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How to stand the heat? Post-stress nutrition and developmental stage determine insect response to a heat wave

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ABSTRACT

Organisms are increasingly confronted with intense and long-lasting heat waves. In insects, the effects of heat waves on individual performance can vary in magnitude both within (e.g. from one larval instar to another) and between life stages. However, the reasons underlying these stage-dependent effects are not fully understood. There are several lines of evidence suggesting that individual ability to withstand a heat stress depends on mechanisms based on nutrition and supporting energetically physiological stress responses. Hence, we tested the hypothesis that the efficiency of these food-based buffering mechanisms may vary between different larval instars of a phytophagous insect. Using larvae of the moth *Lobesia botrana*, we examined the importance of post-stress food quality in insect response to a non-lethal heat wave at two distinct larval instars. Three major conclusions were drawn from this work. First, heat waves induced an overall decline in larval performance (delayed development, depressed immunity). Second, food quality primarily mediated the insect's ability to respond to the heat stress: the reduction in performance following heat wave application was mostly restricted to individuals with access to low-quality food after the heat stress. Third, larval instars differed in their susceptibility to this combination of thermal and food stressors, but conclusions about the instar being the most vulnerable differed in a trait-specific manner. In a global warming context, this study may shed additional light on the combination of direct and indirect (through alteration of plant nutritional value) effects of rising temperatures on the ecology and the evolution of phytophagous insects.

1. Introduction

Ongoing climate change is recognised as a major threat imperilling biodiversity, from individual survival to ecosystem structure and functioning (Bellard et al., 2012; Parmesan, 2006; Walther et al., 2002). This complex phenomenon cannot simply be reduced to an overall rise in mean temperature, but also involves more subtle changes in temperature variability at different temporal scales (Easterling et al., 1997; IPCC, 2014; Meehl and Tebaldi, 2004). Specifically, a widely recognised feature of the predicted climate alterations for the coming century is the considerable increase in the frequency, duration and magnitude of extreme thermal events, especially heat waves (Easterling et al., 2000; Meehl and Tebaldi, 2004; Perkins et al., 2012). Although there is no consensual climatological definition of a heat wave (Meehl and Tebaldi, 2004; Smith et al., 2013), it often refers to as extended periods of exposure to abnormally high temperatures (for definitions and indexes, see Smith et al., 2013).

As ectothermic animals, insects have only a limited capacity to keep their body temperature constant in a changing thermal environment (Angilletta et al., 2010; Huey and Stevenson, 1979). Therefore, they are particularly vulnerable to the threat posed by extreme high temperatures, the effects of which span across communities, populations and individuals (González-Tokman et al., 2020; Ma et al., 2021; Stoks et al., 2017). At the community level, heat waves might affect the species

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assemblage through changes in the relative dominance of competing species and alteration of interspecific relationships (Ma et al., 2015b; Sentis et al., 2013). At the population level, they might lead to a decline in demographic rates and modification of the genetic constitution of natural populations (Chiu et al., 2015; Rodríguez-Trelles et al., 2013). Finally, at the individual level, they may unleash a wide diversity of biological responses (González-Tokman et al., 2020; Ma et al., 2021). Most of the time, this consists of an overall reduction in several traits related to individual performance, such as growth and survival of immature stages (Chiu et al., 2015; Ma et al., 2015a; Potter et al., 2011), adult lifespan and reproductive success (Zhang et al., 2015a, 2015b, 2013), immunity (Fischer et al., 2014) and the accumulation of fat reserves (Fischer et al., 2014).

Within a single insect species, individual responses to a heat wave can vary from a permanent decrease in performance to a total recovery from the physiological damage and fitness costs arising from exposure to high temperature spikes (Zhang et al., 2015a, 2015b; Zhao et al., 2019). This variability can in part be explained by the timing of when the heat wave occurs during insect ontogeny and, subsequently, the life stage affected by this heat event. Indeed, the available body of knowledge indicates ontogenetic variation in heat tolerance both within (e.g. from one larval instar to another) and between developmental stages (Chiu et al., 2015; Klockmann et al., 2017; Zhang et al., 2015a, 2015b). For instance, heat tolerance increases exponentially in larvae of the tropical butterfly *Bicyclus anynana* (Lepidoptera: Nymphalidae) as they develop and get closer to metamorphosis (Klockmann et al., 2017). In the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae), a brief larval exposure to 40 °C for a few hours caused detrimental effects on adult reproduction, and the magnitude of these negative effects was increasingly greater as the heat stress occurred at a developmental stage closer to the adult stage (Zhang et al., 2015a). The modularity of insect development could explain these stage-dependent effects of heat wave (Potter et al., 2011). This hypothesis lies on the discrepancies between insect life stages in terms of morphological (e.g. body size, morphology), physiological (e.g. accumulated resources, heat shock response) and behavioural traits (e.g. thermoregulation) to account for the ontogenetic variation in heat tolerance (Bowler and Terblanche, 2008; Klockmann et al., 2017; Ma and Ma, 2012).

