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Are life-history traits equally affected by global warming? A case study combining a multi-trait approach with fine-grain climate modeling

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ABSTRACT

Predicting species responses to climate change requires tracking the variation in individual performance following exposure to warming conditions. One ecologically relevant approach consists of examining the thermal responses of a large number of traits, both related with population dynamics and trophic interactions (i.e. a multi-trait approach). Based on *in situ* climatic data and projections from climate models, we here designed two daily fluctuating thermal regimes realistically reflecting current and future conditions in Eastern France. These models detected an increase in mean temperature and in the range of daily thermal fluctuations as two local facets of global warming likely to occur in our study area by the end of this century. We then examined the responses of several fitness-related traits in caterpillars of the moth *Lobesia botrana* – including development, pupal mass, survival rates, energetic reserves, behavioral and immune traits expressed against parasitoids – to this experimental imitation of global warming. Increasing temperatures positively affected development (leading to a 31% reduction in the time needed to complete larval stage), survival rates (+19%), and movement speed as a surrogate for larval escape ability to natural enemies (+60%). Conversely, warming elicited detrimental effects on lipid reserves (−26%) and immunity (total phenoloxidase activity: −34%). These findings confirm that traits should differ in their sensitivity to global warming, underlying complex consequences for population dynamics and trophic interactions. Our study strengthens the importance of combining a multi-trait approach with the use of realistic fluctuating regimes to forecast the consequences of global warming for individuals, species and species assemblages.

1. Introduction

Temperature is undeniably one of the most important factors determining individual performance, species evolution and distribution (Angilletta, 2009; Clarke, 2003). In ectothermic animals like insects, whose body temperature and performance are closely related with environmental temperature, the link between a surrogate for individual performance (e.g. development and growth rates, reproductive output) and environmental temperature is typically modeled by an asymmetric and unimodal curve called a thermal performance curve (Martin and Huey, 2008; Sinclair et al., 2016). As temperature increases, individual performance gently rises to a single maximum value associated with a thermal optimum, and thereafter performance declines rapidly.

Thermal performance curves may help to predict how organisms will cope with changes occurring in their thermal environment, such as those related with anthropogenic climate disturbance (Deutsch et al., 2008; Estay et al., 2014; Sinclair et al., 2016). Indeed, one commonly used way to assess the biological consequences of global warming consists of experimentally tracking the variation in organism's performance following exposure to warming conditions designed to simulate future temperatures (for examples, see Bauerfeind and Fischer, 2014; Fischer et al., 2014; Klockmann et al., 2016). In this regard, it is now widely accepted that comparing fluctuating thermal regimes (instead of constant temperatures) significantly improves the ecological realism of global warming experiments, considering the daily variations of temperatures experienced by organisms in their natural settings (Bozinovic et al., 2016, 2011; Colinet et al., 2015; Paaijmans et al., 2013; Zeh et al., 2015). The existing literature suggests complex patterns of biological responses to warmer conditions because of the combined effects of mean temperature and thermal fluctuations on individual performance

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(Colinet et al., 2015; Estay et al., 2014; Kingsolver et al., 2015). Part of this variability in thermal responses might be explained by Jensen's inequality, which applies to non-linear functions such as those inherent to thermal performance curves (Martin and Huey, 2008). Jensen's inequality posits that thermal fluctuations around a mean temperature below the thermal optimum (i.e. in the concave part of the thermal performance curve) will enhance performance, while the reverse trend should be observed once the mean temperature of the thermal regime exceeds the thermal optimum, in the convex part of the thermal performance curve (Bozinovic et al., 2011; Foray et al., 2014; Kingsolver et al., 2015; Paaijmans et al., 2013; Terblanche et al., 2010). In a context of fluctuating thermal environments, an increase in mean temperature attributable to global warming could be expected to depress organism performance through transient or permanent exposure to stressful thermal conditions during the warmest hours of the cycle (Colinet et al., 2015; Paaijmans et al., 2013; Vasseur et al., 2014). This is especially true if the increase in mean temperature is accompanied with a widening of thermal fluctuations (Paaijmans et al., 2013; Terblanche et al., 2010; Vasseur et al., 2014). Indeed, high levels of temperature variance have been shown to decrease an organism's ability to tolerate a thermal stress by limiting the expression of plastic responses and reducing the possibility for appropriate acclimation process to take place (Terblanche et al., 2010).

Responses to warming conditions might, however, greatly vary depending on the biological traits measured to infer individual performance, thereby highlighting the need for a multi-trait approach – involving measurements on a large number of traits – in global warming experiments (Fischer et al., 2011; Karl et al., 2011; Laughton et al., 2017). Indeed, fitness-related traits in a given species might differ regarding their thermal optima and associated thermal performance curves (Laughton et al., 2017; Seehausen et al., 2017). Experimental warming might thus yield different effects on the expression of these traits depending on whether thermal conditions applied encompass supraoptimal temperatures for the expression of the traits considered. For instance, in the Indian meal moth *Plodia interpunctella* (Lepidoptera: Pyralidae), a rise in mean constant temperature from 20 °C to 30 °C led to an almost twofold decrease in developmental time from egg to adult, while reducing adult lifespan after mating by 32% and increasing the count of immune cells (hemocytes) in larvae hemolymph by more than 50% (Laughton et al., 2017). Furthermore, biological traits are linked to each other by energetic trade-offs and thermal conditions are known to influence resource allocation towards different functions. Convincing examples entail trade-offs between immunity and reproduction (Adamo and Lovett, 2011) as well as between immunity and heat tolerance (Fischer et al., 2011; Karl et al., 2011). Experimental studies employing a multi-trait approach usually incorporate both traits linked with population dynamics (e.g. development and growth rates, survival rates, egg production) and interspecific relationships (e.g. defensive traits such as defensive behaviors and immunity) (Iltis et al., 2018; Karl et al., 2011; Laughton et al., 2017). Indeed, the former help to predict the future population dynamics of the focal species, but species will not respond in isolation to climate disturbance as they are tightly connected by ecological linkages, more specifically trophic interactions (Jeffs and Lewis, 2013; Van der Putten et al., 2010). Integrating defensive traits involved in interspecific relationships – such as host-parasitoid associations – is thus crucial to provide significant insight into how a given species might respond to global warming with respect to its associated ecological context (Iltis et al., 2018). In spite of a fairly robust literature about the effects of thermal fluctuations on performance in a climate change perspective, still few studies have to date associated the ecological realism provided by the use of realistic fluctuating regimes with the multi-trait approach, measuring different traits related with both population dynamics and species interaction (for examples, see Fischer et al., 2014; Klockmann et al., 2016). Consequently, little is known about the effects of realistic daily thermal cycles on the expression of a broad series of traits within the framework of global warming. Filling this knowledge gap could improve our mechanistic understanding of the responses of traits, individuals and populations to facets of climate change.

