Influence of photoperiod on thermal responses in body size, growth and development in *Lycaena phlaeas* (Lepidoptera: Lycaenidae)

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ABSTRACT

Many ectotherms species grow faster but attain a smaller body size when reared under warmer conditions, a phenomenon known as the Temperature-Size Rule (TSR). This rule appears to be stronger in aquatic ectotherms than in terrestrial ectotherms. The difference could be related to difficulties for oxygen uptake in water, whereas on land, adaptive responses in body size may relate to seasonal time constraints. To assess the role of seasonal time constraints in temperature size response of terrestrial ectotherms, we reared the small copper *Lycaena phlaeas* at three temperatures (18 ˚C, 23˚C and 28˚C) and two photoperiods (16L: 8D and 12L: 12D). We examined whether differences in body size across treatments was related to (1) differences in growth and development, (2) differences in breakpoints during growth trajectories, or (3) differences in ommatidia size (as a proxy for cell size). We found a weak inverse relationship between developmental temperature and the body size of adult butterflies; adult size decreased by approximately 1% for every degree warmer. Under warmer temperatures, caterpillars developed more quickly and had higher growth rates but reached a smaller body size. Under a short photoperiod, both growth and development were slower, especially at the two lower temperatures, but the body size resulting from slow growth over a longer developmental period did not vary with photoperiod. Breakpoints in growth trajectories occurred when larvae reached ~40% of their maximum mass and these breakpoints were strongly correlated with the size of the resulting adults, suggesting that adult size is predetermined at an early stage. Temperature did not appear to cause reductions in body size through reductions in cell size. Butterflies were largely able to buffer their body size by modulating larval growth and development in tandem. They appear to use photoperiod as a cue to gauge the availability of time (with 12L: 12D indicating less time available) while temperature speeds up growth and development and as such governs the amount of time they need to complete a developmental cycle. Temperature and photoperiod thus induce changes in voltinism to fit a discrete number of generations into a growing season.

1. Introduction

Temperature is one of the main abiotic variables and has a profound effect on the survival and fitness of ectothermic organisms, influencing key traits such as body size, growth rate and development (Bjørge et al., 2018; Ghosh et al., 2013). Most ectotherms show a negative relationship between the size at maturity and developmental temperature, i.e. when reared under warmer conditions they grow faster but reach a smaller final size. This thermal response in body size is known as the temperature-size rule (TSR) (Atkinson, 1994; Verberk et al., 2021). Although the TSR is widely documented across a diverse range of invertebrates and vertebrates, the underlying mechanisms to explain this phenomenon are not fully understood (Angilletta & Dunham, 2003; Forster et al., 2012; Ghosh et al., 2013). Moreover, the temperature-size rule could constitute an adaptive response or results from physiological constraints (van der Have & de Jong, 1996; Walczyńska et al., 2015; Verberk et al., 2021). Under an adaptive framework for TSR, temperatures-size responses are beneficial, increasing the fitness of species. In contrast, non-adaptive mechanisms emphasize temperature-dependent constraints in growth and developmental processes which result in body size variation (Angilletta et al., 2004; Ghosh et al., 2013).

Temperature-size responses can be studied from different perspectives (Fig. 1). At the whole-organism level, body size results from the time spent growing (i.e. development) and the rate at which mass is accrued (i.e. growth rate) and thus, understanding the thermal sensitivity of growth and development may help explain temperature-size responses (Forster & Hirst, 2012; Sibly & Atkinson, 1994; van der Have & de Jong, 1996; Verberk et al., 2021). With increasing temperature, both growth rate and development rate increase, but a smaller size at maturity will result at the warmer temperature if development rate in-

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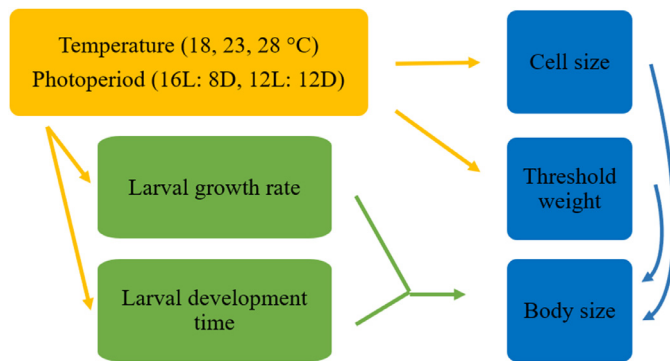
Fig. 1. Schematic illustration of how temperature and photoperiod affect adult body size indirectly, by modulating growth rate, development time, cell size and threshold weight.

creases more than growth rate (Forster et al., 2011). Limits to taking up sufficient oxygen to meet the increased oxygen demand at higher temperatures could constrain growth, thus giving rise to the TSR. This explanation is consistent with observations of stronger size reductions in the warm for aquatic ectotherms than for terrestrial species (Forster et al., 2012; Horne et al., 2015), as oxygen uptake is more difficult in water than in air (Verberk & Atkinson, 2013).

Temperature-size responses can also be investigated from a cellular perspective, as changes in body size arise either by changes in cell number, cell size, or a combination of these (Hessen et al., 2013; Miller, 2013). In eutelic organisms (i.e. species with a constant cell number), thermal responses in body size must arise from changes in cell size, but also in more complex organisms with variable cell numbers, studies have been demonstrated that individuals reared at higher temperatures are composed of smaller cells when compared to their conspecifics reared at lower temperatures (Arendt, 2007; Azevedo et al., 2002; Czarnołęski et al., 2013). Although terrestrial ectotherms may be less vulnerable to oxygen limitation, similar constraints could act at the level of individual cells with smaller cells being better in taking up oxygen (due to favourable surface area to volume ratio's). Thus, better understanding the causes and consequences of differences in cell size may help unravel the puzzling effects of temperature on body size (Hessen et al., 2013; Verberk et al., 2021).

Finally, size adjustments can be investigated from the perspective of hormones controlling insect growth and development. In many insect larvae, the secretion of the ecdysone and juvenile hormone is a signal to stop growing and starting the molting process (Callier & Nijhout, 2011; Miki et al., 2020). In addition, molting from final instar into a pupa depends on achieving a certain threshold weight during the larval stage, which is called critical weight (Ghosh et al., 2013; Sharma et al., 2020). This critical weight represents a "check-point" for body size. Although larvae can and do feed and grow substantially after having reached their critical weight, they are preprogrammed to start initiating metamorphosis even when deprived of food (Callier & Nijhout, 2011; Suzuki et al., 2013). Therefore, the critical weight is considered as an important factor in determining the growth and development duration and appears to act as a regulator of body size in many insects (Davidowitz et al., 2003; Ghosh et al., 2013). In this study, we measured the threshold weight of caterpillars which is relatively comparable to the critical weight. Ghosh et al., (2013) showed that the temperature influences final body size by regulating this critical weight in *Drosophila melanogaster,* while in the larvae of the lepidopteran *Manduca sexta* critical weight did not change with temperature (Davidowitz et al., 2003). Thus, thermal responses in body size could be mediated by thermal effects on critical weight.