The individual ability to recover from heat injuries should hinge on the post-stress acquisition of energetic resources essential for physiological stress responses to take place (Klockmann et al., 2017; Van Dievel et al., 2017; Zhang et al., 2015a). For example, feeding responses (e.g. increased food intake and growth rate) may specifically occur following exposure to heat waves and determine their consequences in terms of energy storage (fat content) and immunity (Van Dievel et al., 2017). In this regard, it is generally understood that food restriction can exacerbate the negative impacts of a heat stress on insect performance (e.g. survival, immunity, reproductive success): performance drops when food is limited while needed the most (Adamo et al., 2012; Karl et al., 2011). Nonetheless, whether or not the efficiency of such buffering mechanisms based on nutrition may differ across ontogenetic stages to account for the stage-dependency of heat wave impacts on individual performance is still an open question. One could expect the efficiency of these mechanisms to vary in a stage-dependent fashion because physiological requirements and feeding activity should be highly variable within and between developmental stages (Zhang et al., 2015a). Furthermore, postulating that recovering from heat stress through nutrition is a time-consuming process, there should be less and less time available to express an appropriate feeding response when the heat wave happens later and later during insect development.

Here, we propose an experimental study to examine the role played by nutrition in the heat stress response at different steps of larval development in a phytophagous insect. Our aim was to test the hypothesis of ontogenetic variation in the efficiency of food-based buffering mechanisms following application of a non-lethal heat wave. This was done by means of a full factorial design, enabling to quantify the fitness impacts of the heat wave relative to a control thermal treatment for different combinations of larval instar and food quality available for post-stress recovery. Indeed, we manipulated the ability of the larvae to recover from heat loads by means of feeding through the variation in the larval instar exposed to the heat stress (determining the time allowed for recovery) and the variation in the nutritional quality of the food available after the heat wave (determining the time needed to regenerate from the heat damage). We used larvae of the European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae), one of the most destructive insect pests for winemaking activities in the Palearctic region (Thiéry et al., 2018). This species is a capital breeder, meaning that its feeding activity is restricted to larvae. As such, feeding during the larval stage represents the unique opportunity to repair the physiological damage induced by exposure to a heat wave. To adopt an integrative approach of individual larval performance, we measured: (i) the body amounts of four major energetic compounds stocked before pupation (proteins, lipids, soluble carbohydrates, glycogen), and (ii) the basal levels of different key immune effectors – haemocyte load and activity of the phenoloxidase/prophenoloxidase system – in the context of host-parasitoid interactions (Carton et al., 2008; Poyet et al., 2013). Indeed, the encapsulation of parasitoid eggs is ensured by a cellular response involving the recruitment of haemocytes (immune cells of invertebrates) (Lavine and Strand, 2002). Then, parasitoid eggs are killed through a melanotic response triggered by the enzyme phenoloxidase (PO), which is produced from its inactive precursor – the prophenoloxidase (PPO) – stored in the haemocytes and the haemolymph (González-Santoyo and Córdoba-Aguilar, 2012).

We expect complex patterns of response to heat wave conditions, depending on the combination of larval instar being affected and the quality of the nutritional diet being provided after the heat wave (i.e. depending on the duration of exposure to the different diets after the heat wave). We expect young larvae (characterised by small body size, low mobility, few accumulated resources) to be the most vulnerable to the heat wave and subsequent heat-driven decline in performance. Indeed, upper lethal temperature increases with ontogeny in *L. botrana*: from 32 °C for eggs to 34 °C and 36 °C for early (first and second) and late larval instars (third to fifth), respectively (Briere and Pracros, 1998). Moreover, survival rates of *L. botrana* cohorts decrease more steeply during the first days of larval development at 30 °C by comparison with cooler conditions (20 °C), thereby indicating the high susceptibility to heat conditions of larvae at their early steps of development in this species (Moshtaghi Maleki et al., 2016). As such, their response to the heat wave should highly depend on the nutritional value of the food available post-stress, especially since they are exposed to post-stress food treatments for a relatively long duration. Specifically, the negative impacts of the heat wave on larval performance should display a lower magnitude for larvae fed with a high-quality diet after the heat wave (most likely to fully or at least partially recover from heat damage) than those provisioned with a low-quality diet. Inversely, we expect larvae closer to pupation to be the most resistant to the heat wave and to be exposed to post-stress food treatments for a relatively short duration, such that larval nutrition should not significantly modulate their response to the heat wave.

