Optimal Temperature for Rearing the Edible Ruspolia differens (Orthoptera: Tettigoniidae)

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Optimal temperature for rearing the edible *Ruspolia differens* (Orthoptera: Tetrigoniidae)

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Abstract

*Ruspolia differens* Serville (Orthoptera: Tettigoniidae) is an insect with significant economic potential in Africa. However, to mass-rear this species on a large scale, the optimal rearing temperature needs to be determined. We assessed multiple performance traits for *R. differens* reared at seven constant temperatures, ranging from 18 to 32°C, from newly hatched nymphs to three weeks after adult molting. The highest observed survival was at 30°C (mean survival of 86.7%), where also the development rate reached its maximum. At this temperature, the development from newly hatched nymphs to adults took approximately 49 days. The weight of individuals at the time of adult molt reached its maximum at 28°C (mean weight of 0.62 g). To maximize the yield from mass-rearing, suggested time to harvest *R. differens* is ten days after the adult molt. According to our results, during this time period *R. differens* individuals can achieve up to 50% higher weight than if harvested immediately after adult molting. For maximal survival and weight gain, we recommend rearing temperature of 28-30°C, whereas a slightly higher temperature of 31°C leads to the shortest development time. Taking into account all the performance traits, the overall optimal temperature is estimated at 29°C. Our results can be used when developing large-scale mass-rearing protocols for *R. differens* in controlled temperatures.

**Key words:** The African edible bush cricket, Brière-1 model, “the edible grasshopper”, edible insects, nsenene
Introduction

*Ruspolia differens* Serville (Orthoptera: Tettigoniidae) (common names include “the edible grasshopper” “nsenene” and “The African edible bush cricket”), is one of the most important edible insects in sub-Saharan Africa (van Huis et al. 2013). It is economically highly valued in East Africa with existing markets, e.g., in Uganda (Okia et al. 2017). *R. differens* could potentially serve as a basis for new food products with considerable nutritional value for humans (Kinyuru et al. 2010, Nyeko et al. 2014). In addition to essential amino acids, this species is rich in essential fatty acids, vitamins and minerals (Kinyuru et al. 2010, Siulapwa et al. 2014). Its fatty acid composition and content can be manipulated to make it even more suitable for humans by modifying its feed (Lehtovaara et al. 2017). Currently, *R. differens* is harvested from the wild during two unpredictable annual swarming seasons (Kinyuru et al. 2010). To improve food security in East Africa, and to create income for local farmers and entrepreneurs, there are currently attempts to develop methods to mass-rear this species (Nyeko et al. 2014, Lehtovaara et al. 2017, Malinga et al. 2018a, 2018b, Ssepuuya et al. 2018). Early studies have shown that *R. differens* can be reared in the laboratory at 30ºC, and in these conditions the development time from egg hatching to adult molting takes approximately 46–56 days (Hartley 1967, Brits and Thornton 1981). However, a detailed understanding of *R. differens* performance across a range of rearing temperatures is lacking.

Temperature determines the survival, development rate, growth rate, and fecundity of insects (Ratte 1984, Honěk and Kocourek 1990, Atkinson 1994). The thermal control of insect performance is determined by enzymatic reactions and can be summarized as a non-linear asymmetric curve across temperatures (Sharpe and DeMichele 1977) which peaks at the optimal temperature (*T*<sub>opt</sub>), i.e., temperature at which the performance occurs at its maximal
rate (Huey and Kingsolver 1989). Performance can take place at temperatures that are above
the critical thermal minimum (the lower developmental threshold; T_{min}) but below the critical
temperature range in which the insect can survive can be wider than the operative range for
certain performance traits (e.g., development and growth rate; Ratte 1984), but sustained
exposure to conditions outside the operative range will result in damage and/or death (Angilletta
2009). Although this general pattern is shared by all insects, the exact shape of the thermal
performance curve varies across species (Deutsch et al. 2008). Furthermore, it may also vary
among different performance traits within the same species (e.g., development rate and growth
rate; Miller et al. 2009). Temperatures that produce the highest development rate typically
produce adults that are less than the maximum size and weight (Atkinson 1994), although
exceptions are found, especially in Orthoptera (Walters and Hassall 2006, Kingsolver and Huey
2008).

Understanding insect performance at different temperatures is crucial in order to find
the optimal rearing temperature for edible insects. The optimal rearing temperature would allow
individuals to maximize development and growth rates while minimizing mortality. Prolonged
exposure to high or low temperatures may alter the lipid metabolism of insects (increased
unsaturation of fatty acids at low temperatures and saturation at high temperatures), which can
significantly alter the ability of the insect to survive (House et al. 1958, Petersen and Holmstrup
2000). This may be important for insect mass-rearing, not only to minimize mortality, but also
to ensure the quality of the fat content. Temperature may also determine food uptake, heating
or air conditioning costs, and emissions in the production process (Roe et al. 1985, Booth and
Kiddell 2007). Further, temperature adjustments might make it possible to manipulate the
development rate in order to synchronize the developmental stages of different-aged individuals
or production units.
The purpose of this work was to evaluate the performance of *R. differens* throughout their nymphal and adult stage at seven constant rearing temperatures: 18, 22, 24, 26, 28, 30, and 32°C. The studied performance traits were: development rate, survival, weight at adult molt, and weight ten days after adult molt. In addition, the amino and fatty acid composition was analyzed from adults reared at five of these temperatures (22, 24, 26, 28 and 32°C).

