change (Andrew et al., 2011; Betini et al., 2013). Within

38 a rapidly changing climate, thermal limits are an essential part of assessing thermal physiology of

39 insects, as they are the critical endpoints that an organism can survive or loses muscular control

40 (Lighton & Turner, 2004; Andrew et al., 2016), quantified in terms of critical thermal maximum

41 (CT_{max}) and critical thermal minimum (CT_{min}) (Kingsolver et al., 2018).

42 Thermal limit measurements like CT_{max} and CT_{min} are commonly used to understand and predict

43 species distributions among populations (Shik et al., 2019) and responses to environmental

44 changes (Angilletta et al., 2003; Angilletta, 2009; Kingsolver et al., 2018). CT_{max} is often aligned

45 to the distribution of species, as it is a biologically significant and ecologically relevant thermal

46 performance trait (Sunday et al., 2014; Shik et al., 2019). However, the CT_{max} of most insect groups

47 is highly plastic, so can change depending on an organism's pre-exposure conditions, making it

48 difficult to forecast their response to a rapidly changing climate (Terblanche et al., 2005; Martínez

49 et al., 2016; Kellermann et al., 2017).

50 CT_{max} has been shown to differ across life stages in some insects e.g. kelp fly (*Paractora dreuxi*),

51 Sirex wasp (*Sirex noctilio*) and mealworm (*Tenebrio molitor*) (Marais et al., 2009; Vorhees &

52   Bradley, 2012; Li et al., 2019), and can be influenced by body mass and surface area of the insect

53   (Lighton & Turner, 2004). CT_{max} values depend substantially on how they are measured (Lighton

54   & Turner, 2004; Shik et al., 2019); it is thus essential to determine a precise scale of temperature

55   exposure for insects (Terblanche et al., 2007). Temperature ramping rates can influence the CT_{max}

56   of an insect (Lighton & Turner, 2004; Terblanche et al., 2007). A slow temperature ramp rate of

57   an organism leads to increased risk of heat shock, which is absent under the natural conditions

58   (Lutterschmidt and Hutchison, 1997). The other process that can occur during a slow ramp is short-

59   term physiological adjustments (e.g. induction of heat shock proteins) that protect cells from heat

60   damage and might increase CT_{max} (Kingsolver, 2009; Shik et al., 2019). Fast temperature ramp

61   leads to delay in thermal equilibrium between the air and the insect's body. Because of this, under

62   a fast temperature ramp, the inner temperature of the insect will be lower than the recorded air

63   temperatures leading to an overestimation of their CT_{max} (Lighton & Turner, 2004; Andrew et al.,

64   2013; Agudelo-Cantero et al., 2019).

65   Intriguingly, only few studies have looked at thermal variation across different life stages (Le Lann

66   et al., 2011). The few studies focused on a single unit of fitness in one environment (Le Lann et

67   al., 2011). Thermal plasticity has been identified in many insect taxa e.g. ants *Iridomyrmex*

68   *purpureus*), *Drosophila* species and parasitic wasps (*Aphidius rhopalosiphi*) (Chown &

69   Terblanche, 2006; Le Lann et al., 2011; Andrew et al., 2013; Shik et al., 2019), but not all

70   (Terblanche et al., 2010).

71   Insect herbivores that are found on the ground and plant surfaces in warmer and drier environments

72   are exposed to substantial microclimate variability including seasonal and daily temperature

73   variations at the soil and plant surface (Hodgson, 1991; Andrew et al., 2016). How organisms

74   respond to the variable microclimates depends on the thermal physiology e.g. CT_{max} (Somero,

75   2010; Shik et al., 2019). The physiology of insects can be modified by the microclimate they

76   experience (Andrew et al., 2013; Andrew et al., 2016). Predicting the future in changing climates

77   is made difficult by our limited understanding of the eco-physiological mechanisms and

78   evolutionary process underlying thermal phenotypic plasticity (Shik et al., 2019).

79   *Helicoverpa punctigera* is native to Australia and widely distributed across the continent (Gregg

80   et al., 2018). It is a notable pest on crops like cotton, oilseeds, legumes and vegetables (Gregg et

81   al., 2019). The adult can travel a long distance and can adapt locally to different environmental

82   conditions including inland Australia (Gregg et al., 2018, 2019). Female adult *H. punctigera* lay

83   eggs on leaves, flower buds and developing fruits of the host plant (Zalucki et al., 1986). The

84   neonate (newly hatched) usually eat soft leaves; as they mature, they move about other feeding

85   sites like flowers or flower buds, pods, fruits and seeds (Gregg et al., 2018). The last larval instar

86   burrows into the soil and pupates. Inland habitats are characterised by extreme climatic conditions

87   like very hot temperatures as high as 50°C during summer (Bureau of Meteorology, 2018). The

88   ability of this insect to survive the extreme summer temperatures in the inland depends on their

89   thermal tolerances and physiological sensitivities e.g., CT_{max} and biochemical shifts that are

90   temperature-dependent (Gaitán-Espitia et al., 2014). Female adults of *H. punctigera* are

91   responsible for host selection and dispersal, whilst feeding activities of the larvae cause economic

92   loss to the host plants, and a complete reorganisation of the body occurs within the pupal stage,

93   understanding the metabolic rates among the different life stages are essential.

94   Energy is the crucial currency of life-history traits of animals including insects (Terblanche &

95   Chown, 2007; Desforges et al., 2019). Metabolism combines organism's energy supply and

96   fitness-related activities (Shah et al., 2021). Measurement of metabolic rate is one way of

97   measuring the energy cost related with different life-functions like growth, development,

98   movement among others (Shah et al., 2021). In insects, metabolic rates can influence not only

99   fitness in individuals but also geographic distributions and abundances (Shah et al., 2021).

100   Therefore, estimating the metabolic rates of organisms will assist in identifying the cost of living

101   and performance in a particular environment (Terblanche et al., 2005; Terblanche & Chown,

102   2007), and can help predict geographic variations in response to global warming (Shah et al.,

103   2021).