Adaptive explanations, although still mediated by thermal effects on growth rate and development rate (Fig. 1), emphasize the benefits of growing smaller in the warm. One such explanation which has been argued to be more applicable to terrestrial ectotherms relates to seasonal time constraints (reviewed in Verberk et al., 2021). Such seasonal time constraints arise as photoperiod and temperature create time windows for insect growth to occur. On land, winter can be more stressful (risks of freezing and desiccation), forcing terrestrial arthropods to reach the developmental stage in which they hibernation before the onset of winter. Horne et al. (2015) reported that the strength and direction of temperature-size responses in terrestrial arthropods was related to voltinism (i.e. the number of generations per year). Univoltine species (which complete a single generation per year) cannot profit from warmer conditions by initiating another generation. Instead, it is more beneficial for them to grow faster and reach maturity at a larger size under warmer conditions. Consequently, they often display weaker or even converse temperature-size responses. In contrast, multivoltine species (completing multiple generations per year) may benefit from maturing earlier and at a smaller size in warmer conditions as this allows them to complete an additional generation. Consequently these species adhere to the TSR more strongly than univoltine species (Fischer & Fiedler, 2002; Horne et al., 2015; Kivelä et al., 2011; Verberk et al., 2021).

Day-length is an important cue for insects to gauge how long the conditions remain favorable to complete their larval development before the growing season ends (De Block & Stoks, 2003; Kutcherov et al., 2011; Lopatina et al., 2011). As growth and development vary with temperature, the time needed to complete development varies with temperature. Photoperiod and temperature may therefore interactively set time windows to complete development to a certain adult size (Verberk et al., 2021). Another factor that may alleviate or exacerbate time constraints is sex as males have the tendency to emerge sooner than females for maximizing the number of mating; a phenomenon known as protandry (Gotthard, 2008). A faster development may result in a smaller size at maturity for males, unless they compensate by also exhibiting faster growth (Fischer & Fiedler, 2000; Gotthard, 2008).

Here, we examine the effect of seasonal time constraints (by using a combination of different temperature treatments and photoperiod treatments) on the temperature size rule in the small copper *Lycaena phlaeas* (Fig. 1). We also assess whether the TSR is linked to physiological mechanisms such as differences in the thermal sensitivity of growth and development, as well as their threshold weight (derived from growth trajectories) and cell size (using the size of ommatidia in butterfly compound eye as a proxy for cell size) (Fig. 1). To explore all these possibilities, we investigated the influence of both photoperiod and temperature on the growth, development time, threshold weight, adult body size, and cell size in both male and female butterflies. We hypothesized that butterflies reared under warm conditions would reach a smaller size than those reared under cool conditions (i.e. adhering to the temperature-size rule). Moreover, we hypothesized that the effect of seasonal time constraints on physiological responses (such as growth rate and development rate) would be magnified under the shorter photoperiod (indicative of autumn) and alleviated by warming. In addition, due to protandry, males are expected to develop faster at the cost of reaching a smaller size. In addition, we expected that differences in adult body size would be related to differences in threshold weight, growth and development and cell size (Fig. 1).

2. Material and methods

2.1. Study species

The small copper butterfly *Lycaena phlaeas* was used in this study for testing the effect of photoperiod on temperature-size responses. *Lycaena phlaeas* is a widespread species and its distribution rang is temperate Holarctic (Bos, 2006). The small copper butterfly is a common butterfly in the Netherlands and it has typically 2-3 generations annually. Depending on location and season the presence of a third generation can vary from year to year. Adults from the first generation fly from late April to

mid-June, those from the second generation from late June to early October and those from the (overlapping) third generation fly from early September to late October (Bos, 2006). With the shortening of the day-length in autumn and decreasing temperatures, caterpillars of the small copper enter into a state of quiescence. The small copper does not go into a real diapause, contrary to the related sooty copper *Lycaena tityrus* (Fischer & Fiedler, 2000). This quiescence is thus adjustable and can be influenced by temperature.

2.2. Host plant cultivation and butterfly maintenance

Adults of the small copper butterflies were collected in May 2015 from a restored heathland towards the village of Wekerom, on the Veluwe in the Netherlands (52°05′21.4"N 5°41′39.0"E). The average temperature in the habitat of the small copper butterflies is around 23°C in July and 18°C in September. Butterflies will experience warmer temperatures in their habitat when basking in the sun and during heat waves. In our experimental design (see below), we therefore also included the third temperature of 28°C. After collecting butterflies, they were brought to the greenhouse facilities of the Radboud University to start a breeding colony. Thus, *L. phlaeas* was maintained at the university for at least 4 years. In the green house, the natural daylight cycle was supplemented with artificial light all year long (16L: 8D) and thermal variation ranged from an average temperature around 20.0°C in winter to an average temperature around 23.8°C in summer. Towards the end of the summer, when the amount of daylight became less, the breeding population in the green house was also provided with UV-light to stimulate mating and to maintain oviposition throughout the whole year. In effect, the butterflies were in a perpetual summer and before the experiment commenced had completed around 25 generations in the greenhouse. Consequently, the experimental short photoperiod is interpreted as an autumn photoperiod.

Common sorrel (*Rumex acetosa*) was used as a host plant for adult oviposition and larval feeding. *Rumex acetosa* was cultivated in the green house of the Radboud University. Seeds were sown and grown on glass beads with water for three weeks in a climate chamber at 25°C during the day and 10°C during the night. The seeds received a photoperiod of 14L: 10D to stimulate germination. Subsequently, five seedlings were planted per pot of 0.75 l, filled with silver sand mixed with plant nutrition and were grown under a light cycle of 16L: 8D until used in the experiments. After the leaves of each plant were eaten by caterpillars, all the stems were cut down. Then, after a few weeks, fresh young leaves had regrown and the plants were used again as a new source of food for caterpillars of the breeding colony, but not for the experiments. Adult butterflies were fed with artificial nectar provided through artificial flowers.