In this context, we here explored the consequences of global warming simulated at a local scale for several aspects of an insect's performance. To realistically simulate global warming under controlled conditions, we built two daily fluctuating regimes: one current regime according to local climate observations and one future regime according to the fine-grain predictions provided by climate models in our focal area (Eastern France). We focused on caterpillars of the European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae), one of the most severe pests for wine-making activity in Palearctic region (Thiéry et al., 2018). We adopted a global view of the insect's performance encompassing a large number of fitness-related traits, such as development time, pupal mass, survival rates, and the storage patterns of four energetic compounds. In a wider ecological context, we also focused on the abilities of the caterpillars to survive in agroecosystems when faced with natural enemies, especially parasitoids, by examining larval defensive strategies that involve behavioral defenses (aimed at avoiding parasitoid oviposition) and immune defenses (aimed at killing parasitoid eggs) (Iltis et al., 2018; Vogelweith et al., 2014).

We hypothesized that biological traits included in this study should respond in different ways to our simulation of future climates that might be experienced in the same location. Given its tight dependence on environmental temperature as a pure physiological rate (Rebaudo and Rabhi, 2018), we expected larval development to be greatly reduced by warming. The thermal response of larval development should in turn determine the time allowed for feeding in this capital breeding species (i.e. only caterpillars feed), thereby negatively impacting pupal mass and energetic reserves. We expected defensive behaviors to display more complex patterns of responses to warming, as the thermal modulation of insect behavior is not purely metabolic, that is, does not only arise from short-term variation in metabolic rates or long-term variation in individual physiological state (e.g. amounts and allocation of energetic reserves) ('kinetic effects', Abram et al., 2017). It also involves intentional behavioral adjustments in response to the perception and the integration of thermal information received by thermosensory organs ('integrated effects', Abram et al., 2017). All of these possible effects were included in our experimental design which sought to investigate the overall impacts of warming on the expression of defensive behaviors to infer the future ability of caterpillars to fend off parasitoids. Finally, we postulated that immunity should be negatively affected by high temperatures because immune function in *L. botrana* caterpillars is known to peak at a relatively low thermal optimum (22 °C or less) (Iltis et al., 2018).

2. Material and methods

2.1. Thermal regimes

We compared the performance of *L. botrana* caterpillars experiencing two daily fluctuating thermal regimes representative of current and future conditions occurring in Burgundy, Eastern France (Longvic-Dijon weather station, 47,27°N; 5,09°E; altitude = 219 m) (www.meteofrance.com). We focused on the summer period extending from 15th July to 15th August because this one-month period is particularly favorable for *L. botrana*, as indicated by one peak of activity (adult moths emergence) over this date range (Barnay et al., 2001; Martín-Vertedor et al., 2010). A curve of current thermal conditions was designed according to a 20-year recording of *in situ* hourly temperatures (from 1995 to 2014). Based on this curve, we built a mean daily cycle divided into six segments, each lasting 4 h and calculated as the mean segment over the 30 days (15th July–15th August) and the 20-year period studied (Table 1). Hereafter, this thermal regime will be referred to as the current regime.

Alongside the current regime, we built the future regime simulating

Table 1
Temperature conditions (°C) for the two daily fluctuating regimes used in this study: the current regime and the future regime, each divided into 6 segments of 4 h. Daily thermal range is defined as the difference between maximum and minimum daily temperatures.

	Current regime	Future regime
0 h–4 h	16.4	21.4
4 h–8 h	17.8	22.9
8 h–12 h	22.3	27.8
12 h–16 h	24.9	30.5
16 h–20 h	22.9	28.3
20 h–0 h	19.0	24.2
Mean temperature	20.5	25.8
Daily thermal range	8.5	9.1

the local conditions that might be found in Burgundy by the end of this century (2081–2100), during the summer period extending from 15th July to 15th August. We selected the most pessimistic of the Representative Concentration Pathways scenarios, called RCP 8.5, which predicts the highest positive radiative forcing (i.e. a high absorbance of solar and infrared radiation) associated with massive and growing emissions of greenhouse gas (www.drias-climat.fr). This scenario was combined with six simulations of high-resolution mesh (8 km) applied to the closest point to Dijon (Burgundy). Each simulation was obtained by downscaling a General Circulation Model (GCM) with a Regional Climate Model (RCM) to increase the spatial accuracy of climate predictions. Both GCMs and RCMs are climate tools that enable to follow the evolution of a climate system over time, at different spatial scales: global scale for GCMs, regional or local scale for RCMs nested within GCMs (for more details about the use of climate models in ecology, see Beaumont et al., 2008; Ziter et al., 2012). The six combinations of GCM/RCM used were: CERFACS-CNRM/RCA4, ICHEC-EC-EARTH/RCA4, MPI-ESM-LR/CCLM4, MPI-ESM-LR/REMO019, MPI-ESM-LR/RCA4, MetEir-ECEARTH/RACMO22E. These combinations have been recently used to generate simulations of climate change at small spatial scales across Europe (Alfieri et al., 2015; Dosio, 2016; Kotlarski et al., 2014). We used GCMs originating from the Coupled Model Intercomparison Project Phase 5 (CMIP5), a recent multi-model ensemble widely used to produce climate projections in the context of anthropogenic climate change (Taylor et al., 2012). All the RCMs used to generate downscaled simulations came from the Coordinated Regional Climate Downscaling Experiment across Europe (EURO-CORDEX) and were adapted to downscale the outputs of the GCMs used (Dosio, 2016; Jacob et al., 2014). All data are available on the Drias portal (www.drias-climat.fr). For each simulation, daily maximum and minimum air temperatures were extracted. We then generated six future mean daily cycles (one for each simulation) based on simulated maximum and minimum temperatures and the present daily cycle. The future regime, as the current regime, was composed of six segments of 4 h, each calculated as the mean segment over the six simulations performed (Table 1). Comparison between current and future regimes reveals that global warming should increase both the mean temperature (+5.3 °C) and the daily thermal range (+0.6 °C) in our focal area (Table 1).