104   Metabolic rate can respond to changes in ambient temperature, activity and body mass; however,

105   age and ontogeny, sex, feeding status, season and time of day, among others, can also exert

106   substantial influence (Terblanche & Chown, 2007; Harrison, 2009; Altermatt, 2010). For example,

107   in the Australian field cricket (*Teleogryllus oceanicus*) and thynnine wasp (*Zaspilothynnus*

108   *nigripes*), males have a higher metabolic rate than females (Kolluru et al., 2004; Tomlinson &

109   Phillips, 2015). Specific dynamic action is the amount of energy expenditure above the basal

110   metabolic rate (RMR) due to the ingestion and digestion of food for use as energy or conversion

111   to a storage form (Secor, 2009). One major metabolic variable is the temporary rise in metabolic

112   rate following feeding status or swallowing food (Karasov & Martinez del Rio, 2007; McCue et

113   al, 2012). This feeding status-associated rise in metabolic rate is a result of food breakdown and

114   processing in the gut of the organism (Karasov & Martinez del Rio, 2007). High temperatures

115   generally produce high metabolic rate, however the total energy assigned for specific dynamic

116   actions is not fully dependent on temperature (McCue et al., 2016a; Wang et al., 2003).

117   The temperature sensitivity (Q_{10}), defined as the rate ratio of a given process taking place at

118   different temperatures, is used to measure the degree of temperature dependence of a given

119   biological process (Davidson et al., 2006; Mundim et al., 2020). Q_{10} is an important parameter in

120   predicting the effects of temperature on carbon dioxide release (Mundim et al., 2020).

121   Previous studies on *Helicoverpa* species (eg. *H. armigera* and *H. punctigera*) focused on the effect

122   of temperature on development rate, survivorship and reproduction (Room, 1983; Qayyum &

123   Zalucki, 1987; Mironidis et al., 2008; Zalucki et al., 2005; Mironidis, 2014) but not on thermal

124   physiology (CT_{max} and metabolic rate) of different life stages. For example, *H. punctigera* are

125   distinctive in terms of their life stage variation and habitat (Gregg et al., 2018, 2019), just as other

126   insects with stage-based niche changes. Across life stages, an individual will be exposed to a range

127   of temperatures (Agudelo-Cantero et al., 2019) with different opportunities to escape extreme

128   exposure. In the field, the larvae and adults of *H. punctigera* live on plant surfaces whiles the pupae

129   lives in the soil. Based on this life stage and habitat variations, their tolerance to extreme

130   temperatures may significantly vary among the different life stages. Our study is the first of *H.*

131   *punctigera* thermal physiology across three life-stages – larvae, pupae and adult.

132   We assessed the thermal physiology (CT_{max} and metabolic rate) of three different life stages ─ late

133   instar larvae, pupae, and adult ─ and sex of *H. punctigera* during temperature ramping using

134   thermolimit respirometry, which employs a flow-through respirometry, with $\acute{V}CO_2$ as a key

135   measure. The technique has an infrared activity detector to monitor moving activity (Lighton &

136   Turner, 2004; Vorhees & Bradley, 2012). One important advantage of this technique is that CT_{max}

137   can be identified directly from respiratory or activity data. In addition, it allows for CT_{max} to be

138   determined without disturbing the organism (Vorhees & Bradley, 2012). The technique, in

139   addition, allows for the measurement of $\acute{V}CO_2$ when exposed to biologically relevant temperature

140   points.

141   The specific questions we asked are:

142 (i) what are the differences in $\dot{V}CO_2$ and CT_{max} across different life stages of *H. punctigera*

143 considering the different field conditions each stage experiences? We predict that, larvae and adult

144 of *H. punctigera*, which are exposed to the many microclimatic conditions on host plants surface,

145 would have a higher metabolic rate and CT_{max} than pupae, because, the soil the pupae is burrowed

146 in is more thermally stable than the leaves.

147 (ii) what are the metabolic rates of the life stages at five different temperatures at 5°C bins: 25°C,

148 30°C, 35°C, 40°C and 45°C? Here, we predict that because exposure temperature has an overwhelming

149 effect on the metabolic rate of insects, the metabolic rate among the life stages would be lower at

150 25°C, 30°C and 35°C compared with 40°C and 45°C.

151 (iii) does sex influence the metabolic rate and CT_{max} of adult and pupae of *H. punctigera*? We

152 predict that because of the differences in activity (e.g., host selection and dispersal in the case of

153 adults) between male and female, sex would influence metabolic rate and CT_{max} of *H. punctigera*

154 pupae and adult.

155

156 **Materials and methods**

157 **Insect Culture and Experimental Animals**

158 A laboratory insect culture was established in April 2018 from pupae provided by Tamworth

159 Agricultural Institute, New South Wales Department of Primary Industries. After emergence, the

160 adults were put into cylindrical framed mating cages containing 5% sugar solution in dental wicks

161 held in 50 ml plastic containers. We left female moths to mate and lay fertile eggs for 72 hours

162 following protocols outlined by Gregg et al. (2016). Eggs were collected by daily scrolling (by

163 way of folding) of the paper towel which acts as the mating cage wall, followed by cutting the

164   towel containing the eggs into pieces measuring approximately 7cm x 21cm x11cm. Eggs were

165   cleaned by brief immersion in 0.2% sodium hypochlorite solution for disease prevention, brushed

166   carefully for one to two minutes and then rinsed two times with distilled water onto filter paper

167   placed in a Büchner funnel. The cleaned eggs were put in plastic eggcups coated with artificial

168   diet and kept at 25°C. Five days after hatching, the larvae were transferred into 35ml plastic cups

169   ventilated with small holes and containing 10-15 ml of soybean-based artificial diet (Greene et al.,

170   1976; Teakle, 1991). The insects were reared in the Agronomy insectary maintained at controlled

171   conditions of 70-75% relative humidity, 14h: 10h light: dark period and 25°C, University of New

172   England, NSW, Australia. For a continuous supply of *H. punctigera* individuals at appropriate life-

173   stages, two cultures were maintained at different developmental stages. Before and after each

174   experiment, the larvae, pupae or adult moths used were weighed on an electronic balance (Mettler

175   Toledo XP 404S, Greifensee, Switzerland) with an adjusted accuracy of 0.1 mg. We used fifth

176   instar larvae in our experiment because they were available and relatively easy to manipulate

177   without damaging their cuticle. The average live body mass of the insects used pre-experiment

178   was 189.09 ± 15.58 mg for adults, 348.26 ± 57.57 mg for larvae and 332.38 ± 18.39 mg for pupae.