2.3. Experimental arrangement and weight measurements

Experiments were conducted from October 2018 to November 2019, using climate cabinets to establish three different rearing temperatures (28, 23, 18°C) and two different photoperiods (16L: 8D, 12L: 12D) at each temperature, giving rise to 6 treatment conditions. Since we could conduct only two treatments at the same time (we only had 2 climate cabinets), we decided to have three, subsequent rearing cycles corresponding to the three temperatures and for each temperature measure both photoperiods. Therefore, the rearing experiments commenced between May and June 2019 for the 28°C treatment, between October 2018 and January 2019 for the 23°C treatment and between July 2019 and November 2019 for the 18°C treatment.

For each temperature, two pots of *Rumex acetosa* were placed in the cage with the breeding population. Female butterflies were allowed to mate randomly and oviposit their eggs on plants for eight hours to minimize variation in egg hatching. Subsequently, the plants containing the eggs were transported to the climate cabinets. A few days after hatching, 30 larvae were haphazardly chosen and placed individually in plas-
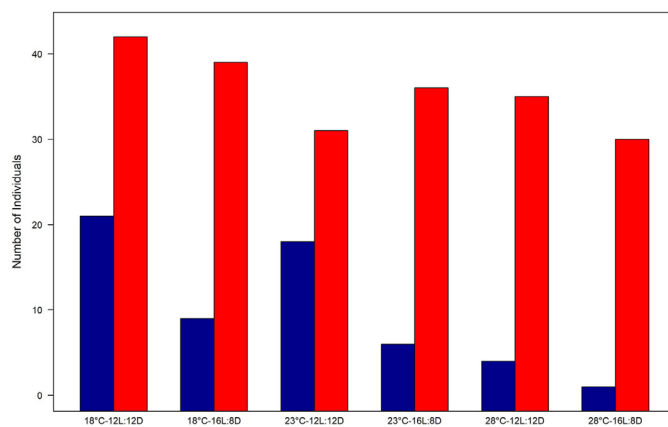

Fig. 2. Total number of caterpillars (blue) used in the experiments and the number of mortalities (red).

tic cups that were attached to the host plants for each temperature and photoperiod. The rest of the caterpillars were kept and maintained in the same climate cabinet to provide potential substitutes in the event that caterpillars that were placed individually in plastic cups died during the experiments. The total number of individuals used for each treatment in this experiment is shown in Fig. 2. Thirty initial number of larvae were considered for each experimental group (temperature, day-length). The initial number of 30 larvae increased during the experiment due to the mortality and subsequent replacement caterpillars. The individual inside each of the plastic cups was checked and weighted regularly to provide regular measurements of growth. Since growth and development varied with temperature, the frequency with which caterpillars were checked was daily for the 28°C treatment, every other day for the 23°C treatment and twice a week for the 18°C treatment. After eclosion, the butterflies were put in the freezer at -21°C within a day of eclosion (except for those individuals that emerged during the weekend) for two hours to ensure their deaths before weighing their fresh mass. The fresh weight of all individuals was measured by the balance with milligram sensitivity (Mettler Toledo XA105DU Dual-Range). The sex of individuals was determined by dissecting the abdomen and checking for male or female reproduction parts.

2.4. Growth rate and development rate

Weight measurements were used to derive growth in two ways. First, since the start weight of freshly hatched larvae was negligible, total growth rate was determined by dividing the maximum weight of the individuals during the larval stage by the age in days of the larvae when they reached maximum weight (see also Fig. 3):

$$Growth\ rate \left(\frac{mg}{day}\right) = \frac{Maximum\ larval\ weight}{age\ at\ \max\ weight}$$

Second, we used growth trajectories of caterpillars (inspired by Kivelä et al., 2018) to derive the threshold weight after which larvae accelerated growth. To this end, the mass gained by an individual between two consecutive measurements (expressed as larval weight gain in mg per day) was plotted against the (ln-transformed) average body weight of that individual during this measurement interval. Next, we combined such data for individuals of the same sex and same treatment (i.e. a given combination of sex, temperature and photoperiod) and fitted a piecewise linear regression to these growth data. Such data showed a clear break point, which corresponded to this threshold weight (see Fig. 8 for an example). The total time spanning from egg hatching to adult emergence was used as a measure of development and was determined for each individual. All individuals were monitored regularly (see above) and the time from hatching to pupation and to adult eclosion were noted. Total development time was converted to development rate
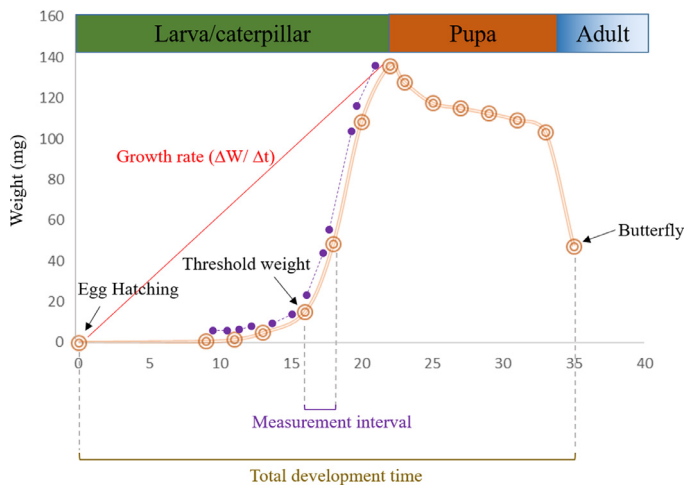
Fig. 3. Schematic illustration of measured traits and different stages in the life cycle of butterfly (the data of 23˚C-16L: 8D group were used in this graph).

by taking the inverse (i.e. development rate = development time $^{-1}$). An overview of different measured traits in this study is shown in Fig. 3.

2.5. Ommatidia measurements

To determine whether changes in cell size contributed to body size variation, we measured cell size. Counting and measuring all adult body cells was not feasible, therefore we required a measurable tissue or organ (such as wing or eye), where individual body cells are easily discerned. Previous studies have used the size of the ommatidia that make up the eye as a proxy for cell size (e.g Schramm et al., 2015., 2021). Here, we measured the ommatidia of adult eyes (as a proxy for cell size), using nail polish to create an imprint from the external surface of the eye (Arya & Lakhotia, 2006; Schramm et al., 2015). Butterflies were decapitated with a sharp needle under a binocular stereo microscope, and a small drop of transparent nail polish was placed on the right eye of the butterflies. After 3-5 min at room temperature, the dried layer of nail polish was gently peeled-off with the help of thin needles. The resulting imprint of the eye was mounted in imsol-mount on a slide and a cover slip. These prepared slides were dried at room temperature for 24 h before being photographed. Digital images were taken at two optical magnifications (5× and 10×), using a Leica DM-RBE FL4 microscope mounting a Leica DFC450 C camera. The 5× magnified pictures were measured in the ImageJ software. The length of a row of 10 ommatidia was measured in three separate locations and along the same direction. The distance was divided by 10 to estimate the single ommatidium diameter. Then the three measurements were averaged to provide an average ommatidium diameter for that individual. All the individuals used in this measurement were frozen between 1-2 years at -21˚C prior to examination.