2.2. Insect stock and larvae collection

We used larvae originating from an inbred and diapause free strain of *L. botrana* maintained at the French National Institute for Agricultural Research (INRA, Villenave d'Ornon, France) for several years under controlled conditions. This stock consists of a large number of caged adults (several thousand per week), to which wild individuals were regularly added to preserve genetic diversity. Hence, larvae coming from this artificial stock display similar patterns of defensive behaviors and basal immunity compared with wild individuals collected in French vineyards (Vogelweith et al., 2014). Moths were reared under standard conditions (20 ± 0.5 °C, 60 ± 5% relative humidity, photoperiod of L17: D6 and 1 h of dusk). Luminosity was 600 lx during photophase and 100 lx at dusk. Two bands of waxed paper were provided as a support for oviposition. Every 2 days, these papers were renewed and transferred to a plastic box covered by a moist piece of paper towel to avoid egg desiccation until hatching.

A total of 2246 freshly-hatched larvae (aged < 24 h) were collected throughout the experiments with a fine brush and individually reared in Eppendorf tubes filled with 1.5 ml of artificial medium (composition for 1000 ml: 1000 ml water, 15 g agar, 86.6 g maize flour, 41.3 g wheat germ, 45.5 g beer yeast, 6 g ascorbic acid, 3.4 g mineral salt, 0.32 g Scala®, 2.7 g benzoic acid, 2.8 g Nipagin® and 5 ml 95% ethanol) (Thiéry and Moreau, 2005). Neonate caterpillars were isolated in tubes so that no larval competition could occur and introduce an experimental bias (Thiéry et al., 2014). Tube caps were pierced and covered by a piece of fine mesh fabric to allow air circulation while preventing larvae from escaping. Individuals were randomly assigned to two incubators (ST 2/2 BASIC, Pol-Eko Aparatura), each simulating one of the two thermal regimes described above (temperature ± 0.1 °C, 50 ± 10% relative humidity, L18: D6, 650 lx). In order to avoid a potential incubator effect, larvae and their associated thermal regime were switched between climate chambers two times a week, as well as the location of individuals inside the new incubator. Temperatures within the climate chambers were tracked weekly with an independent data logger (Hobo, Onset Computer Corporation), and did not deviate from the thermal regimes programmed during the whole course of the experiments. Larval development was surveyed when caterpillars were swapped between incubators (i.e. two times a week) to minimize disturbance. Once they reached their fifth – and final – instar stage, larvae were randomly assigned to one of three experimental groups all along the experiments, because the different measurements of biological traits involved in this study cannot be taken on a single individual. We focused on the fifth instar because the energetic resources accumulated during the whole larval stage can be quantified at this late larval instar that directly precedes pupation. In addition, measurements of behavioral and immune defenses against parasitoids are classically performed at the end of larval development in this species (Iltis et al., 2018; Vogelweith et al., 2014). In Group 1, the fifth instar larvae were left until pupation in order to measure development time, pupal mass, survival from first instar larva to adult stage, and sex ratio. In Group 2, the fifth instar larvae were frozen and stored to quantify later their energy budgets. In Group 3, the behavioral and immune traits involved in defense of fifth instar caterpillars against larval parasitoids were measured.

2.3. Group 1: development time, pupal mass, survival and sex ratio

A total of 2043 freshly hatched larvae were initially assigned to Group 1 in order to develop until pupation (n = 981 individuals for current regime, n = 1062 individuals for future regime). We recorded the time (in days) elapsed between hatching and pupation (i.e. the development time of the larval stage), which corresponds to the time during which caterpillars are able to feed. The pupae were collected carefully with fine forceps and weighed (± 0.1 mg) with a balance (Pioneer PA214C, OHAUS). Development time and pupal mass were only recorded for individuals that reached the adult stage to discriminate the results between males and females, which were sexed upon emergence. We also calculated survival (number of emerged adults relative to the number of freshly hatched larvae initially assigned to each thermal regime), and sex ratio (expressed as the proportion of males in the adult population).

2.4. Group 2: energetic assessments

These measurements initially involved 59 fifth instar larvae (n = 29

for current regime, $n = 30$ for future regime). Caterpillars were cleaned with a fine brush to remove any residual of artificial medium from the surface of their body, transferred to Eppendorf tubes, frozen in liquid nitrogen and kept at −80 °C for latter quantification of their energy budgets. They were subsequently weighed to obtain fresh body mass (± 0.01 mg) with a microbalance (Quintix 35–15, Sartorius), before being crushed with stainless steel beads (tubes shaken for 90 s at 25 Hz) in 180 µl of aqueous lysis buffer solution (composition: 100 mM KH_2PO_4, 1 mM dithiothreitol DTT, 1 mM ethylenediaminetetraacetic EDTA, pH = 7.4). Biochemical assays were run on four metabolites potentially involved in energy production: proteins, lipids, soluble carbohydrates and glycogen. The total amount of soluble proteins available was estimated using a DC Protein Assay kit (Bio-Rad). We used the spectrophotometry method described by Foray et al. (2012) for the quantification of lipids, soluble carbohydrates and glycogen.

Crushed samples were placed on ice for 30 min to ensure proteins solubilization, centrifuged (4000*g*, 5 min, 4 °C) and 5 µl of supernatant from each sample were pipetted to microplate wells together with 35 µl of lysis buffer to obtain diluted samples. Particular attention was paid to avoid collecting lipids (which often form a thin surface layer on the top of the sample) when pipetting the 5 µl of supernatant for protein assays, because lipids might disturb the absorbance readings in the Bradford approach used to quantify proteins (Foray et al., 2012). Then, 5 µl of these diluted samples were transferred to new microplate wells and supplemented with 225 µl of Bradford micro-assay reagent (DC Protein Assay Kit, Bio-Rad). Reaction was allowed to proceed for 15 min at room temperature, after which absorbance readings were taken at 750 nm. Protein concentration in the samples was estimated with a standard curve based on a dilution-series of bovine gamma globulin standard (Pierce, Thermo Fisher Scientific) dissolved in lysis buffer. This dilution-series was constituted by the following protein concentration values: 0, 0.062, 0.125, 0.25, 0.5, 1 and 2 $mg.ml^{-1}$. Finally, 5 µl of lysis buffer, 20 µl of 20% sodium sulphate solution and 1.5 ml of chloroform–methanol mixture (1:2 v/v) were added to each crushed sample to solubilize total lipids and water-soluble carbohydrates. These samples were then conserved at room temperature for 24 h before biochemical assays of the amounts of lipids, soluble carbohydrates and glycogen.