**Material and Methods**

*Study insects and their rearing*

We used 258 newly hatched nymphs produced by 13 females from a laboratory population at the University of Eastern Finland. The population originated from individuals collected at Makerere University Agricultural Research Institute (Kabanyolo, Uganda) in 2015 and 2016. The experiments were conducted between January and April 2016, and June and September 2016. During experiments, the nymphs were reared in groups of three until they reached the second instar (staging criterion by Brits and Thornton 1981: the species has six to seven instars in total) after which they were reared individually. Rearing took place in 0.75 l plastic boxes (17 × 12 × 14 cm) with netted holes on the top for air circulation. The insects were fed with oatmeal, oat grain and reindeer pellet (Poro-Elo 1; Suomen Rehu, Finland). Sprouting oat shoots in plastic jars (5 × 3 × 3 cm) were made available during the first three instars. Upon passing into the fourth instar, the live oat shoots were replaced with a piece of cucumber. Insects had *ad libitum* access to feed throughout the experiment, and both the cucumber and dry food were replenished every second or third day. In each box, insects had access to water that was absorbed in cotton wool, and a piece of hanging paper was provided as a place for molt.
Experimental design

In the experiment, 15 to 52 randomly selected newly hatched individuals were randomized to each temperature treatment (18, 22, 24, 26, 28, 30, and 32°C (±1°C)) where they were reared individually from second instar on in rearing boxes (Table 1). Due to high mortality at 22 and 24°C, these two treatments were repeated twice. Each temperature treatment was carried out in a growth chamber (Snijders Scientific Microclima Climate Chamber Model No. MC1000HE-EVD, Tilburg, Netherlands) or in rooms where temperature was manipulated with heating lights and radiators. In each temperature treatment, insects were kept at a photoperiod of 12:12 (L:D) at 65-75% RH (measured from inside the rearing box; as an exception, during the first three instars, the humidity was 75-90% due to the fresh oat). When replenishing feed, locations of rearing boxes were randomized within the temperature chamber to avoid potential shelf effects.

Individual insects were reared from egg hatching until three weeks after adult molting (except in the two repeated treatments of 22°C and 24°C, where individuals were only reared until adult molting). The weight of each individual was measured every second or third day for two weeks after the adult molt. No adult emergence was recorded at 18°C, and at 22°C only a few individuals had reached adulthood at a time when all the other temperature treatments had ended. In these cases, the experiment was terminated 16 weeks after the last newly hatched nymphs were placed in the experiment. Thus, the experiment lasted from 11 weeks (at 30°C) to 16 weeks (at 18°C and 22°C) depending on the treatment. At the other temperature treatments, all individuals either emerged as adults or died during the experiment. All individuals were frozen at the end of the experiment.

Amino and fatty acid analysis
Two individuals (regardless of sex) from each temperature treatment (22, 24, 26, 28, and 32°C) were randomly selected for amino acid analysis (N=10). The amino acid analysis was performed in Competence Center of Food and Fermentation Technology (Estonia) with hydrolysis by liquid chromatography-UV absorption (LC-UV). Analyses were performed with Acquity UPLC and an AccQ-Tag Ultra column. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. A photodiode array (PDA) detector was used for detection and data was processed with Empower software.

Three individuals (regardless of sex) from each temperature treatment (22, 24, 26, 28, and 32°C) were randomly selected for fatty acid analyses (N=15). Fatty acid analysis was performed in Bio-Competence Centre of Healthy Dairy Products (Bio-CC) (Estonia) using a direct transmethylation method reported by Sukhija and Palmquist (1988) with minor modifications as described by Lehtovaara et al. (2017). Fatty acid methyl esters (FAME) were quantified with gas chromatography with flame ionization detection (GC-FID). Identification of common fatty acids was performed by comparing sample peak retention times with FAME standards and the quantification of fatty acids was done based on peak areas in relation to the internal standard. Further details are provided in the Supplementary Material.