2.6. Statistical Analyses

To test our hypotheses (see Figure 1), we fitted a series of linear models. These models fall into two broad sets of analyses, each addressing a different research question. In one set of analyses category, we asked how the treatments (temperature, photoperiod) affected butterfly performance at different levels of biological organization (from cells to adult mass). In the other set of analyses, we asked how the highest level of biological organization (i.e. adult size) was related to lower levels of biological organization (e.g. cell size, larval development). Note that finding strong relationships at one level of biological organization does not invalidate causality at another level of biological organization. For example, adult size may be significantly related to temperature yet differences in adult size must logically arise via the effects of temperature

on growth and the time spent growing (i.e. development). As such these analyses provide complementary views on the same phenomenon (the TSR).

When investigating the effect of our treatments on the different performance metrics, we started with a full model, relating temperature, photoperiod, sex and all interactions (including 3-way and 2-way) as predictor variables to the response variable. As response variables we included adult mass, development rate, growth rate and ommatidia size. Next, we reduced the full model by step-wise removing the least significant parameter if this reduced the AIC value of the resulting model. For our analysis of adult body size, the natural logarithm of adult mass was included as the response variable, as previous studies found that the relationship between temperature and body size was not linear, but rather that, temperature reduces body size by a certain percentage for a given degree warming (Forster et al., 2012).

In addition to relating the effects of treatment conditions to response variables, we also tested whether differences in adult body size could be explained directly from differences in either cell size, threshold size and the combination of larval mass (as a measure of larval growth) and development time (as a measure of the time spent growing) (Fig. 1). Threshold weight was calculated by combining data for all the caterpillars of a given sex and in a given treatment as explained above. The R package {segmented} was used to fit a piecewise linear regression model and calculate the break point, which represented threshold weight. Thus, threshold weight was not measured on an individual basis, but rather calculated separately for males and females in a given treatment group (i.e. a single value for each of the 12 sex by treatment combinations). Because of this low sample number, we did not statistically analyse how threshold weight varied with sex, temperature and photoperiod. Instead, we related these related these values for threshold weight in each of the 12 sex by treatment combinations, to the average adult mass observed in each of these 12 groups. To relate differences in adult size to differences in cell size, we performed a partial regression. Such a partial regression can account for potentially confounding effects. In our case, we observed opposite relationships between temperature and either adult size (negative) or cell size (positive). In addition, males and females showed opposite patterns. The partial analysis accounted for these effects of temperature and helped establish whether and to what extent cell size and adult size were correlated in males and females by focusing on within-treatment variation. Finally, to test to what extent adult mass could be related directly to the rates underlying body mass (i.e. growth rate and development rate), we performed a linear model, including the natural logarithm of adult mass as a response variable. As explanatory variables we included sex, development time and mass at a standardized time point (maximum larval weight). Note that we did not include growth rate as an explanatory variable as this has development time included in its calculation, and a model that includes development time twice would be difficult to interpret. All analyses and figures presented in the paper were performed in RStudio Version 1.2.5019 (Team, 2019), using the packages "segmented" (Muggeo, 2008), "visreg" (Breheny & Burchett, 2017) and "tidyr" (Wickham & Henry, 2020).

3. Results

3.1. Temperature-size response

Adult butterflies reached a smaller size when reared at higher temperatures ($F = 9.2$, $P = 0.003$; Fig. 4, Table 1). For a given temperature, female butterflies reached a greater body mass than the males ($F = 16.1$, $P < 0.0001$). The model that included photoperiod showed no effect of photoperiod on either adult mass ($F = 0.0001$, $P = 0.99$) or thermal responses in adult mass (temperature*photoperiod: $F = 2.4$, $P = 0.125$) and this model received less support (AIC for the model including photoperiod: -70.60; AIC for the model excluding photoperiod: -72.15). Similarly, models that included the interaction between tem-
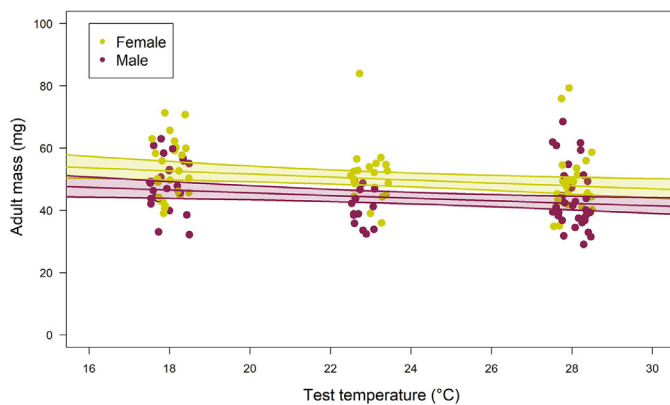
Table 1

Anova table for adult mass as a function of: (A) test temperature and sex; (B) growth rate and development rate; (C) threshold weight and sex.

Response variable	Factor	DF	Sum Sq	Mean Sq	F-value	P- value
A) Adult mass (natural logarithm-transformed) (Adjusted R^2=0.14)	Temperature	1	0.3	0.3	9.2	0.003
	Sex	1	0.6	0.6	16.1	<0.0001
	Residuals	142	4.9	0.03		
B) Adult mass (natural logarithm-transformed) (Adjusted R^2=0.33)	Maximum larval weight	1	1.3	1.3	47.9	<0.0001
	Development time	1	0.1	0.1	3.5	0.06
	Sex	1	0.6	0.6	21.8	<0.0001
	Residuals	140	3.7	0.03		
C) Adult mass (Adjusted R^2=0.67)	Threshold weight	1	143.1	143.1	16.3	0.003
	Sex	1	67.7	67.7	7.7	0.02
	Residuals	9	78.9	8.8		

Table 2

Anova table for development rate (natural logarithm-transformed), growth rate (natural logarithm-transformed) and ommatidia size as a function of test temperature, photoperiod, sex and the interaction effects.