179   One-week-old pupae were sexed into male and female under a microscope using the genital scars

180   (Kirkpatrick, 1961) and used in pupal assays. Finally, virgin adults that were one to two days post-

181   emergence were used for the experiment: these adults were not fed. Because of the polyphagous

182   nature of the insect, larvae would always have a bite of a meal or would always have eaten in the

183   field, so all measures of metabolic rate include the specific dynamic action (roughly, the cost of

184   digestion) of the larvae. Larvae were fed with the same amount of food and quality ad-libatum,

185   until it was time to use for the experiment.

186

1

Experimental setup

We used standard thermolimit respirometry protocols (Lighton & Turner, 2004; Terblanche et al., 2007; Boardman & Terblanche, 2015; Andrew et al., 2016; Ghaedi & Andrew, 2016). In brief, using a HiBlow HP40 air pump, room air was pushed into sodalime to remove CO_2 and Drierite to remove moisture. Air was set to flow at 180 ml min^{-1} using a mass flow controller (Sable MFC-2, Sable Systems) and flow control valve (Side-Trak 840L; Sierra Instruments Inc., Monterey, CA, USA) through Bev-a-line tubing to a 30ml glass cuvette. Carbon dioxide and water in the air that was flowing before and after the glass cuvette was analysed by a two-channel calibrated infrared Li-7000 analyser (Li-Cor, Lincoln, NE, USA). The scrubbed airstream flowed across the experimental organism in the 30ml glass cuvette, which was held underwater in a water bath (Grant, GP200-R4), programmed (resolution ± 0.1°C) with LABWISE software. Cuvettes were placed into two plastic bags to stop any water entering the cuvette. We warmed insects from 25°C to 55°C, at 0.25°C min^{-1} (Andrew et al., 2016), over 120 minutes in the 30ml glass cuvette. The outgoing air from the cuvette then enters the infrared Li-cor analyser again: the CO_2 concentration difference of the air before and after it flowed through the cuvette, at one-second intervals is then recorded. A type-T thermocouple attached to a data logger (PicoTech TC-08 data logger with resolution ± 0.025°C, accuracy ± 0.5°C) was used to record the temperature in the glass cuvette. The two data recordings (Licor and data logger) were combined using Microsoft excel, aligned using the computer time stamp. An infrared activity detector (AD-1; Sable Systems, Las Vegas, NV, USA) recorded the movement of the test insect (see trace in supplementary).

Data Extraction and measurement of CO_2 emission rate (metabolic rate) and CT_{max}

209   Using Expedata (Sable Systems International, Las Vegas, NV, USA) version 1.9.2 analysis

210   software, data on CO_2 emission rate was extracted and measured from 25°C to 55°C (Boardman

211   et al., 2015; Andrew et al., 2016). We drift-corrected our recordings to baseline recordings from

212   an empty 30ml glass cuvette made ten min before and after trial, and converted to ml CO_2 h^{-1}

213   (Andrew et al., 2016; Ghaedi & Andrew, 2016). We controlled chamber switching by recording

214   from the cuvette by manually adding/removing the insect from the same cuvette as the baseline.

215   For each replicate insect, $\acute{V}CO_2$ (n = 10 larvae, 10 pupae, 10 adults) were extracted at these

216   temperature bins (25, 30, 35°C, 40°C, 45°C and CT_{max} ±0.25°C). The overall $\acute{V}CO_2$ (in ml CO_2

217   h^{-1}) was calculated by extracting the area under the curve (integral of CO_2 ml h^{-1} vs hours) in total

218   over the ramping period from 25°C to CT_{max} by transforming ppm concentration of CO_2 to CO_2

219   fraction and then multiplied by the flow rate. This area was equal to the volume of CO_2 produced

220   by each replicate insect in the 30ml glass cuvette (Stevens et al., 2010; Andrew et al., 2016). The

221   $\acute{V}CO_2$ for each replicate insect at each temperature point was extracted and multiplied by 1,000 to

222   give µl CO_2 h^{-1} per insect per hour. CT_{max} (n = 10 larvae, 10 pupae, 10 adults) was determined by

223   identifying the point at which the spiracular activity ceases i.e. the inflection point in the absolute

224   difference sum (ADS) residuals (Lighton & Turner, 2004; Vorhees & Bradley, 2012; Andrew et

225   al., 2016; Ghaedi & Andrew 2016). Because of differences in activity among the life stages, to

226   standardise our analysis, we used $\acute{V}CO_2$ data to determine CT_{max} value not activity. Other studies

227   showed that $\acute{V}CO_2$ and activities measurements produced similar estimates of CT_{max} in insects

228   (Lighton & Turner, 2004; Vorhees & Bradley, 2012). Metabolic rates at 25°C, 35°C and 45°C for

229   Q_{10}, were calculated as the average $\acute{V}CO_2$ over the five-minute period at each temperature e.g. start

230   at 25°C and go to 26.25°C. The inflection point for activity ADS residuals where motor activity

231   ceases is the activity CT_{max}. Lighton & Turner (2004) defined ADS as the cumulative sum of the

232   absolute difference between adjacent sampling points.

233

234   **Data analysis**

235   We considered our measures of $\acute{V}CO_2$ as a proxy for metabolic rate (Terblanche et al., 2010).

236   Statistical analyses were carried out in SAS® 9.4 software (SAS Institute Inc., 2013) using the

237   GLIMMIX procedure. CT_{max} and total metabolic rate differences between the life stages were

238   determined using generalised linear models (GLM). Differences in metabolic rate for different

239   temperatures were assessed using generalised linear mixed models (GLMM) with individuals as

240   random factors to balance for the repeated measurement. A one-way analysis of variance

241   (ANOVA) was used to compare the overall $\acute{V}CO_2$ and CT_{max} among the life stages. The Q_{10} (25–

242   35 °C) and Q_{10} (35–45 °C) for the life stages were also recorded similar to the above described

243   procedure. Data were reported as means plus or minus standard error (mean ± SE). In all the

244   models CT_{max} and $\acute{V}CO_2$ of individuals were the response variables with life stage as the

245   explanatory variables. Data was checked for normality and homogeneity of variance. The factors

246   in the model include life stage, sex and body mass as a covariate for metabolic rate. Because sex

247   was not significant for CT_{max} and metabolic rate, data on sex for those parameters were combined

248   in the subsequent analysis. Mass was removed from the final model for CT_{max} since it was not

249   significant. Where there were significant differences, post-hoc analysis was performed using least-

250   square means (LSmeans) and the differences compared, α=0.05 (Tukey-Kramer Adjustment).