**Statistical analyses**

Temperature treatment 18°C has been excluded from analyses as none of the individuals reached adulthood during the experiment. For the rest of the temperature treatments (and each replica of 22 and 24°C treatment), we calculated the mean development rate (1/duration of development in days), mean survival (percentage of individuals that survived to adulthood) and mean weight at the time of adult molt and ten days after the molting.
For each of these four performance traits, we fitted a Brière-1 model (Brière et al. 1999), which is commonly used in the literature (Lachenicht et al. 2010, Shi and Ge 2010). It is a simplified model of development that describes the nonlinear relationship of developmental rate to temperature for insects (Brière et al. 1999). Other nonlinear models that are often used, like the one by Sharpe and DeMichele (1977), which is based on enzyme kinetics, and model by Logan (1976), are relatively complex containing four to six parameters to estimate developmental rate (Brière et al. 1999). Brière-1 only requires three parameters that also allow descriptive interpretation like the Logan model, and even though they do not have biochemical meaning like in the Sharpe and DeMichele model, they have graphical interpretation (Brière et al. 1999). Brière-1 also provides upper and lower estimated developmental thresholds, whereas when using Sharpe and DeMichele and Logan models, these estimates are usually calculated with linear functions. The equation was:

\[ D = aT(T - T_{\text{min}})\sqrt{T_{\text{max}} - T} \]

where \( D \) is the trait (survival, development rate, or weight), \( a \) is an empirical constant, \( T \) is the rearing temperature (°C), \( T_{\text{min}} \) is the lower temperature threshold, and \( T_{\text{max}} \) is the lethal temperature (upper threshold). The model was fitted by iterative nonlinear regression based on the Levenberg-Marquardt algorithm (IBM SPSS Statistics version 24) using mean values for the traits in each treatments or replica.

We illustrated weight gain after adult molting for each experimental temperature by plotting the weight (mean across individuals) as a function of time. The weight was generally measured every second day, but in rare cases weight was measured only every third day. In these instances, we estimated the weight (for every second day) by calculating the mean weight measurements from the previous and subsequent days.
To test for a directional change in the amino and fatty acid compositions along the temperature gradient (22, 24, 26, 28, and 32°C), distance-based linear models (DistLM) were fitted. With this multivariate method, the Bray-Curtis similarity matrix (generated from data describing the amino or fatty acid compositions of the studied individuals) can be modelled with the rearing temperature as a continuous predictor. If a gradient was found, to elucidate which amino or fatty acids most contributed to the observed dissimilarities, we ran a similarity percentage analysis procedure (SIMPER). The differences in amino and fatty acid compositions for individuals reared in the experimental temperatures were visualized with non-metric multidimensional scaling (NMDS). All multivariate analysis were conducted with Primer-E, version 6 (Clarke and Gorley 2006, Anderson et al., 2008). Further details are provided in the Supplementary Material (Tables S1-S2 and Figures S1-S3).

Results

Development rate

The fastest development rate was observed at 30°C (Fig. 1A) where, on average, *R. differens* developed to adulthood in 49 days. At 22°C, the development time was twice as long (96 days), and at 32°C it took 54 days (Table 1). Based on the fitted Brière-1 model, the lower developmental threshold occurs at 14.6°C and upper developmental threshold at 36.6°C (Table 2). When females and males were considered separately, males developed faster at all temperatures except at 26 and 32°C (Table 3). At 30°C, females developed to adulthood in 51 days compared to 46 days for males.

Survival rate
The highest survival rate was observed at 30°C, where 86.7% of individuals reached adulthood (Fig. 1B). At temperatures 22°C and at 32°C, only 36% or less of individuals survived. The Brière-1 model estimates the lower temperature threshold at 20.1°C even though at the end of the experiment (terminated after 16 weeks) at treatment 18°C, 25.6% of individuals were still alive as larvae (Table 2). Also, model estimates peak survival at slightly lower temperature (29.0°C) than the observed peak.

**Weight at adult molting and weight gain as an adult**

The highest weight at adult molting was observed at 28°C when individuals were on average 0.62 g (Fig. 2A). At 22 °C the mean weight was 0.52 g and at 32°C it was 0.48 g.

Weight increased for six to ten days after the adult molt (Fig. 2B) and subsequently plateaued. The weight plateaued earlier for males compared to females (Fig. 2C and 2D). Male weight gain was more strongly and negatively modified by the highest experimental temperature (32°C), compared to females. Ten days after adult molting, the highest weight was observed at 28°C (mean 0.88 g; Fig. 2A), while at 22°C, the observed weight was only 0.62 g, and at 32°C it was 0.67 g. Based on the fitted Brière-1 models (Table 2), the weight gain between adult molt and ten days after molting is highest at 29.2°C being 51.2% (Fig. 2A).

Our results indicate that different performance traits have different optimal temperatures ranging from 28 to 31°C (Fig. 1–3). Considering the fitted Brière-1 models for development rate, survival and weight ten days after adult molting, the overall optimum rearing temperature can be estimated at 29.1°C (Fig. 3).

**Amino and fatty acid composition**

There was a statistically significant directional change in the amino acid composition of *R. differens* along the temperature gradient (22-32°C) (DistLM; Pseudo-$F_{1,8} = 5.75$, $P = 0.005$, n
Although the gradient was visible in NMDS ordination (Supp. Fig. S1A), however there was no clear pattern in the proportions of amino acids along the studied temperature gradient (Supp. Fig. S2).