At the end of this 24 h duration, homogenates were centrifuged at room temperature (4800*g*, 6 min). Centrifugation enabled to isolate lipids and soluble carbohydrates (supernatant) from glycogen (pellet). Determination of lipid body amount was performed with a colorimetric method involving vanillin reagent (Alfa Aesar, Thermo Fischer Scientific). For each centrifuged sample, 75 µl of supernatant were transferred to an Eppendorf tube and heated at 90 °C until complete solvent evaporation. Then, each tube was supplemented with 40 µl of 95–98% sulphuric acid and incubated at 90 °C for 10 min. For lipids revelation, 960 µl of vanillin solution (composition: 100 ml ultrapure water, 400 ml 85% orthophosphoric acid, 600 mg vanillin) were then deposited in each tube. After vigorous vortexing, 200 µl of each tube were pipetted to microplate wells for absorbance measurements, which were taken at 525 nm. The dilution-series used to assess lipid concentration in the samples involved glyceryl trioleate as a standard (Sigma-Aldrich) dissolved in chloroform, and was comprised by the following concentration values: 0, 2.5, 5, 10, 20, 40 and 80 $\mu g.ml^{-1}$. The body amounts of both soluble carbohydrates and glycogen were determined with a colorimetric method based on anthrone reagent (Alfa Aesar, Thermo Fisher Scientific). For soluble carbohydrates, 750 µl of supernatant from each centrifuged sample were collected and deposited in an Eppendorf tube, before being allowed to fully evaporate at 95 °C. For glycogen, the remaining supernatant in the centrifuged samples was removed and the pellet was left at room temperature during 30 min for complete solvent evaporation. Then, 2 ml of anthrone solution (composition: 150 ml ultrapure water, 380 ml 95–98% sulfuric acid, 750 mg anthrone) were added to each tube (containing either soluble carbohydrates or glycogen), and all these tubes were heated at 95 °C for 17 min. Tubes were subsequently vortexed and 200 µl of each were withdrawn and deposited in microplate wells before readings of optical density, which were performed at 625 nm. The concentration of these two energetic compounds in the samples was estimated through comparison with a dilution-series constituted by the following concentration values of glucose (Sigma-Aldrich): 0, 6.25, 12.5, 25, 50, 100, 200 and 400 $\mu g.ml^{-1}$.

For proteins and lipids, two replicates were performed and individuals for which the coefficient of variation exceeded 30% were excluded from further analysis. Repeatability was relatively high, as indicated by the low percentages of individuals removed from the protein (8.5%) and the lipid datasets (5.1%). At the end of this selection procedure, we retained 54 larvae from the protein dataset ($n = 27$ for current regime, $n = 27$ for future regime) and 56 larvae from the lipid dataset ($n = 29$ for current regime, $n = 27$ for future regime).

2.5. Group 3: behavioral and immune defenses

A total of 144 fifth instar larvae were assigned to measurements of defensive traits ($n = 73$ individuals for current regime, $n = 71$ individuals for future regime). Three successive behaviors were quantified, always in the same order ('flee', 'twisting' and 'dropping'), in accordance with the sequence involved in defense against larval parasitoids in natural conditions (Vogelweith et al., 2014). Additionally, these behavioral tests were performed at an ambient temperature strictly identical to the mean temperature of the regime considered: 20.5 °C if the tested individual was reared in current regime, or 25.8 °C if it was reared in future regime (Table 1). Temperature in the testing rooms was controlled with Hobo data loggers. After the completion of each behavioral test, 20 min of rest were given to each larva in a clean Eppendorf tube drilled to allow air circulation before the next test started.

The first behavior studied, called 'flee', refers to larvae escape ability by moving away from a generic threat without leaving the bunch they feed on. Here, we estimated the locomotor activity (movement speed) of caterpillars as a proxy of 'flee' behavior, to infer their ability to evade parasitoids in natural conditions. For this purpose, caterpillars were installed on a plastic squared sheet (84 × 116 cm, squares size: 1 × 1 cm) and acclimated for 30 s under the lid of a Petri dish. The lid was then removed and each larva was observed to count the total number of lines crossed during 60 s (the minimum duration required by a larva to exit the sheet). As this behavior occurs before physical contact between the caterpillar and the parasitoid, no simulation of a parasitoid attack was needed to cause the larvae to flee. Locomotor activity was naturally induced by the stress associated with the manipulation of caterpillars with a fine paintbrush during the extraction from their rearing tube, and their exposure to a new environment during behavioral tests.

The second behavior, 'twisting', consists of multiple and rapid torsions in response to contact between a larva's body and a parasitoid sting. We mimicked a parasitoid attack by touching the dorsal part of the caterpillar with a fine brush, after 30 s of acclimation under the lid of a Petri dish. Each individual was touched four times in 1 min. The entire sequence was video recorded (HDR-CX220E, Sony) and further analyzed in slow motion with Kinovea 0.8.15 software to score the total number of torsions over the four touches.

Finally, we examined 'dropping' behavior, which describes how caterpillars faced with a foraging parasitoid can drop and weave a silk thread to return to their living patch once the parasitoid has left it. Larvae were installed at the top of a 150 cm high bracket. After an acclimation of 30 s under a reversed Eppendorf tube, each larva was touched with a fine brush (as described in the previous paragraph) until it fell. For caterpillars weaving a thread, we measured its length with a ruler (precision ± 3 mm) after 5 s of stabilization. Thread length is supposed to be positively linked to larval escape ability (Vogelweith et al., 2014).

Once the three behavioral tests were carried out, larvae were returned to their rearing tube and associated thermal regime for 3 h before the immune measurements were taken. The ability of the larvae to kill parasitoid eggs was estimated by measuring hemocyte concentration and activity of the phenoloxidase (PO). Hemocytes are immune cells that freely circulate in hemolymph of insects and bind to parasitoid eggs. Hemocytes release the PO enzyme that catalyzes melanization reactions occurring around parasitoid eggs as part of the immune response (Lavine and Strand, 2002). Caterpillars were not challenged prior to these immune tests, because they naturally display high levels of basal immunity and do not significantly respond to an infection (Vogelweith et al., 2014). Larvae were chilled on ice for 30 min and then photographed using a stereomicroscope at 12.5x magnification (Stemi 508, Zeiss) to measure the distance between the most distant margins of the head capsule (precision: ± 0.1 μm) as a reliable estimator of larval body size (Delbac et al., 2010). For this experimental group, we measured larval body size rather than body mass since body size is known to correlate with some of the larval defenses in this species (Iltis et al., 2018) and because caterpillars cannot be accurately weighed while alive (Muller et al., 2015). One 2 μl sample of hemolymph was then collected from a prick in the dorsal part of the abdomen with a cold sterile glass capillary (Hirschmann Laborgeräte, Eberstadt). This sample was diluted in 20 μl of cold phosphate-buffered saline (PBS, 8.74 g NaCl, 1.78 g Na_2HPO_4, 1000 ml distilled water, pH 6.5). A volume of 10 μl of this solution was immediately withdrawn to estimate hemocyte concentration with a Neubauer improved hemocytometer under a phase contrast microscope at 400x magnification (Primo Star, Zeiss). The remaining 12 μl of diluted hemolymph were supplemented with 10 μl of PBS. These samples were frozen in liquid nitrogen and stored at −80 °C for the latter estimation of PO activity.