Response variable	Factor	DF	Sum Sq	F-value	P- value
Development rate	Temperature	1	38.2	2340.5	<0.0001
	Photoperiod	1	1.7	102.8	<0.0001
	Sex	1	0.2	12.5	0.0005
	Temperature: Photoperiod	1	1.1	66.5	<0.0001
	Residuals	149	2.4		
Growth rate	Temperature	1	32.5	619.7	<0.0001
	Photoperiod	1	4	75.8	<0.0001
	Sex	1	0.01	0.2	0.670
	Temperature: photoperiod	1	1.3	25.5	<0.0001
	Residuals	148	7.8		
Ommatidia size	Temperature	1	1.8253e-05	22	<0.0001
	Photoperiod	1	4.0470e-06	4.9	0.029
	Sex	1	5.1895e-05	62.7	<0.0001
	Residuals	150	1.2423e-04		

Fig. 4. Adult mass (mg) of butterflies at different rearing temperatures for males (purple circle) and females (yellow circle). The lines with confidence intervals represent the best performing model (Table 1).

perature and sex indicated that the effect of temperature did not vary between males and females (temperature*sex: $F = 0.1$, $P = 0.75$). The decline in body mass with temperature (expressed as % change in mass per°C) for butterflies was relatively small (-0.95%), and together, temperature and sex explained 13.94% (Adjusted R^2) of the variation in adult body mass.

3.2. The response of life history traits to the seasonal changes

3.2.1. Temperature and photoperiod effect on developmental rate and growth rate

Temperature stimulated both development and growth (Development: $F = 2340.5$, $P < 0.0001$; Growth: $F = 619.7$, $P < 0.0001$; Fig. 5, Table 2). Photoperiod also influenced growth and development, but only

at the lower temperatures (Development: temperature*photoperiod: $F = 66.5$, $P < 0.0001$; Growth: temperature*photoperiod: $F = 25.5$, $P < 0.0001$). Caterpillars grew and developed faster under the long photoperiod typical of summer, except at the highest temperature, where growth and development were similar for the two photoperiods. For development, there was a small effect of sex, with males developing slightly faster than females ($F = 12.5$, $P = 0.0005$). However, no significant differences of growth rate were observed for different sexes ($F = 0.2$, $P = 0.670$). Interestingly, for the short photoperiod at 23°C we observed a dichotomy in development: caterpillars either developed rapidly or they took longer to develop (Fig. 5A).

Adult mass was strongly related to maximum larval weight, which combines growth rate and the time spent growing ($F = 47.9$, $P < 0.0001$; Table 1). Caterpillars with a given maximum weight had the tendency to develop into heavier adults if they had taken longer to develop ($F = 3.5$, $P = 0.06$, Figure 6), individuals with longer development time (i.e. under cool conditions). In addition, males developed into smaller adults regardless of larval weight and development time ($F = 21.8$, $P < 0.0001$).

3.2.2. Temperature and photoperiod effect on cell size

Both temperature and photoperiod had a significant effect on the ommatidia size in butterflies, but not their interaction (temperature: $F = 22$, $P < 0.0001$; photoperiod: $F = 4.9$, $P = 0.029$). Contrary to our expectations, butterflies reared at higher temperatures exhibited larger ommatidia (Fig. 7A, Table 2). Ommatidia size was slightly larger in the long photoperiod. We also found male butterflies to exhibit larger ommatidia across the treatments compared to females ($F = 62.7$, $P < 0.0001$), contrasting with their smaller body mass. A partial regression between body size and ommatidia size revealed that when accounting for the effect of temperature, adult mass was weakly, but positively related to cell size in both males and females ($F = 8.27$, $P = 0.0004$, R-squared: 0.11, Fig. 7B)
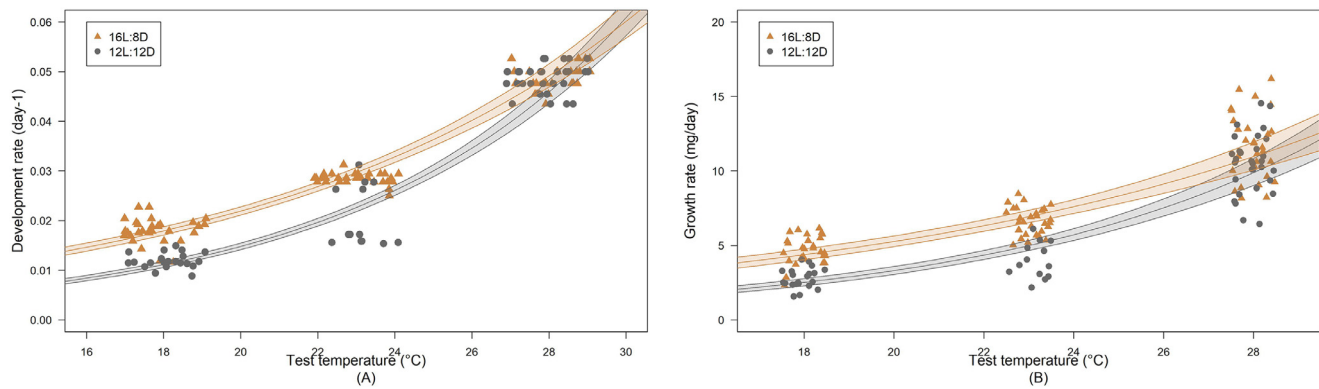
Fig. 5. Changes in development rate (day^{-1}) (A) and growth rate (mg/day) (B) of butterflies at three different test temperatures and two photoperiods (summer photoperiod: brown triangles, autumn photoperiod: grey circle). The lines with confidence intervals represent the best performing model (Table 2).

Table 3

The threshold weight of different treatments (temperature-photoperiod) and different sex.