There was no directional change in the fatty acid composition of *R. differens* along the studied temperature gradient (DistLM; Pseudo-F$_{1,13}$ = 1.01, P = 0.418, n = 15, Supp. Fig. S1B, S3). Total fatty acid content (TFA), saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acid (PUFA) contents are presented in Supp. Table S2.

**Discussion**

The developmental rate of *R. differens* peaks at 30°C, with $T_{\text{min}}$ estimated at 15°C and $T_{\text{max}}$ estimated at 37°C. These parameters represent a typical thermal performance curve for tropical insects (Deutch et al. 2008). At temperatures higher than optimal, insect development rates are slowed down due to a lack of energy available for larval growth after respiration energy needs are met (Frouz et al. 2002). At temperatures lower than optimal for development, the development rates increasingly decline due to slower metabolic reactions (Sharpe and DeMichele 1977). The mean development time from hatching to adult molting (at 30°C, 46 and 51 days for males and females, respectively) was similar to that reported by Hartley (1967) and Brits and Thornton (1981). Males develop faster as they have one less instar than females (Brits and Thornton 1981), but at high temperatures (32°C) male development becomes markedly slower than that for females, suggesting that males are more sensitive to elevated temperatures. In general, the development time for *R. differens* is comparable to other common edible insects: *Acheta domesticus* (L.) (Orthoptera: Gryllidae) reaches adult molting in 45 days at 30°C (Clifford and Woodring 1990), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) in 20-40 days at 28°C (Oonincx et al. 2015) and *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) in 70-125 days at 30°C (Weaver and McFarlane 1990, Prokkola et al. 2013).
The survival rate of *R. differens* peaks at 30°C, with $T_{\text{min}}$ estimated at 20°C and $T_{\text{max}}$ estimated at 32°C. At temperatures above or below the optimum, the survival of insects typically decreases due to a lethal imbalance between various temperature-dependent components of an insect’s metabolism (Huey and Kingsolver 1989). At high temperatures, the supply of food and oxygen may not be sufficient for the increased metabolic rate (Harrison and Fewell 1994, Hochachka and Somero 2002) and a faster reduction in necessary substrates and accumulation of excessive metabolic products may inhibit enzyme activity (Shi et al. 2011). At the highest experimental temperature (32°C), 69% of the individuals that reached adulthood had deformed wings or a failed final molting (data not shown). Also, the individuals were generally paler in color indicating potential metabolic problems. At high temperatures the breakdown of the wax layer of the cuticle and/or desiccation may explain molting difficulties (Hepburn 1985).

Our results therefore indicate that the survival rate of *R. differens* is more sensitive to high temperatures than development rate. When the temperature is increased from 30°C to 32°C, the development rate decreases only by 8% (from 0.021 to 0.019) whereas the survival is decreased by 58% (from 86.7 to 36.1%). As is typical for poikilotherms, temperatures only slightly over the optimum for metabolism can cause near total mortality (Ghouri and McFarlane 1958, Ratte 1984).

Low temperatures both increase mortality and delay development of *R. differens*. These phenomena are likely to be related, because the lethal effects of extreme temperatures typically depend upon the time of exposure, and insects with prolonged development are the ones most likely to die (Howe 1967). At 18°C, individuals survived on average for 1.5-2 months before most of them died prematurely. The insects that survived at 18°C developed extremely slowly, and their size did not markedly increase between molts. To some extent, the decelerated development in lower than optimum temperatures could be beneficial when managing large-scale rearing units of insects. For example, if the development needs to be prolonged, i.e., for
synchronization of production patches, it can be achieved by placing individuals in temperatures slightly cooler than the optimum (but above 22°C to prevent increased mortality) for short periods of time.

As typical for many insect species (Atkinson 1994; but see Walters and Hassall 2006, Kingsolver and Huey 2008), the temperatures that promoted the maximum development rate of *R. differens* (30°C), did not promote maximal adult weight; maximum weight was reached at 28°C. At temperatures lower or higher than optimum, insect weight is typically decreased by effects of body temperature on food and energy intake or on metabolic rate (Harrison and Fewell 1994). Further, at lower temperatures, insects can fill their crop more quickly than they are able to process the ingested food, leading to slower weight gain (Harrison and Fewell 1994). At extreme temperatures, lipid metabolism may be hindered, but in this study we found no effect of temperature on fatty acid composition. The weight of *R. differens* continued to increase approximately ten days after the adult molt, suggesting that *R. differens* continues to accumulate lipids in its fat body, as previously reported for the locusts e.g. *Locusta migratoria* (L.) (Orthoptera: Acrididae) (Loveridge 1973). In *R. differens*, this fat provides energy for swarming and long flights (Karuhize 1972).