We distinguished between functional PO naturally activated in hemolymph (PO activity) and total PO including functional PO and proenzymes (total PO activity). These measurements were based on the spectrophotometry method described by Vogelweith et al. (2013). Hemolymph samples were allowed to thaw very slowly on ice and centrifuged (4000*g*, 15 min, 4 °C) before 5 μl of supernatant were transferred to microplate wells. These samples were supplemented with either 160 μl of diluted PBS solution (35 ml ultrapure water, 5 ml filtered PBS) for PO activity measurement, or with 160 μl of chymotrypsin solution (35 ml ultrapure water, 5 ml filtered PBS, 2.45 mg trypsin) for total PO activity measurement. Finally, 20 μl of L-Dopa solution (40 ml ultrapure water, 160 mg L-Dopamine) were deposited in each well as a colorimetric substrate. An enzymatic reaction proceeded for 40 min at 30 °C in a microplate reader (Versamax, Molecular Devices), during which the readings were taken every 15 s at 490 nm. Data were subsequently analyzed with the SOFT-Max Pro 4.0 software. We recorded maximum enzyme activity (V_{max}), corresponding to the slope of the absorbance curve during its linear phase. All immune assessments were reported to 1 μl of pure hemolymph.

2.6. Statistical analyses

Development time and pupal mass were normally distributed and displayed homogenous variances (homoscedasticity) between thermal regimes and sexes. Consequently, they were analyzed by means of two-way Analyses of Variance (ANOVAs) integrating thermal regime, sex and their interaction. Survival and sex ratio were expressed in proportions and therefore compared among thermal regimes with Generalized Linear Models (GLMs)-binomial error. To control for allometric effects on larval physiological and behavioral traits, we included either larval body mass (for energetic reserves) or larval body size (for defensive behaviors and immunity) as a covariate in the statistical models, alongside thermal regime as an explanatory variable. Most of the data were normally distributed (the reserves of the four energetic compounds, hemocyte concentration, total PO activity) or required a logarithm transformation to satisfy normality assumption (thread length and PO activity), and their variances were homogenous between thermal regimes. Accordingly, all these data were studied with analyses of covariance (ANCOVAs) testing for homogeneity of slopes and intercepts, as these models integrated the effects of the explanatory variable (thermal regime), the associated covariate (larval body mass or larval body size) and their interaction. As overdispersed count data, the number of lines caterpillars crossed and the number of twists were analyzed with GLM-negative binomial error. Normality and homoscedasticity were assessed with a Shapiro-Wilk and a Levene test, respectively. For count data, overdispersion (observed variance greater than the theoretical one predicted by the fitted model) was detected with a postregression likelihood ratio test (Cameron and Trivedi, 1990). All statistical analyses were carried out using R 3.5.2 software.

3. Results

3.1. Group 1: development time, pupal mass, survival and sex ratio

Neither development time (two-way ANOVA: $F_{1,832} = 0.04$, $P = 0.84$) nor pupal mass (two-way ANOVA: $F_{1,832} = 0.39$, $P = 0.53$) was impacted by the interaction between thermal regime and sex. Development time was modulated by thermal regime ($F_{1,832} = 639$, $P < 0.001$) and sex ($F_{1,832} = 26.5$, $P < 0.001$) in an additive way: individuals reared in future, warmer conditions needed less time to complete their larval development than those reared in current, cooler conditions, and females developed slower than males (Table 2). Pupal mass was affected by sex ($F_{1,832} = 1308$, $P < 0.001$) but not by thermal regime ($F_{1,832} = 0.67$, $P = 0.41$), as females being significantly heavier than males (Table 2). Survival was influenced by thermal regime (GLM-binomial error: $\chi^2_1 = 10.6$, $P < 0.001$): larvae reared in warmer conditions were more prone to survive until the adult stage than those held in cooler conditions (Table 2). Adult sex ratio did not differ between thermal regimes (GLM-binomial error: $\chi^2_1 = 0.02$, $P = 0.88$) (Table 2).

3.2. Group 2: energetic assessments

None of the four energetic compounds was affected by the interaction between thermal regime and larval body mass (Table 3). Among these compounds, only the body amount of lipids was influenced by thermal regime, while the amounts of proteins, soluble carbohydrates and glycogen remained the same across thermal regimes (Table 3, Fig. 1A–D). Caterpillars reared in warmer conditions stocked less lipid reserves at the end of their larval development relative to their counterparts reared in cooler conditions (Fig. 1B). Protein and lipid body amounts were both affected positively by larval body mass (Table 3, Fig. 1A, B): heaviest larvae accumulated more reserves than lightest ones (proteins: slope = 44.1; lipids: slope = 32.1).

Table 2
Mean values and 95% confidence intervals of development time (time between egg hatching and pupation), pupal mass, survival (from larval to adult stage), and sex ratio (proportion of males in the adult population) for the two thermal regimes. Different letters indicate significant differences for a given trait ($P < 0.05$).

		Current regime	Future regime
Development time (days)	Males	24.6 [24.0; 25.2]^a	16.9 [16.4. 17.3]^b
	Females	26.3 [25.5; 27.0]^c	18.4 [17.8; 19.0]^d
Pupal mass (mg)	Males	9.00 [8.9; 9.2]^a	9.00 [8.9; 9.1]^a
	Females	12.8 [12.5; 13.1]^b	12.7 [12.4; 12.9]^b
Survival (%)		43.5 [38.4; 48.6]^a	48.4 [43.9; 52.9]^b
Sex ratio (%)		59.3 [54.2; 64.4]	58.5 [54.0; 63.0]

Table 3
Effects of thermal regime, larval body mass and their interaction on the total body amounts of the four energetic compounds. All data were studied with analyses of covariance testing for homogeneity of slopes and intercepts. Bold values indicate significant effects ($P < 0.05$).