251   Mass was log-transformed before analysis. T-test was used to compare treatments under two

252   treatments.

253

254 **Results**

255 **Metabolic rate (overall $\dot{V}CO_2$ (ml h^{-1}) across life stage of *H. punctigera***

256 There was a significant effect of life stages on overall $\dot{V}CO_2$ (ANOVA, DF=2, F=31.52,

257 p<0.0001). Pupae (n=10) had an overall $\dot{V}CO_2$ of 41% lower than adults (n=10) and 50% lower

258 than larvae (n=10) (p<0.0001, and p<0.0001, respectively; Figure 1). The $\dot{V}CO_2$ of larvae was 9%

259 higher than adults, but not statistically significant (p=0.20; Figure 1).

260

261 **Upper thermal tolerance (CT_{max}) across life stages of *H.punctigera***

262 There was a significant life stage effect on CT_{max} (ANOVA, DF=2, F=43, p<0.0001). Adult (n=10)

263 and pupae (n=10) had a similar CT_{max} (p=0.12; Figure 2). However, larvae (n=10) had a CT_{max} on

264 average 2.2°C higher than adults and 1.8°C higher than pupae (p<0.0001; p=<0.0001, respectively;

265 Figure 2). Activity CT_{max} and CO_2 CT_{max} for adults were similar and not significant; unlike larvae,

266 which had significantly higher CO_2 CT_{max} (Table 1).

267

268 $\dot{V}CO_2$ of *H. punctigera* **life stages at different temperature points**

269 Temperature effect on mean $\dot{V}CO_2$ was high in adults and larvae compared to pupae (Table 2) at

270 the different temperature points. The mean $\dot{V}CO_2$ of pupae was significantly different from that of

271 adult and larvae at all temperature points except at 25°C (Table 2). The mean $\dot{V}CO_2$ of each of the

272 life stages doubled at both 40°C and 45°C (Table 2) before $CT_{max.}$ Although the mean $\dot{V}CO_2$

273 between adults and larvae was not significantly different at 40°C, adults had a 0.01 µl h^{-1} higher

274 mean $\dot{V}CO_2$ than larvae (Table 2). At equilibrium (25°C) and 30°C-35°C larvae had 0.19 µl h^{-1} ,

275 0.13 µl h^{-1} and 0.16 µl h^{-1} higher than adult (Table 2) respectively. The Q_{10} 25–35 °C values for

276 larvae and adults were lower compared to Q_{10} 35–45°C – 28% lower for larvae (Q_{10} :25–35 °C

277   1.40 ± 0.06 to Q_{10} 35–45 °C 1.95 ± 0.14), 41% lower for adults (2.01 ± 0.22 to 3.42 ± 0.24) but 32%

278   higher for pupae (1.74 ± 0.21 to 1.42 ± 0.98) (Figure 3). Among life stages the Q_{10}: 25–35 °C for

279   adults was significantly higher (p= 0.04) compared with larvae, but not pupae (p=0.53; Figure 3).

280   The Q_{10}: 25–35 °C was not significant between larvae and pupae (p=0.35; Figure 3). The Q_{10}:35–

281   45 °C values were not statistically different among larvae and pupae (p=0.07; Figure 3) but

282   significant among larvae and adults as well as pupae and adults (p=0.00, p=0.00; Figure 3)

283   respectively.

284

285   **The influence of sex on upper thermal limit (CT_{max}) and metabolic rates $\dot{V}CO_2$ (ml h^{-1}) of**

286   **adult moth and pupae**

287   There was no sex effect (ANOVA, DF=3, F=0.15, p=0.92; Figure 4) on the CT_{max} of adults (males:

288   46.92°C $\pm$ 0.29°C and females: 46.72°C $\pm$0.29°C) and pupae (males: 47.47°C $\pm$ 0.29°C and

289   females: 47.32°C $\pm$ 0.29°C). For metabolic rate assessments, there was no significant difference

290   between female and male adults as well as between female and male pupae (t= -0.57, p=0.93;

291   Figure 5) and (t=1.38, p=0.52; Figure 5) respectively.

292

293   **Discussion**

294   Here, we measured the upper thermal tolerance limits (CT_{max}) and metabolic rates at different

295   temperature exposures across three life stages larvae, pupae, and adults and between sexes of *H.*

296   *punctigera* using thermolimit respirometry. These life stages experience different temperatures

297   because of the different habitats they naturally occupy, enabling an ecologically relevant

298   investigation of their metabolic rates at different temperatures. We found that CT_{max} differed

299   among the life stages of the insect with larvae exhibiting significantly higher CT_{max} compared with

300   pupae and adults (Figure 2). Similar to the findings presented here, studies by other authors have

301   shown that CT_{max} differs among life stages of insects including *Paractora dreuxi*, *Sirex noctilio*

302   and *Tenebrio molitor* (Marais et al., 2009; Vorhees & Bradley, 2012; Li et al., 2019). The higher

303   CT_{max} of the larvae compared with adults however, contradicts the findings of Vorhees & Bradley,

304   (2012) and Li et al., (2019) in *T. molitor* and *S. noctilio* respectively, where adults had a higher

305   CT_{max} than larvae, although, the insects are of different taxa. The differences in the CT_{max} between

306   the life stages suggest differences in upper thermal tolerance in this species with larvae having the

307   highest thermal tolerance to high temperature (Marais et al., 2009).

308   The CT_{max} differences among the life stages could be caused by several different factors such as

309   interactions of the microclimatic conditions experienced by the life stages and the level to which

310   they compensate their physiology to avoid harsh or extreme temperatures (Vorhees & Bradley,

311   2012). However, this would not be the case here, as all insects were kept at a constant 25°C, so a

312   more evolutionarily relevant mechanism must be in train here. The results imply that, larvae, pupae

313   and adults have the ability in the wild to adapt and cope differently.