If the target of *R. differens* mass-rearing is to maximize weight, our results suggest that weight can be manipulated by both rearing temperature and duration. *R. differens* weight can increase rapidly during the first days of adulthood. The optimal harvest time would be when the weight reaches its maximum and prior to a decrease due to e.g., egg laying or elevated energy expenditure for male singing (Stevens and Josephson 1977, Lorenz and Anand 2004). For *R. differens*, this time period would be between six to ten days after adult molting, when up to a 51.2% increase in weight gain can be achieved leading to a higher yield. However, larger scale rearing experiment with estimation of feed conversion efficiencies are needed to further verify at which temperature highest yield can be achieved. This experiment was conducted at
constant temperatures, but the effect of daily fluctuating temperatures should be assessed as it could be beneficial for production processes. Slightly lower than optimal temperatures during the night might lower the heating costs without drastically changing the development time (Ratte 1984).

Temperature modifies the weight gain of males and females differently, where males are more sensitive to higher temperatures. In its natural environment, (e.g. Uganda), daily temperatures range from 23-32°C (mean maximum) during the day and 14-19°C (mean minimum) during the night (World Meteorological Organization 2018). Feeding, flying and singing activities take place during the cooler night time. Since male sound formation generates heat (Stevens and Josephson 1977), a high constant temperature could more negatively impact males since they will more likely face overheating.

Although prolonged exposure to high or low temperatures may alter the lipid metabolism of insects (increased unsaturation of fatty acids at low temperatures and saturation at high temperatures; House et al. 1958, Petersen and Holmstrup 2000), we found no evidence of modified fatty acid compositions in our study. This finding is relevant for the mass-rearing of insects, because the fat quality does not seem to be sensitive to rearing temperature. Furthermore, although diet and/or stress may alter amino acid composition of insects (Jabbar and Strang 1985, Ramos-Elorduy 2002), the composition is generally considered to be relatively constant (Finke and Oonincx 2014). This fact possibly explains why we observed no clear patterns in the composition of the most common amino acids along the temperature gradient in our study (Supp. Fig. S2).

An improved understanding of the thermal performance of R. differens enables us to predict its possible responses to climate change in natural populations. This prediction is vital since the current use as edible insect is completely based on collection from the wild.
Developmental rate peaked at 30°C, which is typical for tropical insects, but higher than the ambient temperatures typically experienced in nature by this species (see above). This finding suggests that increased temperatures due to climate change could speed up the development rates of this species, and possibly allow the production of more generations per year and higher population densities, unless limited by precipitation and the availability of green foliage for food. However, our results also suggest that the mortality of *R. differens* is likely to increase rapidly if temperatures increase even moderately by climate warming, since with respect to survival, *R. differens* is currently living very close to its thermal optimum (see also Deutsch et al. 2008).

In conclusion, considering all the measured performance traits, the optimal rearing temperature for *R. differens* is estimated at 29.1°C. The development rate for *R. differens* peaked at 30°C, with $T_{\text{min}}$ estimated at 15°C and $T_{\text{max}}$ estimated at 37°C. The survival rate also peaked at 30°C. Temperatures that produced the maximum development rate did not produce maximum adult weight; the maximal weight of *R. differens* was reached at 28°C. The weight of *R. differens* continued to increase approximately ten days after adult molting, indicating that this could be the optimal time point for harvesting. Indeed, by rearing these insect for ten days after adult molting, up to a 50% gain in weight can be achieved. Our results can be used when developing large-scale mass-rearing protocols for *R. differens* in the future.

**Acknowledgements**

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(Uganda) for permission to export the insects. Funding was provided by the Academy of Finland (Project no 14956 to HR) and Joensuu University foundation (to VJL).

**Author contribution**

Conceived and designed the experiments: VJL, HR and AV. Performed the laboratory study: VJL. Conducted statistical analysis: VJL and AV. Drafted the manuscript: VJL. All authors contributed to the interpretation of the data, writing and editing of the manuscript.

**Literature Cited**


Table 1. Measured performance trait values for *R. differens* reared in seven constant temperatures