Treatments (temperature/photoperiod)	Sex	threshold weight break point ± ST err (Natural logarithm scale)	threshold weight (mg)	The growth curve slope after passing the threshold weight break point
28°C/ 16L:8D	M	3.24 ± 0.067	25.6	47.74
28°C/ 16L:8D	F	3.3 ± 0.09	24.1	27.77
28°C/ 12L:12D	M	3.18 ± 0.071	24.1	39.60
28°C/ 12L:12D	F	3.16 ± 0.077	23.6	43.47
23°C/ 16L:8D	M	2.89 ± 0.1	17.9	22.22
23°C/ 16L:8D	F	2.92 ± 0.096	18.5	20.95
23°C/ 12L:12D	M	3.37 ± 0.156	29.2	12.22
23°C/ 12L:12D	F	3.58 ± 0.06	35.7	22.33
18°C/ 16L:8D	M	3.51 ± 0.072	33.5	19.01
18°C/ 16L:8D	F	3.61 ± 0.074	37	19.97
18°C/ 12L:12D	M	3.27 ± 0.199	26.3	4.85
18°C/ 12L:12D	F	3.32 ± 0.12	27.7	6.90

3.2.3. Threshold weight variation under different temperatures and photoperiods

Growth rates varied with larval weight in a non-linear way. Larvae first grew slowly up until a threshold weight was reached, after which growth was much faster (Fig. 8). We characterized these growth trajectories for each of our six treatments in both males and females (Table 3). We observed pronounced variation across treatments in both this threshold weight as well as the growth rate exhibited beyond the threshold weight: in the coldest treatment (18°C), threshold weight was largest in caterpillars reared under a long photoperiod, whereas at 23°C, the threshold weight was smallest in caterpillars reared under a long photoperiod. Males and females generally showed similar threshold weight at each treatment, with the possible exception of caterpillars reared at 23°C under a short photoperiod, where males exhibited a smaller threshold weight. Growth rates of caterpillars that surpassed their threshold weight mostly reflected the effects of temperature, with faster growth in warmer conditions, with the exception of the coldest temperature where caterpillars continued to grow very slowly under the short photoperiod (Fig. 8).

The threshold weight of a treatment group was a good predictor of the average adult mass in that group ($F = 16.3$, $P = 0.003$) (Fig. 9, Table 1). In addition, this relation was different between males and females ($F = 7.7$, $P = 0.02$). Together, 66.7% of the variation in average adult mass across treatments for males and females could be explained by variation in threshold weight.

4. Discussion

4.1. Body size & underlying physiological processes

In the current study, we investigated how temperature and photoperiod modify body size in the small copper, and whether size re-

sponses are related to responses in growth and development, threshold weight and cell size. When comparing adult mass at different temperatures we found some support for the temperature-size rule (Atkinson, 1994) as butterfly mass decreased with higher rearing temperatures. The 0.95% decrease in body mass per degree Celsius agrees well with the average temperature-size response for Lepidoptera reported by Horne et al. (2015). This small effect of temperature for butterflies contrasts with the response for other arthropod groups, indicating that butterflies can buffer their body mass against perturbations in temperature quite well. Together, growth rate and development time, two distinct processes, control the size at maturity (D'Amico et al., 2001; Stern, 2001; van der Have & de Jong, 1996) and also in our results, growth and development exhibited during the larval stage corresponded to adult mass (Fig. 6). Photoperiod affected both growth and development, especially at lower temperatures. Growth and development are tightly linked in insects since moulting is essential for growth to occur (Kivelä et al., 2018; Miki et al., 2020) even though moulting and growth may be differentially affected by temperature and photoperiod, each acting on specific signaling pathways (Miki et al., 2020). The shorter autumn photoperiod reduced both growth and development at the lower temperatures, which resulted in slower life cycles. Still, because both processes were similarly affected, variation in development was compensated for by variation in growth (Blanckenhorn & Demont, 2004) and the resulting body mass did not vary consistently with photoperiod MacLean & Gilchrist, (2019). reported that development time in *Drosophila subobscura* is dependent on both temperature and photoperiod, with photoperiod also having stronger effects in the colder treatment. However, their fruit flies (who hibernate as adults) sped up development under short photoperiods, whereas our butterflies (who hibernate as caterpillars) developed slower. Higher temperatures appear to override effects of photoperiod, leading to faster growth rates and shorter development times which resulted in faster life cycles and a somewhat smaller body mass.
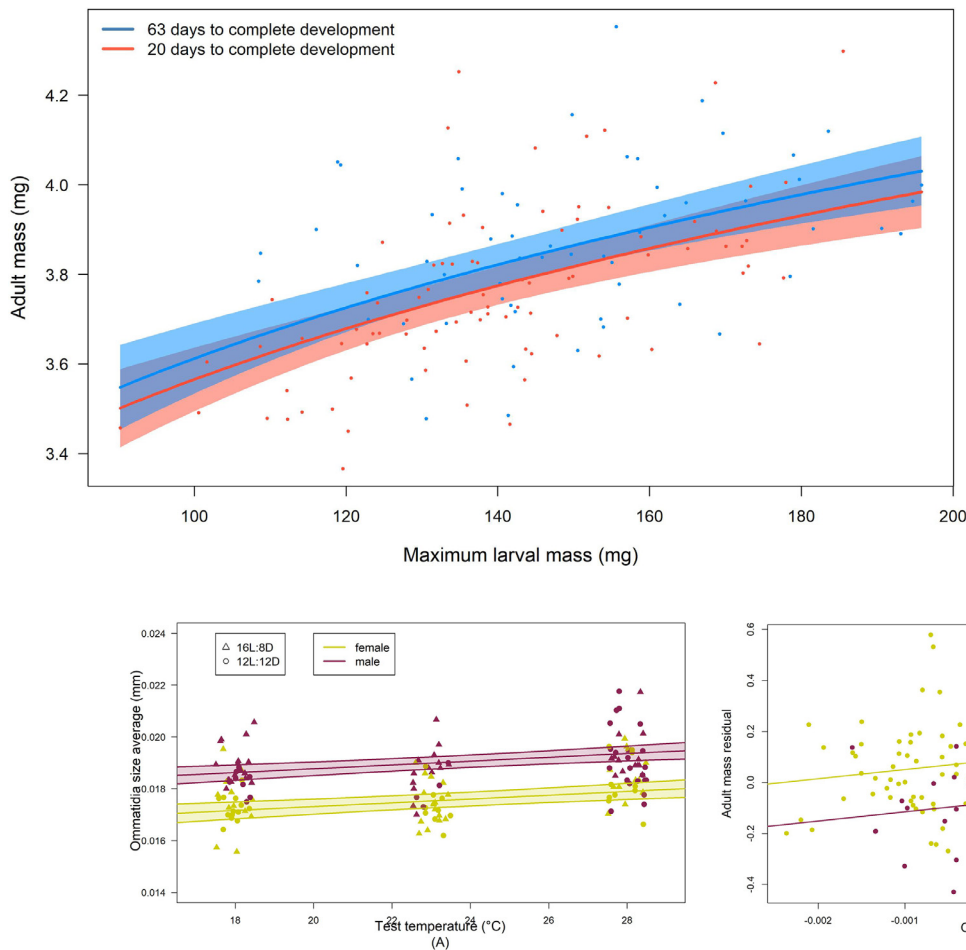
Fig. 6. Partial residuals plot, showing the relationship between (natural logarithm transformed) adult mass and maximum larval mass. Colors indicate model predictions for different development time.