314   The significantly higher CT_{max} exhibited by larvae is not surprising because larvae in their natural

315   environment are generally more exposed to extreme/hot temperatures, just above the soil surface

316   and while feeding on plants, and, less able to behaviourally adapt to extreme periods. Hence the

317   need to modify their physiological response to such extreme periods of heat conditions for survival

318   (Andrew et al., 2013a; Andrew et al., 2013b; Andrew et al., 2016). Even though the insects used

319   in the study were laboratory-reared, they were most likely to exhibit similar characteristics like

320   those in the field, because there is little genetic variability among *H. punctigera* population (Daly

321   et al., 1985). The limitation of our study is that; the low genetic diversity in the wild in the insect

322   does not imply that laboratory populations will not be adapted after many generations. We

323   acknowledge that the climatic variability experienced in the wild could further influence in these

324   thermal tolerance traits, but is unlikely to affect the clear life-stage differences.

325   The decline in the upper thermal limit between larvae, pupae and adults in that order (Figure 2)

326   suggests that the insects upper thermal limit could be influenced by ontogeny as observed in

327   *Drosophila melanogaster* (Bowler & Terblanche, 2008), although we did not test for age effects

328   in a single life stage. This upper thermal limit decline across the life stages however, does not

329   always follow a logical order as in some insect's e.g. *Drosophila buzzatii*; heat tolerance was high

330   in pupae followed by eggs and larvae (as cited in Vorhees & Bradley, 2012). Therefore, caution

331   must be made against the generalisation concerning the effects of ontogeny on heat resistance

332   (Vorhees & Bradley, 2012), although we should expect ontogenetic variation (Bowler &

333   Terblanche, 2008). One would have expected in part, no significant difference in the CT_{max}

334   between larvae and adults because of the several factors like the microclimate conditions they

335   experienced and the extent to which they can use their physiology to avoid the extreme

336   temperatures. The lower CT_{max} of the adults compared with the larvae may be because the adults

337   have a higher ability to avoid extreme temperatures by flying away from areas of extreme

338   temperatures compared to larvae. The results suggest that there are ecological reasons for different

339   thermal preferences: larvae are more sedentary so it is harder for them to move away from hotter

340   temperatures they may be exposed to; adults are more mobile, so can move to preferred

341   temperatures refuges in the evening so have a lower thermal threshold. Because of the ectothermic

342   and regional heterothermic nature of insects, high temperatures could speed up growth and

343   development of insects (Andrew et al., 2011; DeVries et al., 2016). The higher CT_{max} of the larvae

344   could assist them to grow faster in warmer conditions like summer if food, and most importantly

345   water, are not limiting factors.

346 The CT$_{max}$ exhibited by the life stages is significantly greater than the highest recorded maximum
347 daily mean ambient temperature in Tamworth region where the population originated from
348 (40.9°C) (NicheMapR). However, the microclimatic conditions that *H. punctigera* could be
349 exposed to may be much higher than this, particularly during the middle of the day (Andrew et al.,
350 2013), and can put the insects under physiological stress that can change their interactions under
351 changing climate. The higher CT$_{max}$ exhibited by the insect could be the reason for which it is able
352 to occupy a wide range of climatic conditions including inland of Australia, and spread in all states
353 of Australia (Gregg et al., 2018, 2019).

354 Our results show that upper thermal limits were not significantly different between males and
355 females. The similar thermal limits demonstrated by males and females indicates that sex has
356 minimal influence on CT$_{max}$ (Figure 4), similar to the findings in bumble bees (*Bombus terrestris*)
357 (Oyen et al., 2016); suggesting that *H. punctigera* sex ratios will not change with adult exposure
358 to climatic extremes.

359 Insects as other animals require energy to forage, survive, grow and reproduce (Terblanche et al.,
360 2004; White et al., 2019). The processes that cause variation in metabolic rate apart from ambient
361 temperature, body mass is also an important factor that contributes to metabolic rate of insects
362 (White et al., 2019). Insects show a range of thermoregulatory capacities: ectothermic, regional
363 heterothermic, poikilothermic and endothermic (Heinrich, 1993; Gullan & Cranston, 2014) their
364 activity and physiological processes are primarily influenced by ambient temperature (Nguyen et
365 al., 2014). Our results revealed $\dot{V}CO_2$ varied among the life stages at the different temperature
366 points. The differences in the mean $\dot{V}CO_2$ among the life stages suggests that the metabolic
367 demands of the life stages differ. Several factors may account for the differences in the metabolic

368   rates among the life stages; including ontogeny, upper thermal tolerance, activity, body mass and

369   feeding (Terblanche et al., 2005).

370   Fat body tissues play crucial roles in the life of insects, as they are involved in multiple metabolic

371   functions like energy storage and utilisation in reply to the energy call or request of the insect

372   (Arrese & Soulages, 2010). Muscle tissue usually has a higher metabolic rate than fat body tissue.

373   Muscle tissues are active and burns calories even at rest unlike fat body for storing excess energy.

374   The significantly higher $\dot{V}CO_2$ of larvae and adult compared with pupae could be due to low

375   amount of fat body tissues in the larvae and high muscle tissues in the case of adult. Another reason

376   for the high metabolic rate may be due to the high activities of larvae and adult compared with

377   pupae, similar to that reported by DeVries et al. (2013) for the bed bug, *Cimex lectularius.* Size

378   also affects the metabolic rate of an organism (Terblanche et al., 2004; Quinlan et al., 2006;

379   DeVries et al., 2013), and was similar in our case, as size was significant (Figure 8 in

380   supplementary material). Pupae, which has a higher CT_{max} than adults, had a comparative lower

381   $\dot{V}CO_2$. The lower $\dot{V}CO_2$ may be an indication of lower stress at the higher temperatures and or

382   maybe due to more fat tissues in pupae. It is plausible that it may be an adaption strategy for

383   survival to dry conditions (Terblanche et al., 2004; Quinlan et al., 2006). The high amount of fat

384   tissues in the pupae because of lipid reserves needed to provide energy during the extended non-

385   feeding periods could be the reason for the low metabolism (Arrese & Soulages, 2010). Because

386   pupae do not have a source of free water underground, they must conserve water by reducing

387   respiration (Table 3 in supplementary material) (Harrison, 2009).