<table>
<thead>
<tr>
<th>Temperature treatment (°C)</th>
<th>Survival % (alive at final molting)</th>
<th>Individuals placed in experiment (total N)</th>
<th>Total developmental time (days)</th>
<th>n for total development</th>
<th>Adult weight (g) at adult molt</th>
<th>n for total adult weight</th>
<th>Adult weight (g) at 10 days after adult molt</th>
<th>n for total adult weight 10 d</th>
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</thead>
<tbody>
<tr>
<td>18</td>
<td>25.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>31.6;31.3b</td>
<td>19;33</td>
<td>95.1;96.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6;5</td>
<td>0.52</td>
<td>11</td>
<td>0.62</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>21.2;41.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16;31</td>
<td>68.6;87.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5;11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56</td>
<td>13</td>
<td>0.82</td>
<td>5</td>
</tr>
<tr>
<td>26</td>
<td>71.4</td>
<td>35</td>
<td>69.4</td>
<td>25</td>
<td>0.58</td>
<td>23</td>
<td>0.88</td>
<td>23</td>
</tr>
<tr>
<td>28</td>
<td>61.8</td>
<td>34</td>
<td>55.3</td>
<td>21</td>
<td>0.62</td>
<td>21</td>
<td>0.88</td>
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<tr>
<td>30</td>
<td>86.7</td>
<td>15</td>
<td>48.8</td>
<td>13</td>
<td>0.55</td>
<td>13</td>
<td>0.81</td>
<td>13</td>
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<tr>
<td>32</td>
<td>36.1</td>
<td>36</td>
<td>54.3</td>
<td>13</td>
<td>0.48</td>
<td>5</td>
<td>0.67</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>258</td>
<td>99</td>
<td>86</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> For treatment 18 survival at the end of the experiment (16 weeks after starting the experiment).

<sup>b</sup> Values for two separate replicates for treatments at 22°C and 24°C.
Table 2. Parameter estimates and $R^2$ of the Briére-1 models for describing performance of $R. \text{differens}$.

<table>
<thead>
<tr>
<th>Performance trait</th>
<th>$a$</th>
<th>$T_{\text{min}}$ (°C)</th>
<th>$T_{\text{max}}$ (°C)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development rate</td>
<td>0.00001647</td>
<td>14.57</td>
<td>36.59</td>
<td>0.92</td>
</tr>
<tr>
<td>Survival</td>
<td>0.16126217</td>
<td>20.09</td>
<td>32.39</td>
<td>0.82</td>
</tr>
<tr>
<td>Adult weight</td>
<td>0.00026811</td>
<td>-2.85</td>
<td>34.55</td>
<td>0.90</td>
</tr>
<tr>
<td>Weight ten days after adult molt</td>
<td>0.00079143</td>
<td>10.81</td>
<td>33.47</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 3. Mean development rate, time and adult weight of $R. \text{differens}$, shown for females and males at six temperature treatments.

<table>
<thead>
<tr>
<th>Rearing temperature (°C)</th>
<th>Development rate</th>
<th>Development time (days)</th>
<th>n</th>
<th>Adult weight (g)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.010</td>
<td>97.3</td>
<td>6</td>
<td>0.54</td>
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Fig. 1. (A) Development rate and (B) survival for *R. differens* over a range of experimental temperatures. Black dots represent the observed means of development rate and survival in each temperature treatment, and the curve represents the fitted Brière-1 model.
Fig. 2. (A) Mean weight of *R. differens* at the time of adult molting and ten days after adult molting at the six experimental temperature treatments, (B) weight increase during the two weeks following adult molting for all individuals, and for (C) females and (D) males separately. The dotted lines in panel A represent extrapolated curves.
Fig. 3. Brière-1 models of the three performance traits (survival, development rate and weight at ten days after adult molting), scaled so that the maximum value of each at optimum temperature is one. The sum of scaled values reaches its maximum at 29.1°C, indicating that this is the overall optimum rearing temperature for *R. differens*. 
Supplementary material (Supplementary Tables S1-S2 and Supplementary Figure S1-S3)

Optimal temperature for rearing the edible *Ruspolia differens* (Orthoptera: Tettigoniidae)

V.J. Lehtovaara, H. Roininen and A. Valtonen

*Ruspolia differens* adults from five temperature treatments (22, 24, 26, 28, and 32°C) were analyzed for their amino acid and fatty acid content. After the experiments (see Material and Methods), randomly selected adult individuals were kept frozen at -18°C, until chemical analysis. Samples were first freeze-dried (Christ ALPHA 1-4 LD Plus; main drying for 24 h + final drying for 6 h), and the drying was finalized with a sorption dehumidifier (DST Aquasorb A-30) for 48 h. To ensure dryness, the abdomen of females was cut open. Wings were also removed prior to analysis.

**Methods**

**Amino acid analysis**

Two individuals (regardless of sex) from each temperature treatment (22, 24, 26, 28, and 32°C) were randomly selected for amino acid analysis (N=10). The amino acid analysis was performed at the Competence Center of Food and Fermentation Technology (Estonia) with hydrolysis by liquid chromatography-UV absorption (LC-UV). The samples were ground with a mortar and pestle before analysis. The samples were hydrolyzed by weighing 10 mg of sample into glass vials, adding 200 μl of 4M methanesulfonic acid and heating the sample at 105°C for 22 h. After hydrolysis, the samples were diluted 200 times with MilliQ water and derivatized with 6-aminooquinolyl-N-hydroxysuccinimidyl carbamate at 55°C for 10 min. Analyses were performed with Acquity UPLC and an AccQ-Tag Ultra column at 55°C. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. A photodiode array (PDA) detector was used for detection and data was processed with Empower software. All the equipment, reagents, and eluents were obtained from Waters Corp. (Milford, MA, USA).