	Thermal regime		Larval body mass		Regime * Body mass	
	Test value	*P*	*Test value*	*P*	*Test value*	*P*
Proteins	$F_{1,50} = 1.72$	0.20	$\mathbf{F_{1,50} = 50.0}$	**< 0.001**	$F_{1,50} = 0.001$	0.98
Lipids	$\mathbf{F_{1,52} = 5.69}$	**0.02**	$\mathbf{F_{1,52} = 5.34}$	**0.02**	$F_{1,52} = 1.63$	0.21
Soluble carbohydrates	$F_{1,55} = 0.03$	0.86	$F_{1,55} = 1.50$	0.23	$F_{1,55} = 1.64$	0.20
Glycogen	$F_{1,55} = 2.36$	0.13	$F_{1,55} = 1.98$	0.16	$F_{1,55} = 0.52$	0.47

3.3. Group 3: behavioral and immune defenses

None of the behavioral or immune traits was impacted by the interaction between thermal regime and larval body size (Table 4). Among the behavioral traits examined, only the number of lines crossed by caterpillars during 'flee' tests was influenced by thermal regime: larvae reared and tested at warmer conditions crossed significantly more lines relative to individuals originating from cooler conditions (Table 4, Fig. 2A). Thermal regime did not affect 'twisting' (number of twists) and 'dropping' (length of the silk thread woven) behaviors (Table 4, Fig. 2B, C). From an immunological perspective, hemocyte concentration and PO activity were not impacted by thermal regime (Table 4, Fig. 3A, B). However, total PO activity was significantly modulated by thermal regime: larvae reared in warmer conditions displayed lower levels of total PO activity compared with those held in colder conditions (Table 4, Fig. 3C). None of the behavioral and immune traits was modulated by larval body size (Table 4).

4. Discussion

Within the framework of global warming, we here examined the responses of different fitness-related traits in caterpillars of the moth *L. botrana* to realistic daily fluctuating regimes reflecting current and future conditions in Eastern France. Many of the traits inspected were affected by our experimental imitation of climate change. Warming positively influenced caterpillars' development (leading to a 31% reduction in the time needed to complete all five larval instars), survival from first instar larva to adult stage (+19%) and larval ability to escape natural enemies through the expression of 'flee' behavior (+60%). Conversely, rising temperatures led to depletion of lipid reserves (−26%) and reduced total PO activity (−34%). Such a diversity of thermal responses among traits strengthens the importance of an integrative approach involving measurements on a wide array of traits to accurately forecast the consequences of global warming for an organism's performance, and the way it will interact with adjacent trophic levels.

Some traits related with population dynamics were positively

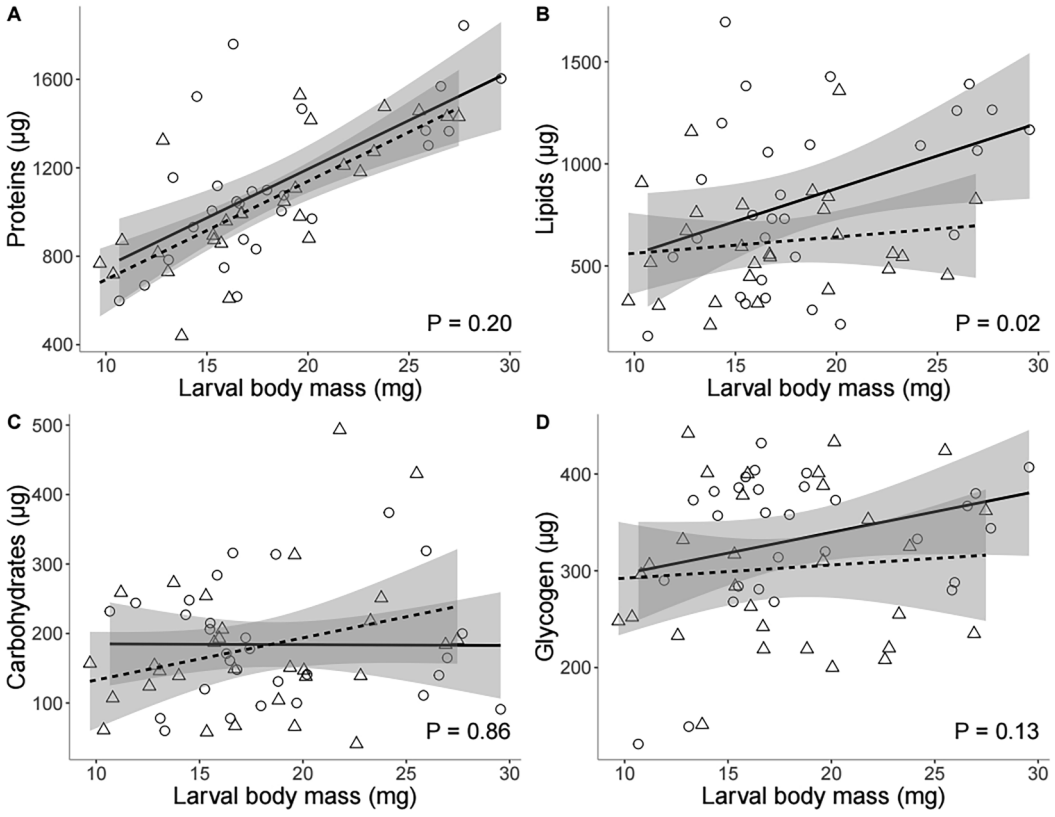

Fig. 1. Effect of thermal regime (circles and solid lines: current regime, triangles and dashed lines: future regime) in relation with larval fresh body mass on the total body amounts of proteins (A), lipids (B), soluble carbohydrates (C), and glycogen (D). Shaded area around each line corresponds to the 95% confidence interval for the predicted values of the linear regression. Reported *P* values refer to the simple effect of thermal regime on each energetic compound (testing homogeneity of intercepts between solid and dashed lines).

Table 4
Effects of thermal regime, larval body size (head capsule width) and their interaction on different defensive behaviors and immune traits involved in defenses of caterpillars against larval parasitoids. Bold values indicate significant effects ($P < 0.05$).

	Thermal regime		Larval body size		Regime * Body size	
	Test value	*P*	*Test value*	*P*	*Test value*	*P*
Defensive behaviors						
Number of lines[a]	χ^2_1 = **6.88**	**0.02**	χ^2_1 = 0.24	0.67	χ^2_1 = 0.50	0.54
Number of twists[a]	χ^2_1 = 0.64	0.35	χ^2_1 = 4.10^{-5}	0.99	χ^2_1 = 0.80	0.30
Thread length[b]	$F_{1,96}$ = 1.58	0.21	$F_{1,96}$ = 1.59	0.21	$F_{1,96}$ = 0.05	0.82
Immune traits						
Hemocyte concentration[b]	$F_{1,72}$ = 0.64	0.43	$F_{1,72}$ = 0.08	0.78	$F_{1,72}$ = 0.67	0.42
PO activity[b]	$F_{1,72}$ = 3.71	0.06	$F_{1,72}$ = 0.89	0.35	$F_{1,72}$ = 0.70	0.41
Total PO activity[b]	$\boldsymbol{F_{1,72}}$ = **9.66**	< **0.001**	$F_{1,72}$ = 1.28	0.26	$F_{1,72}$ = 0.39	0.53

[a] Generalized linear model-negative binomial error.
[b] Analysis of covariance.