388   As the effect of temperature on metabolic rates of insects is widely reported (Terblanche et al.,

389   2004, 2007; Hill et al., 2016), only a few studies looked at that at different temperature points for

390   different life stages for *H. punctigera.* Our results on metabolic rate between life stages considering

391   the different temperatures (Table 2) and Q_{10} (Figure 3) indicates that there was a correlation

392   between temperature rise and $\dot{V}CO_2$ release on the life stages during the thermolimit respiratory

393   (Table 2). The Q_{10} values for all the life stages except pupae almost doubled from Q_{10}: 25–35 °C

394   to Q_{10}: 35– 45 °C, with adults having the highest thermal sensitivity. This increase in metabolic

395   rate as temperature increases is consistent with other studies (Terblanche et al., 2005; Terblanche

396   & Chown 2007; Andrew et al., 2016). However, the negative effects associated with increasing

397   temperatures on metabolic efficiency could be reduced on adults, as they are able to compensate

398   for short-term extreme microclimate temperatures by flying away. Although, increase in

399   temperatures result in high metabolic rate, the total energy assigned for certain actions, as specific

400   dynamic actions are not dependent on temperature (McCue et al., 2016a; Wang et al., 2003). It is

401   possible that as insects enlarge their geographic radius or scale, they will face abiotic and biotic

402   stress.

403   For better comparison among life stages, we measured $\dot{V}CO_2$ at CT_{max}, and in all cases, $\dot{V}CO_2$

404   among life stages was higher. Even though the $\dot{V}CO_2$ at CT_{max} increased in all the life stages, adults

405   $\dot{V}CO_2$ were higher than larvae and pupae. Harrison (2009) reported that tracheated insects have the

406   ability to carry or move gases by opening their spiracles. Short periods of spiracles closure follow

407   a continuous carbon dioxide exchange (Harrison, 2009). The higher metabolic rates due to higher

408   stress levels may be due to a decrease between spiracle opening and closing. Therefore, the higher

409   $\dot{V}CO_2$ of adults at CT_{max} may be due to their high activity rate and a decrease between its spiracle

410   opening and closing compared to larvae and pupae (Harrison, 2009; Boardman et al., 2015). The

411   lower metabolic rate of pupae at CT_{max} compared to larvae and adults could not be explained

412   beyond a physiological adaption strategy to reduce physiological cost by reducing respiration and

413   conserve water at this stage (Harrison, 2009), similar to what Merkey et al. (2011) reported in

414   *Drosophila melanogaster* pupae.

415   The lower $\dot{V}CO_2$ of adults compared to larvae at 25°C could suggest that adults have a lower

416   resting metabolic rate than larvae at that point. The higher resting metabolic rate of larvae could

417   be the result of modification of the energy allocation between competing functions such as

418   maintenance for continuous development. Additionally, the lower $\dot{V}CO_2$ of adults may be as result

419   of the occurrence of discontinuous gas exchange in the adults at 25°C. Discontinuous gas exchange

420   is associated with low metabolic rates. In contrast, age, as proven to affect metabolic rates in some

421   insects like *Glossina pallidipes* (Terblanche et al., 2004) did not apply in our case, as larvae had a

422   lower $\dot{V}CO_2$. The high $\dot{V}CO_2$ produced by the larvae could imply a high-energy utilisation

423   (Terblanche et al., 2004) for development. This high-energy utilisation can provide a deep

424   understanding into the life histories of the life stage across a variety of environments. Therefore,

425   this high-energy utilisation may translate to an increase in food consumption and more

426   considerable plant damage (Deutsch et al., 2018). The metabolic rate of pupae and adults was not

427   influenced by sex, suggesting that the cost of living of the dispersing adults and pupae of both

428   sexes are similar. Similar work by Terblanche et al. (2004) found out that metabolic rate did not

429   differ between males and females of *Glossina pallidipes* and *G. morsitans*.

430

431   **Conclusion**

432   The present study provides vital information on how the different life stages of *H. punctigera* will

433   physiologically perform in a changing thermal environment. It also provides data for predictions

434   about metabolic rates of *H. punctigera* among life stages. The study revealed that CT_{max} of larvae

435   was significantly higher than those of pupae and adults, and the metabolic rate of pupae was

436 significantly different from those of larvae and adults. Also, sex did not influence CT_{max} and

437 metabolic rate. The results suggest that the performance and survival of the different life stages of

438 *H. punctigera* could differ. This indicates that life-stage needs to be taken into account when

439 assessing responses to a rapidly changing change, as ontogeny influences vulnerability and

440 exposure. This finding can be used to predict the potential spread of the insect to other new

441 environments and can indirectly help in management planning.

442

443 **Acknowledgements**

444 Sarah Hill assisted with the setup, calibration, maintenance and protocols of the thermolimit

445 respirometry system. Zac Hemmings assisted in the data extraction, analysis and initial

446 interpretations of the raw-data outputs. We thank Bianca Boss-Bishop, Sarah Hill, James

447 O'Hanlon, Zac Hemmings, Alva Rebecka Curtsdotter and Behnaz Ghaedi for their comments on

448 earlier versions of the manuscript. Also NSW DPI for providing the insects for the culture.

449

450 **Declarations of interest**: Nigel R. Andrew is an Academic editor of PeerJ

451

452
453 **Funding**

454
455 University of New England (UNE) provided funds as scholarship to Samuel Abukari Bawa as well

456 as for equipment and consumables used in the study. The funders had no role in the study design,

457 data collection and analysis, decision to publish, or preparation of the manuscript.

458

459 **Author Contributions**
460

461   All authors conceived and designed the experiment. The first author (Samuel Abukari Bawa)

462   performed the experiment. The data was analysed by Samuel Abukari Bawa and Cara Miller.

463   Samuel Abukari Bawa wrote the paper, prepared figures and tables. Nigel R. Andrew, Peter C.

464   Gregg and Alice P.Del Socorro reviewed the drafts of the paper and contributed to writing the

465   paper.