**Fatty acid analyses**

Three individuals (regardless of sex) from each temperature treatment (22, 24, 26, 28, and 32°C) were randomly selected for fatty acid analyses (N=15). Fatty acid analysis was performed at the Bio-Competence Centre of Healthy Dairy Products (Bio–CC) (Estonia), using a direct transmethylation method reported by Sukhija and Palmquist (1988) with minor modifications as described by Lehtovaara et al. (2017). Fatty acid methyl esters (FAME) were quantified with gas chromatography with flame ionization detection (GC-FID). Identification of common fatty acids was performed by comparing sample peak retention times with FAME standards and the quantification of fatty acids was done based on peak areas in relation to the internal standard.
**Statistical analyses**

We used multivariate methods to model patterns in the amino and fatty acid compositions of *R. differens* individuals reared from neonate nymph to three weeks after adult molt at the five experimental temperatures (22-32°C). As the response datasets, we used the proportions of each amino acid or fatty acid (out of the total content) in each studied individual. Altogether 16 amino acids and 49 fatty acids were detected in the studied individuals. Non-metric multidimensional scaling (NMDS) ordinations were first used to illustrate the patterns of amino and fatty acid compositions among the individuals (50 restarts). We then used a distance-based linear models (DistLM: 9999 permutations), with temperature as the predictor, to test for linear patterns in the amino or fatty acid compositions along the temperature gradient. If a gradient was found, to elucidate which amino or fatty acids contributed most to the observed dissimilarities, we ran a similarity percentage analysis procedure (SIMPER) (Clarke & Warwick 2001). The multivariate analyses were run with the software Primer-E, version 6 with PERMANOVA+ add-on (Clarke and Gorley 2006, Anderson et al. 2008). In all multivariate analyses Bray-Curtis was used as a measure of similarity.

**Results and discussion**

**Amino acids**

There was a statistically significant linear gradient in the amino acid compositions of *R. differens* along the studied temperature gradient (DistLM; Pseudo-F$_{1,8}$ = 5.75, P = 0.005, N = 10). Glutamine and glutamic acid, alanine, arginine, methionine and tyrosine accounted for 52.8% of the dissimilarity between the lowest and highest studied experimental temperatures (22 vs 32°C). Although the gradient was visible in NMDS ordination (Supp. Fig. S1A), there was no clear pattern in proportions of amino acids along the studied temperature gradient (Supp. Fig. S2). Amino acid content in *R. differens* reared at temperatures between 22-32°C are shown in Supp. Table S1.

Our results corroborate earlier findings suggesting that even though diet and/or stress may alter insect amino acid composition (Auclair 1959, Jabbar and Strang 1985, Ramos-Elorduy 2002), the composition in general is considered relatively constant (Finke and Oonincx 2014) since it is species-specific (Chen 1985) and specific parts of the insect body are made from certain amino acids. This relative constancy explains why no clear pattern in the amino acids was visible along the temperature gradient. In extreme temperatures certain proteins, such as antifreeze proteins and heat shock proteins, (Denlinger et al. 1991) are formed, which could explain the differences between the amino acid composition at low and high temperatures. However, in our experiment the gradient of studied temperatures was rather modest (covering only 10°C), exposure lasted most of the insect’s life-time and our sample size was very small, so further studies are needed to clarify this.

*R. differens* appears to be a good source of essential amino acids for humans, as it contains eight out of the nine essential amino acids. Humans require histidine, isoleucine, leucine, lysine, methionine + cysteine, phenylalanine + tyrosine, threonine, tryptophan and valine (WHO/FAO/UNU 2007). Requirement estimates for adults vary from 4 to 39 mg/kg per day (i.e. 0.28 - 2.73 g/day for a 70 kg adult) depending on the indispensable amino acid. In this experiment, cysteine and tryptophan were not found in the samples. Previously, however, Siulapwa et al. (2014) reported contents of 0.07±0.02g/100g and 0.03±0.01g/100g (mean±SE on a dry weight basis) for cysteine and threonine, respectively, in *R. differens* samples collected from the wild.
**Supplementary Table S1.** Amino acid content (mean ± within sample SD on a dry weight basis) of *R. differens* reared at five different temperatures within the range of 22-32°C (n=2 for each temperature). The estimated requirement of essential amino acids for humans is shown in the leftmost column (expressed as amount per kg of body weight).