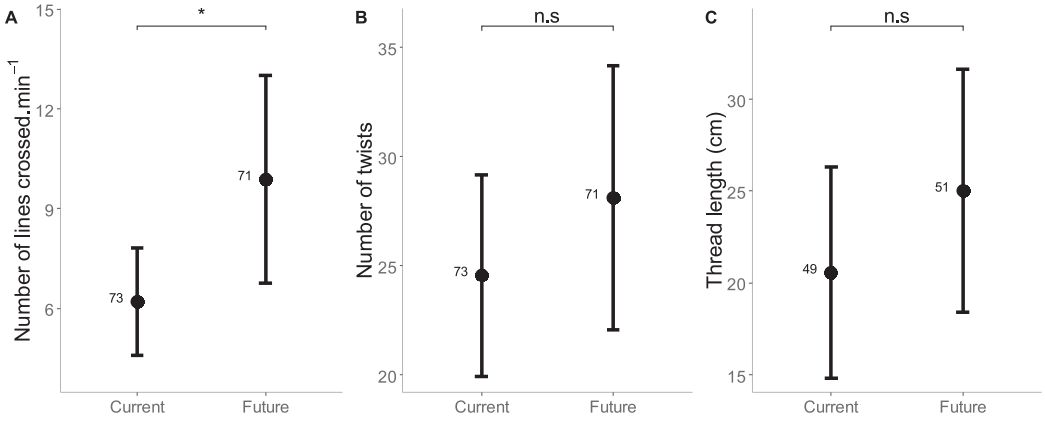

Fig. 2. Effect of thermal regime on the mean (± 95% c.i.) of the number of lines crossed by larvae during 'flee' tests (A), the number of twists over four touches (B), and the length of the silk thread spun (C). Asterisks highlight significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. non-significant) and numbers refer to sample sizes.

affected by rising temperatures, presumably indicating an overall increase in caterpillars' performance under a simulated global warming scenario. Indeed, caterpillars experiencing higher, future temperatures developed faster and showed increased survival rates compared with individuals kept under thermal conditions similar to those currently recorded in Eastern France. These findings remain in accordance with previous studies showing that the thermal optimum for the development of *L. botrana* larvae – including the laboratory strain used in this study – ranges from 28 °C to 30 °C (Briere and Pracros, 1998; Iltis et al., 2018). Hence, the experimental warming applied here (mean temperature increasing from 20.5 to 25.8 °C) positively impacted development rates by bringing environmental temperatures closer to the thermal optimum of *L. botrana*. Similarly to a previous work conducted on the same species (Thiéry and Moreau, 2005), we found that faster

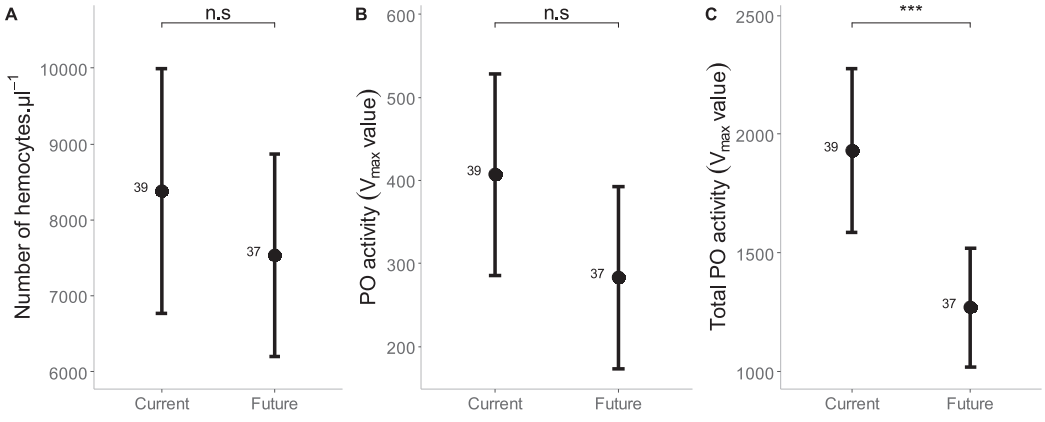

Fig. 3. Effect of thermal regime on the mean (± 95% c.i.) of hemocyte concentration (A), phenoloxidase activity (B), and total phenoloxidase activity (C). All these immune measurements were reported to 1 µl of pure hemolymph collected from fifth instar caterpillars. Asterisks highlight significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, n.s. non-significant) and numbers refer to sample sizes.

development was accompanied with higher survival rates. One plausible explanation to these results could involve a phenomenon known as 'developmental resistance', whereby disease resistance usually increases throughout larval stages in some butterfly species (McNeil et al., 2010; Shikano et al., 2018). Hence, caterpillars may benefit from a faster development under warmer conditions by spending shorter in the early ontogenetic stages, which are the most susceptible to mortality driven by pathogenic infections (Valadez-Lira et al., 2012).

As a capital breeding species, we would have expected *L. botrana* individuals to incur costs arising from a shrunk larval stage (i.e. a reduced time allowed for feeding) in warmer conditions, especially regarding pupal mass. Contrary to these initial expectations, pupal mass did not differ between thermal regimes. These results confirm that *L. botrana* caterpillars could compensate for a shortened feeding period under hotter conditions through an increase in daily gain of body mass (i.e. growth rate) (Iltis et al., 2018). The underlying mechanisms to support a high growth rate in warm conditions could involve an increased food intake rate and/or an enhanced efficiency at converting food into body matter when exposed to hotter conditions (Bauerfeind and Fischer, 2013; Lee and Roh, 2010; Lemoine et al., 2014). Nonetheless, the 26% decrease in the body amounts of lipids observed in warmer conditions seems to indicate that larvae cannot compensate for the effects of warming on energetic budgets, with costs being specifically apparent on fat reserves (the three other energetic compounds remaining unaffected by temperature). Interestingly, previous work also recorded a reduced lipid storage efficiency for fast developing caterpillars in *Spodoptera exigua* (Lepidoptera: Noctuidae), suggesting that high development rates induced by warming may be accompanied with a decline in lipid reserves (Lee and Roh, 2010). These findings indicate that the lipid energetic compartment could be particularly susceptible to the detrimental effects of a reduced feeding duration caused by an accelerated development under warmer conditions.