Fig. 7. Butterfly ommatidia size (mm) (A) comparison between male (purple) and female (yellow) at three different test temperatures and two photoperiods (summer photoperiod: triangles, autumn photoperiod: circle). The lines represent the best performing model (Table 2). The residual partial regression plot between adult mass of both male (purple) and female (yellow) and ommatidia size within each treatment (B).

Across treatments, averages for threshold weight and adult mass were very strongly related, suggesting that adult size is predetermined at a stage where larvae have yet to accrue more than 60% of their body mass. Since we could only derive an average threshold weight for groups of individuals, we cannot use threshold weight to explain individual variation in body mass. The hormonal controls on moulting (Callier & Nijhout, 2011) seem to be in place to predetermine adult mass, and these have been suggested to be shaped by the temperature and oxygen conditions that can be anticipated (Verberk et al., 2021; Walczyńska et al., 2015). Earlier findings by Davidowitz et al., (2003) reported that critical weight (i.e. the minimal weight at which further growth is not necessary for a normal time course to pupation) does not vary with rearing temperature in tobacco hornworm *Manduca sexta*. In *Manduca sexta*, the critical weight occurred at 55% of peak larval mass, so quite similar to our measure of threshold weight and the two measures could thus be related. However, our threshold weight was affected by temperature (and photoperiod) in the small copper. In addition, our results suggest that time constraints, forcing animals to complete development sooner but at the cost of a smaller body mass, were stronger under a long photoperiod at 23°C, but being weaker at this photoperiod at 18°C. Thus, the decision to grow to a certain size may involve time constraints as perceived by the caterpillars under a given photoperiod and temperature (Verberk et al., 2021).

To test whether thermal responses in body mass reflect thermal responses in cell size, we assessed the ommatidia size as a proxy for cell size. Interestingly, we found opposite effect of temperature on ommatidia size; butterflies reared at higher temperature exhibited larger om-

matidia. The negative relationship between body mass and ommatidia size contrasts with work in other species which showed cell size to partly explain the temperature-size rule (Leinaas et al., 2016; Partridge et al., 1994; Verberk et al., 2021). Although we did find a positive relationship between body size and ommatidia size within a treatment, our results across treatments suggest that butterflies at colder temperatures attain larger bodies despite being composed smaller cells and it is thus reasonable to assume that butterflies at colder temperatures are composed of more cells. Thus, our results do not lend support to constraints in oxygen delivery at the cellular level at high rearing temperature. One resolution could be that different cell types may exhibit different responses to temperature as has been reported by Czarnołęski et al., (2015) who compared cell size in the epithelium, muscle, and hepatopancreas and found that temperature differentially affects the cell size of different cell types. We also found opposite effects of sex on ommatidia size and body mass: compared to females, the smaller males had larger ommatidia. Generally, males with better vision are expected to have an advantage to locate females which may have selected for larger ommatidia in males, consistent with previous studies that showed males to have generally larger eyes and ommatidia than females in Lepidoptera (Lund et al., 2001; Rutowski, 2000; Ziemba & Rutowski, 2000).

4.2. Sex differences

Although responses to temperature and photoperiod were mostly consistent between males and females, we did observe differences with females exhibiting in general a greater threshold size and final body
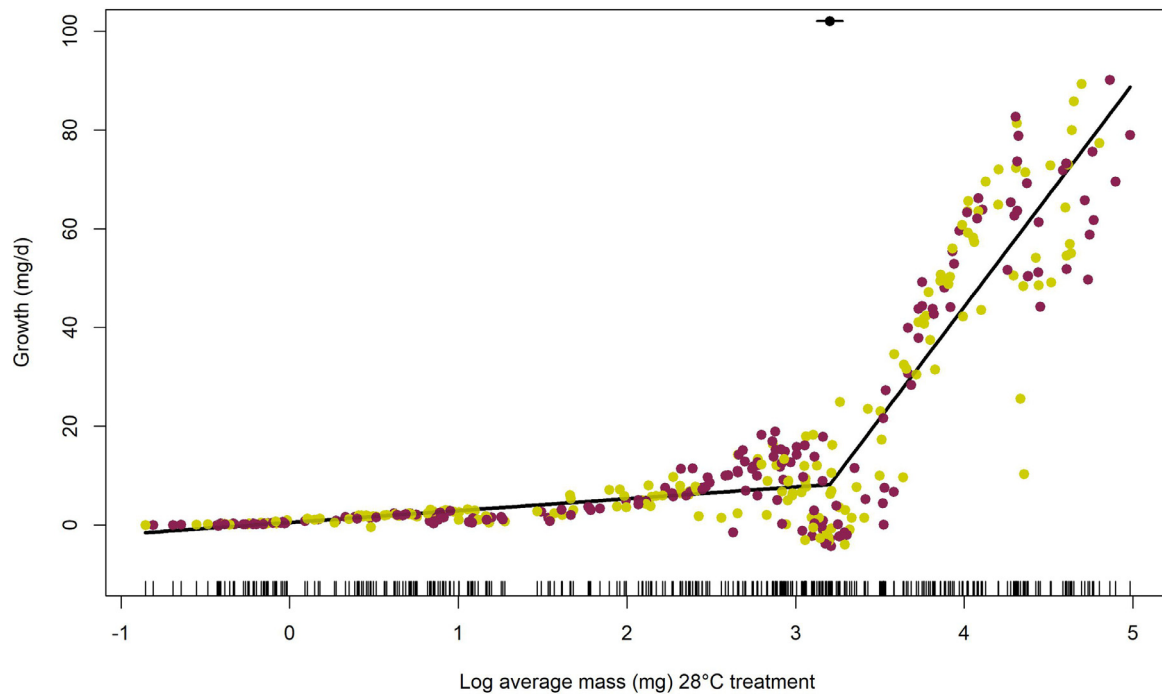
Fig. 8. Example of the non-linear growth trajectories for all the individuals in the warmest treatment, combining males (purple) and females (yellow), as well as short and long photoperiods. The growth rate varied with average mass in a non-linear manner and was better explained by a piecewise linear regression (R^2=0.88) than by a linear relationship (R^2=0.53).
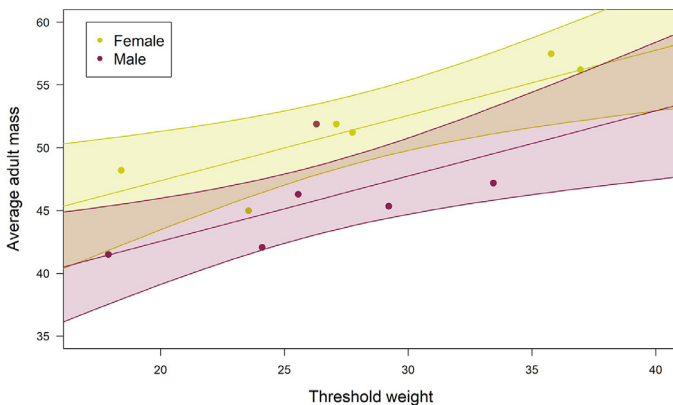
Fig. 9. The relationship between adult mass and the threshold weight in male (purple circle) and female (yellow circle) of butterflies. The lines represent the best performing model (Table 1).
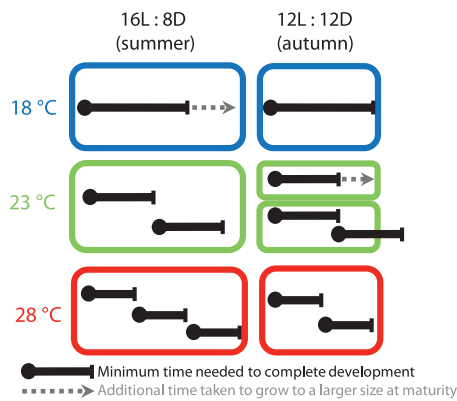
mass and males showing faster development, indicating that the need for speed and the need for mass differed between the sexes. Body size caries selective advantages for females, such as greater potential fecundity (Honěk, 1993; Nakamura, 2002; Nylin & Gotthard, 1998). Rapid development caries selective advantages for males as protandry (i.e. maturing earlier than females) enables them to maximize the number of mating (Nylin et al., 1993). Moreover, a lower male adult mass may result in lower energy requirement during flight allowing them to spend more time on mate searching and as a result gain higher mating success (Bennik et al., 2020; Kelly et al., 2008).