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729

Figure 1

Box plot showing the Total mean $\dot{V}CO_2$ ml h^{-1} among life stages (larvae, pupae and adult) of *H. punctigera* over complete ramping period of about 120mins from 25°C to 55°C following thermolimit respirometry

The dark line is the median in each group
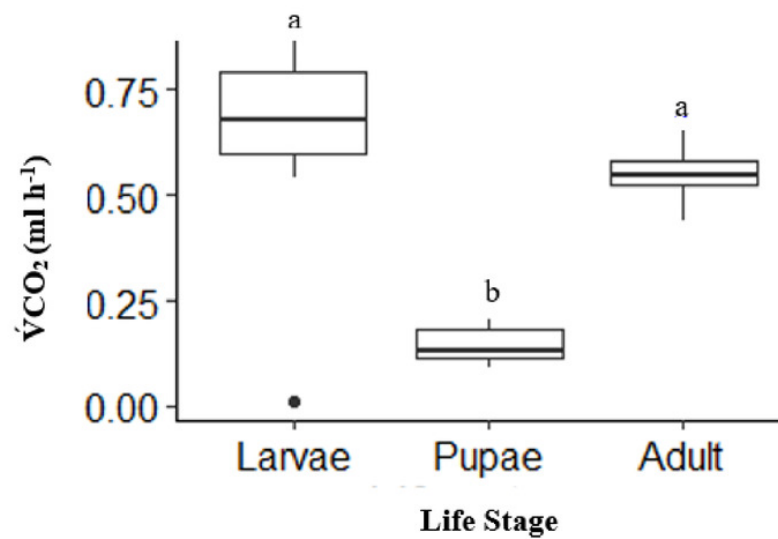
Figure 1: Metabolic rate $\acute{V}CO_2$ (ml h^{-1}) boxplot of *H. punctigera* life stages (larvae, pupae and adult; n = 10 for all stages) over the complete ramping period of about 120mins from 25°C to CT$_{max}$ following thermolimit respirometry. Significant differences (p>0.05) indicated by the letter above each boxplot. The box represents the lower and upper quartiles with the dark line representing the mean. The bars represents the minimum and maximum whiskers, the dot represents an outlier.

Figure 2

Box plot showing the Mean CT_{max} identified as the inflection point in the absolute difference sum (ADS) residuals among life stages (larvae, pupae and adult) of *H. punctigera* following thermolimit respirometry.

The dark line is the median of the data in each group
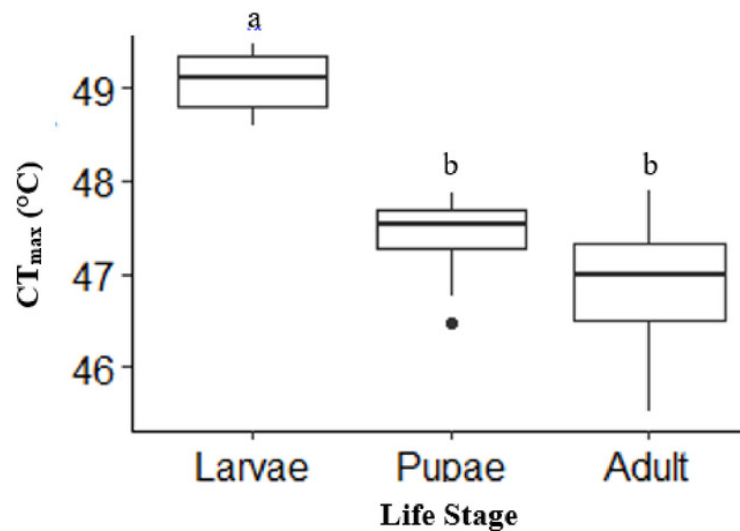
Figure 2: Upper thermal limit (CT_{max}) identified as the inflection point in the absolute difference sum (ADS) residuals among life stages (larvae, pupae and adult; n = 10 for all stages) of *H. punctigera* following thermolimit respirometry. Box plots with the same letter are not significantly different (p>0.05). The box represents the lower and upper quartiles with the dark line representing the mean. The bars represents the minimum and maximum whiskers, the dot represents an outlier.

Figure 3

Box plots comparisons of *H. punctigera* life stages (larvae, pupae and adult; n = 10 for all stages) Q_{10} (25–35 °C) and Q_{10} (35–45 °C).

The box represents the lower and upper quartiles with the dark line representing the mean.

The bars represents the minimum and maximum whiskers, the dots represent outliers.
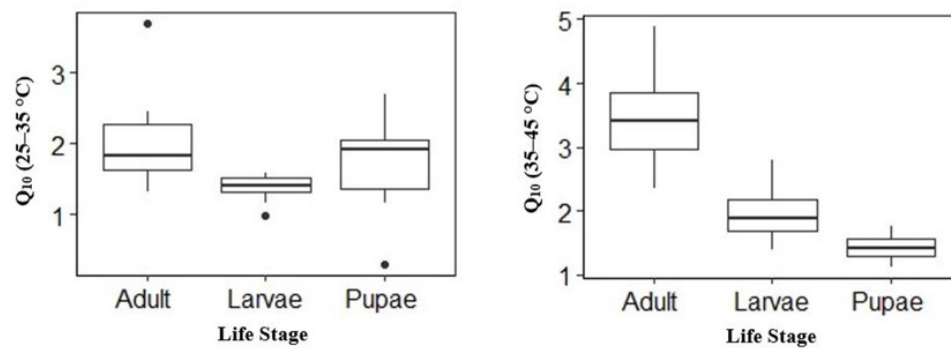
Figure 3: Box plots comparisons of *H. punctigera* life stages (adult, larvae and pupae; n = 10 for all stages) Q_{10} (25–35 °C) and Q_{10} (35–45 °C). The box represents the lower and upper quartiles with the dark line representing the mean. The bars represents the minimum and maximum whiskers, the dots represent outliers.