<table>
<thead>
<tr>
<th>Amino acid (g/100g dry weight)</th>
<th>Rearing temperature (˚C)</th>
<th>Requirement estimate mg/kg per day*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.96±0.31</td>
<td>3.42±0.14</td>
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<tr>
<td>Arginine</td>
<td>2.71±0.03</td>
<td>2.25±0.12</td>
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<tr>
<td>Asparagine and Aspartic acid</td>
<td>3.86±0.04</td>
<td>3.29±0.18</td>
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<td>Glutamine and Glutamic acid</td>
<td>5.26±0.06</td>
<td>4.41±0.25</td>
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<tr>
<td>Glycine</td>
<td>2.44±0.11</td>
<td>2.04±0.04</td>
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<tr>
<td>Histidine</td>
<td>1.14±0.05</td>
<td>0.92±0.14</td>
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<tr>
<td>Isoleucine</td>
<td>1.44±0.03</td>
<td>1.18±0.04</td>
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<tr>
<td>Leucine</td>
<td>3.49±0.05</td>
<td>2.94±0.06</td>
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<td>Lysine</td>
<td>2.52±0.04</td>
<td>2.04±0.12</td>
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<tr>
<td>Methionine</td>
<td>0.95±0.06</td>
<td>0.65±0.06</td>
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<td>Phenylalanine</td>
<td>1.32±0.06</td>
<td>1.09±0.07</td>
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<td>Proline</td>
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<td>2.05±0.04</td>
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<td>Serine</td>
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<td>Threonine</td>
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<tr>
<td>Valine</td>
<td>1.98±0.04</td>
<td>1.62±0.05</td>
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</table>

*(WHO/FAO/UNU 2007)

* Requirement estimate for both methionine and cysteine

b Requirement estimate for both phenylalanine and tyrosine
Supplementary Figure S1. The non-metric multidimensional scaling (NMDS) ordination (using Bray-Curtis similarity) showing patterns in (A) amino acid and (B) fatty acid compositions of *R. differens* individuals reared at five different temperatures (22-32°C). The colors illustrate “low”, “medium”, and “high” temperatures. Each symbol represents an individual *R. differens*; the closer the individuals are in the NMDS ordination space, the more similar their amino or fatty acid composition.
Supplementary Figure S2. Mean proportions of amino acids of *R. differens* reared at the five temperature treatments. The total amino acid content (mean as g/100g dry weight) for temperature treatments is shown on the top of each bar.

**Fatty acids**

There was no linear gradient in the fatty acid composition (%) of *R. differens* along the temperature range (DistLM; Pseudo-F$_{1,13}$ = 1.01, P = 0.418, N = 15; Supp. Fig. S1B, Supp. Fig. S3). The found fatty acid composition was very similar to those of wild *R. differens* reported by Opio (2015) and with those reared on carbohydrate and protein rich diets by Lehtovaara et al. (2017). The total fatty acid content (TFA) and contents of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) of *R. differens* reared at temperature range 22-32℃ are shown in Supp. Table S2.

Our results, suggesting that rearing temperature does not strongly modify the fatty acid composition of *R. differens*, was somewhat unexpected, because it is known that prolonged exposure to high or low temperatures may alter the lipid metabolism of insects (increased unsaturation of fatty acids at low temperatures and saturation at high temperatures; House et al. 1958, Hazel 1995, Petersen and Holstrup 2000). The phospholipid fatty acids in membranes of ectotherms become more unsaturated as a response to exposure to low temperatures in order to maintain the membrane homeoviscosity (Hazel 1995, Hochachka and Somero 2002). In addition, the storage fatty acids may show increased saturation as a response to warmer conditions (Ohtsu et al. 1993, Haubert et al. 2008, van Dooremalen and Ellers et al. 2010). Our results do not support this theory on homeoviscous adaptation. This discrepancy may be due...
to limitations in the methods used to analyze the overall fatty acid composition and content. Phospholipids (membrane lipids) and triacylglycerol fatty acids (storage lipids) were not analyzed separately, and thus the possible changes in either group were not visible. Additionally, in our study, where the insects were reared at a certain temperature through their entire postembryonic life cycle, the range of rearing temperatures may not have been large enough to cause detectable changes.

**Supplementary Table S2.** Fatty acid content (mean as mg/g dry weight) of *R. differens* reared at the five temperatures (n=3 for each temperature).

<table>
<thead>
<tr>
<th>Rearing temperature (°C)</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>32</th>
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<td>Total fatty acids</td>
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<td>Saturated fatty acids</td>
<td>191.85</td>
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<td>Mono unsaturated fatty acids</td>
<td>208.68</td>
<td>223.84</td>
<td>275.01</td>
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<td>190.06</td>
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<td>Poly unsaturated fatty acids</td>
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<td>63.66</td>
<td>75.75</td>
<td>61.40</td>
<td>65.45</td>
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<tr>
<td>n6/n3</td>
<td>17.60</td>
<td>18.48</td>
<td>16.91</td>
<td>18.20</td>
<td>15.54</td>
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</table>

**Supplementary Figure S3.** Mean proportions of the seven most common fatty acids of *R. differens* in the five temperature treatments. Fatty acids with a mean composition of > 0.5% are shown. The total fatty acid content (mean as mg/g dry weight) is shown on the top of each bar.
Literature cited