4.3. Fitness implications

From an ecological viewpoint, growth and development are not only tailored to ensure reaching an adequate body size, but also to ensure that the life-cycle is completed before the end of the season (Blanckenhorn & Demont, 2004; Buckley et al., 2015; Kivelä et al., 2011). Species thus need to gauge time available and respond appropriately in terms of growth and development. Above, we have seen that growth and development tend to be matched such that changes in body size are buffered, likely to maintain fitness (fecundity in females, competitiveness/ mobility in males). We hypothesized that a short photoperiod would speed up the caterpillars, accelerating development and growth, but instead found that development and growth were slower at the short photoperiod. Small coppers can complete 1-3 generations depending on how favourable their conditions are and hibernate as half grown or fully grown caterpillars. The short autumn photoperiod used in our experiment coincided with mid-September and at this time point, there could still be sufficient time for growing, reproducing and producing a new generation of caterpillars that then hibernate, but only if temperatures are sufficiently warm (Fig. 10). This could explain why we observed a clear dichotomy under a short photoperiod at 23°C: caterpillars either grew very fast and matured early at a small size (aiming at reproducing the same year) or they grew slower to a larger size (aiming to reproduce next year). No such dichotomy was observed under a long photoperiod (indicative of summer) at 23°C; the caterpillars likely aimed to reproduce at least once this year, possibly twice and they all developed fast and exhibited small threshold weight (Table 3). At 18°C the effect of photoperiod on threshold weight was reversed compared to 23°C. Likely, cold temperatures slowed growth and development such that the caterpillars at 18°C were more time constrained and exhibited smaller threshold weight than those at 23°C (Fig. 10). Caterpillars at 18°C under a long photoperiod likely aimed to reproduce only once this year and were least time constrained and exhibited the largest threshold weight. The caterpillars reared at higher temperature developed rapidly, irrespective of photoperiod. Possibly, the high temperature overruled photoperiod as an indicator for season length, or the butterflies were primed to go equally fast, aiming to reproduce twice (short photoperiod) or thrice (long photoperiod) this year (Fig. 10). In both photoperiods, reducing the development time and increasing growth rates enable them to complete an additional generation(s) before the end of the growing season. The decision to either reproduce now or to reproduce later, reflects fit-

16L : 8D (summer)   12L : 12D (autumn)

18 °C   23 °C   28 °C

Minimum time needed to complete development
Additional time taken to grow to a larger size at maturity

Fig. 10. Schematic showing putative decisions to either reproduce now or later in order to fit a discrete number of generations into a growing season. Perceived time available is shown as a rectangle and is longer in the long photoperiod. Black lines indicate the minimum amount of time needed to complete a generation and at higher temperatures less time is needed (shorter lines). When season length (perceived time available) is longer, animals may either add an additional generation, or when time is too short, extend the development period allowing them to grow larger (indicated by grey dashed arrow). Note that at the short photoperiod, indicative of autumn, we observed a dichotomy with some butterflies extending the development period, while others grew very fast, attempting to fit another generation into the growing season.

ting a discrete number of generations into a growing season (Fig. 10). Complex interactions between temperature and photoperiod were also found in the multivoltine mayfly (Cabanita & Atkinson, 2006) and voltinism appears to integrate different life-cycle regulating mechanisms to mount an adaptive response to environmental variation in temperature and photoperiod (Lindestad et al., 2020). Indeed, if the season ends before they complete development to the overwintering stage, then there will be the risk for high mortality or losing this generation (Van Dyck et al., 2015).

5. Conclusion

In summary, *Lycaena phlaeas* follows the temperature-size rule: cool conditions slow down growth and development, and adults were slightly larger. Such differences in mass could be most consistently related to treatment-differences in threshold weight. Individual differences in growth and development were also significantly related to adult mass, but differences in cell size were only weakly correlated to adult mass and could not explain the observed responses in adult size. Our results suggest that thermal responses and their modulation by photoperiod are adaptive responses aimed to fit a discrete number of generations into a growing season (fewer generations under cold temperatures, amplified by short photoperiods, and more generations under either a long photoperiod or high temperatures). Developing caterpillars thus use information on temperature and photoperiod to gauge the time needed and the time available for completing development and adjust growth and development accordingly.

Authors' contributions

Designed the study: M.S. and W.C.E.P.V.; Provision of study material: H.S.; Collected the data: M.S.; Analyzed the data: M.S. and W.C.E.P.V.; M.S. led the writing of the manuscript. All authors contributed critically to the draft and gave final approval for publication.

Data availability statement

All data files are available from the DANS EASY archive (DOI: https://doi.org/10.17026/dans-22w-ygkz).

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