Aside from traits related with population dynamics, thermal conditions modulated the expression of several defensive traits involved in trophic interactions between *L. botrana* caterpillars and their natural enemies, especially the guild of parasitoids. Concerning behavioral traits, it is not possible to identify the mechanisms underlying the thermal response of 'flee' behavior at this stage. It may arise from long-term effects, whereby temperature met throughout larval development might have impacted the physiological state of caterpillars and their resource partitioning between competing defensive traits (Abram et al., 2017). It could also be attributable to short-term effects related with temperature prevailing during behavioral tests, which might have influenced caterpillars' metabolic rates and/or triggered thermosensory processes leading to behavioral adjustments following perception of heat conditions (Abram et al., 2017; Soto-Padilla et al., 2018). In terms of trophic dynamics, our findings indicate that increasing temperatures should enhance the ability of *L. botrana* caterpillars to escape from natural enemies through upregulation of 'flee' behavior. This does not, however, necessarily imply a lower larval susceptibility to parasitism, provided that the behaviors specifically expressed in case of an attempt of parasitoid oviposition ('twisting' and 'dropping') remained unaffected by thermal conditions.

Concerning immune traits, total PO activity was the only immune trait affected (negatively) by temperature, congruently with a previous work on *L. botrana* (Iltis et al., 2018) and our hypothesis that global warming could induce immunosuppressive effects in larvae of this species. Intriguingly, the decrease in total PO activity was not explained by a concomitant lowering of hemocyte concentration, which remained constant across thermal treatments in our study. Hemocyte populations are composed of different cell types (four types have been identified in larvae of the moth *Eupoecilia ambiguella*, a relative of *L. botrana*), each having a particular role in immunity (Lavine and Strand, 2002; Vogelweith et al., 2016). In this regard, we could postulate that thermal conditions might influence the relative abundances of hemocyte types in *L. botrana* larvae, tending to decrease the proportion of cells involved in synthesis of PO zymogens (e.g. oenocytoids). Our findings also indicate that, despite no effect on basal immunity (PO activity), warming could significantly reduce the ability of *L. botrana* caterpillars to respond to a parasitic infection because hotter rearing conditions decreased total PO activity. Indeed, the activation of PO cascade system is required to catalyze melanization of parasitoid eggs (González-Santoyo and Córdoba-Aguilar, 2012; Kanost and Gorman, 2008). However, it is worth noting that, in our immunological assays, immune reactions were allowed to proceed at standard temperature. Considering that, in lepidopteran species, the kinetics of PO enzyme will likely increase alongside larval body temperature up to a certain limit (Xue et al., 2006), it remains possible that larvae reared in warmer conditions invested less in the production of the active form of PO as an adaptive choice to keep the overall enzymatic activity constant across temperatures. Further insights into the functional consequences of this variation in larval immunity – for instance by measuring the *in vivo* ability of caterpillars to encapsulate and melanize foreign bodies at different temperatures – would be highly valuable to predict more thoroughly the impacts of global warming on caterpillars' survival in context of high parasitism pressure as recorded in vineyards.

5. Conclusion

In summary, our study brings new lines of evidence to suggest that multiple traits in a given species may differ in their sensitivity to global warming, underlying complex consequences for population dynamics and trophic interactions. Here, we found that, in terms of population dynamics, warming brought *L. botrana* closer to its thermal optimum for larval development. This should lead to a shorter generation time and therefore a higher future voltinism (number of generations per growing season) of *L. botrana* in Eastern France, as recently observed in meridional areas (Martín-Vertedor et al., 2010). To what extent these benefits might be mitigated by the reduction of lipid reserves observed under warmer conditions remains not well appreciated for now. A decline in lipids stock might, for instance, negatively impact the survival of the naturally diapausing generation of *L. botrana*, because fat reserves are crucial to survive extended non-feeding periods (Sinclair and Marshall, 2018). It may also persist into the adult stage and adversely impact adult dispersal ability and reproduction (Vande Velde and Van Dyck, 2013). Finally, we found that key traits involved in the trophic interactions between *L. botrana* caterpillars and their larval parasitoids were altered by warming. At this stage, predicting the consequences for future larval mortality induced by natural enemies in agroecosystems is somewhat challenging, provided that the direction of the effect of warming differed among defensive traits, and that parasitoids will also be impacted by rising temperatures, from both physiological and behavioral perspectives (Hance et al., 2007).

Several considerations should, however, be carefully examined to provide a more integrated picture of the impacts of warming for *L. botrana*, with respect to its evolutionary and ecological context. First, our study specifically investigated the plastic response occurring within one generation of *L. botrana* larvae exposed to an abrupt environmental warming. In natural conditions climate disturbance will more likely translate into a gradual shift in thermal conditions whose effects will spread across insect life stages and generations. For this reason, it is now widely acknowledged that the response of insect species to global warming will not solely be determined by intragenerational plasticity, but also by transgenerational plasticity (whereby temperatures faced by one generation will influence the expression of a phenotype in the subsequent generations) in concert with evolutionary processes (Donelson et al., 2018; Sgrò et al., 2016). It would therefore be worth investigating whether these different types of response might interact to shape the insect phenotype when experiencing gradual warming over several successive generations. Second, to what extent the mosaic of microclimatic conditions existing at the vine plant and grape bunch levels might create opportunities for larval behavioral

thermoregulation (through microsite selection) to alleviate the effects of warming must be taken into consideration. Apparently homogenous landscapes are actually composed of an important diversity of thermal conditions at much smaller, biologically relevant spatial scale matching the body size of the insect (Potter et al., 2013; Terblanche et al., 2015). Thus, variation in air temperature recorded in a given site at a macroclimatic scale may bear little relationship to the variation in the operative body temperature of the insect experiencing a set of contrasting microclimatic conditions. In vineyards, grape berry temperature displays an important variability both between grape bunches and within a single bunch, in relation with factors determining direct solar radiation (e.g. bunch exposure, shading provided by leaf canopy), bunch compactness and architecture (Haselgrove et al., 2000; Kiaeian Moosavi et al., 2018; Tello and Añez, 2017). Small scale variation in berry temperature due to sunlight exposure has been shown to deeply impact *L. botrana* performance, particularly in the early ontogenetic stages (egg hatching, larval settlement) (Kiaeian Moosavi et al., 2018). Incorporating this natural thermal complexity at microhabitat scales and the way it would be altered by a rapidly changing climatic context could therefore become a next step towards a more thorough understanding of the consequences of global warming for this insect pest.

Declaration of Competing Interest

The authors declare no conflict of interests.

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