Figure 4

Box plots of upper thermal limit (CT$_{max}$) identified as the inflection point in the absolute difference sum (ADS) residuals among Life Stage (pupae and adult) and sex (male – M and female – F) of *H. punctigera* following thermolimit respirom

The box represents the lower and upper quartiles with the dark line representing the mean. The bars represent the minimum and maximum whiskers, the dots represent outliers.
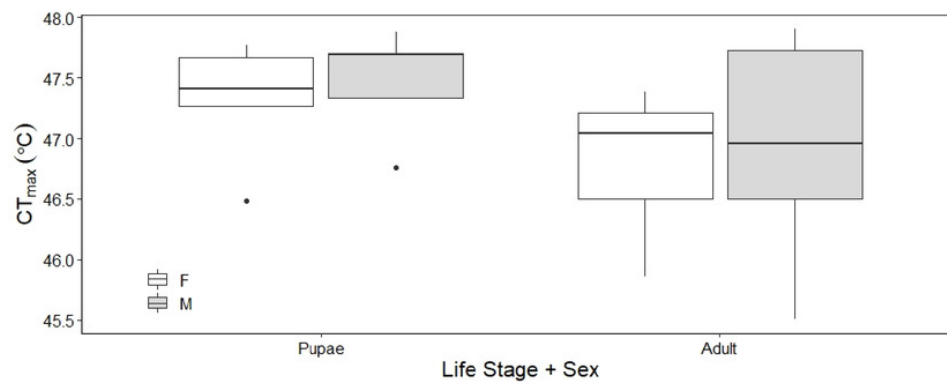
Figure 4: Box plots of upper thermal limit (CT_{max}) identified as the inflection point in the absolute difference sum (ADS) residuals among Life Stage (pupae and adult) and sex (male – M and female – F) of *H. punctigera* following thermolimit respirometry ; n = 5 for all sexes. The box represents the lower and upper quartiles with the dark line representing the mean. The bars represent the minimum and maximum whiskers, the dots represent outliers.

Figure 5

Box plots of V́CO$_2$ ml h^{-1} among Life Stage (pupae and adult) and sex (male – M and female – F) of *H. punctigera* over the complete temperature ramping period of about 140mins from 25°C to CT$_{max}$ following thermo

The box represents the lower and upper quartiles with the dark line representing the median. The bars represents the minimum and maximum whiskers, the dots represent outliers.
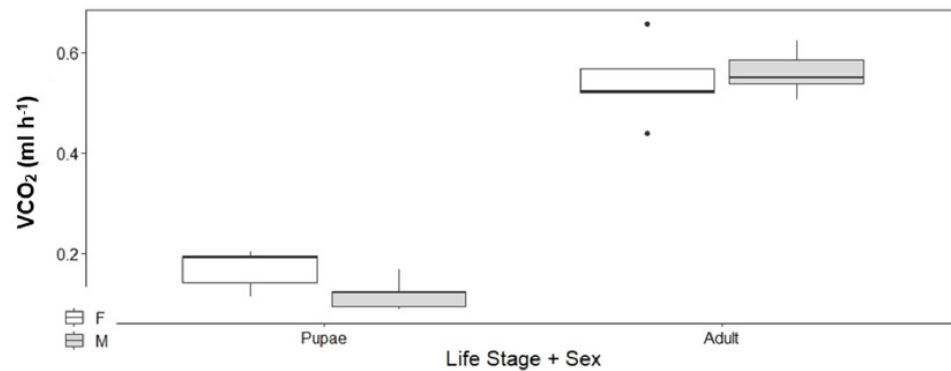
Figure 5: Box plots of $\acute{V}CO_2$ ml h^{-1} among Life Stage (pupae and adult) and sex (male – M and female – F) of *H. punctigera* over the complete temperature ramping period of about 140mins from 25°C to CT_{max} following thermolimit respirometry. n=five in all cases; n = five for all sexes. The box represents the lower and upper quartiles with the dark line representing the median. The bars represents the minimum and maximum whiskers, the dots represent outliers.

Anova summary information for Activity CT_{max}, CO_2 CT_{max} and mean CT_{max} between two life stages (Larvae and Adults) of *H. punctigera* following thermolimit respirometry

2 Table 1: Anova summary information for Activity CT_{max}, CO_2 CT_{max} and mean CT_{max} between two life stages (Larvae
3 and Adults) of *H. punctigera* following thermolimit respirometry. n=10 for each life stage

| | | | | | | Mean CT_{max} | |
| | | | | | | Life Stage | |
Stage	DF	Sum Sq.	Mean Sq.	F	p	Larvae	Adults
Activity CT_{max}	1	8.3	8.28	0.21	0.65	44.1±1.3	43.1±2.5
CO_2 CT_{max}	1	133.1	133.08	4.27	0.05	49.0±0.3	43.5±2.4

Mean ± s.e. V́CO$_2$ (µl CO$_2$ h^{-1}) over different temperature points among life stages (adult, larvae, and pupae).

Values in bold indicate significant differences. Letters indicate significant differences among life stages. The 95% CL is 95% confidence limit

1   Table 2: Mean ± s.e. V̇CO$_2$ (μl CO$_2$ h^{-1}) over different temperature points among life stages (adult, larvae, and pupae). Values in bold indicate significant differences.

2   Letters indicate significant differences among life stages. The 95% CL is 95% confidence limit

Life Stage	Mean V̇CO$_2$ (μl CO$_2$ h^{-1})											
	25°C	95% CL	30°C	95% CL	35°C	95% CL	40°C	95% CL	45°C	95% CL	CT$_{max}$	95% CL
Larvae	**0.40±0.04a**	0.31-0.50	**0.44±0.04a**	0.43-0.62	**0.53±0.04a**	0.57-0.76	**0.70±0.06a**	0.54-0.81	**1.03±0.22a**	0.83-1.78	**1.03±0.14a**	0.90-1.50
Pupae	**0.08±0.03b**	-0.03-0.11	**0.11±0.03b**	0.02-0.15	**0.14±0.03b**	0.08-0.22	**0.19±0.04b**	-0.01-0.16	**0.24±0.16b**	-0.09-0.60	**0.23±0.10b**	-0.05-0.37
Adult	**0.21±0.04b**	0.16-0.36	**0.31±0.04c**	0.21-0.41	**0.37±0.04c**	0.22-0.43	**0.71±0.06a**	0.71-1.01	**1.47±0.24c**	0.91-1.92	**1.23±0.15a**	1.01-1.65