2. Materials and methods

2.1. Insect stock and rearing methods

This study was conducted using a laboratory stock of *L. botrana* maintained without diapause under standard controlled conditions (temperature: 20 ± 0.5 °C, relative humidity: 60 ± 5%, photoperiod: L17:D6 and 1 h of dusk, luminosity: 600 lx during photophase and 100 lx at dusk). All individuals used in the experiments originated from a large stock with a high density of adults (several thousands of caged moths per week) supplemented annually with wild individuals sampled in nearby vineyards (Semillon cultivar: 44.46°N; 0.32°E) to enhance the genetic

Table 1
Composition of the two larval diets used in the experiments (for a final volume of 1000 ml). Freshly collected larvae were fed with the augmented medium (AM) as a highly nutritive food until they were distributed among the two food treatments (at a larval age of 7 days or 14 days). One subset was kept on AM, and another group of larvae was transferred to depleted medium (DM) as a poorly nutritive food until the end of the experiments. The recipe of DM was elaborated by halving the quantity of plant material (maize flour and wheat germs) by comparison with AM, without further modification regarding the other ingredients.

	Augmented medium (AM)	Depleted medium (DM)
Water (ml)	**1000**	**1000**
Agar (g)	**15**	**15**
Maize flour (g)	**86.6**	**43.3**
Wheat germs (g)	**41.3**	**20.6**
Beer yeast (g)	**45.5**	**45.5**
Ascorbic acid (g)	**6**	**6**
Mineral salt (g)	**3.4**	**3.4**
Pyrimethanil (mg)	**128**	**128**
Benzoic acid (mg)	**2.7**	**2.7**
Nipagin® (mg)	**2.8**	**2.8**
95% ethanol (ml)	**5**	**5**

diversity. In this regard, it has been shown that larvae from this laboratory stock display similar patterns of investment in basal immunity and ability to respond to a bacterial infection compared with field-collected individuals (Vogelweith et al., 2014).

During a one-month period, a total of 2843 larvae were continuously collected from the insect stock. Throughout the experiments, these newly born individuals (age < 24 h) were regularly and haphazardly assigned to one of the eight experimental blocks (each defined by the combination of one thermal treatment, larval instar, and food treatment) constituting the full factorial design involved in this work (see the subsection 2.4.). Using a fine paintbrush, they were delicately transferred to microtubes filled with 1.5 ml of rearing medium for which the recipe was developed from Thiéry and Moreau (2005) (Table 1). Hereafter, this nutritional diet will be referred to as the augmented medium (AM) considering its high nutritive value for *L. botrana* larvae, since it provides higher larval and adult performance compared with semi-synthetic food derived from plant material (grapevine or alternative hosts) (Moreau et al., 2006; Muller et al., 2015; Thiéry and Moreau, 2005; Vogelweith et al., 2011). Feeding the freshly collected larvae with AM was a way to limit larval mortality and possible artificial selection. Moreover, it ensured that individuals assigned to the heat wave treatment were in good health before being subjected to the heat wave. Larvae were raised individually in microtubes to avoid larval intraspecific competition for food (Thiéry et al., 2014). Once deposited in their rearing tube, all larvae were immediately transferred to the normal thermal regime described below (see the subsection 2.2.). Thermal regimes were run with climate chambers (ST 2/2 BASIC, Pol-Eko Aparatura). Abiotic conditions inside these devices were controlled (temperature ± 0.1 °C, 50 ± 10% relative humidity, L18: D6, 650 lx). Temperatures were checked once a week with independent data loggers (Hobo, Onset Computer Corporation) to ensure they conformed to the daily thermal cycle programmed during the entire experiments.

2.2. Thermal treatment: Temperature regimes

Thermal treatment was comprised of two levels labelled as control and heat wave. As a first step, all individuals used in the experiments were placed in the normal thermal regime immediately after their initial deposition in rearing tubes. This daily fluctuating temperature regime simulated a mean summer day (from 15 July to 15 August) likely to be observed in Burgundy (Eastern France, 47.27°N; 5.09°E; altitude = 219 m) by the end of the century (2081–2100) (for more details, see Iltis et al., 2019). It was constituted by a daily thermal cycle comprised of six temperature segments (each lasting 4 h) and included a photoperiod of

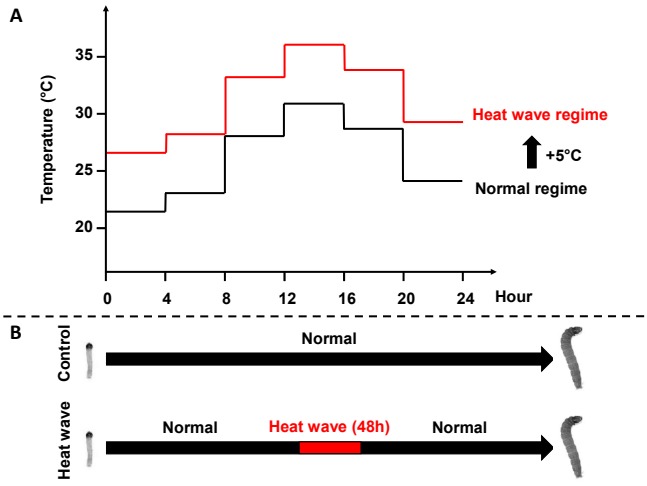

Fig. 1. Description of the thermal treatments (temperature regimes) applied. Two daily fluctuating regimes were used in the experiments: the normal regime (black) and the heat wave regime (red), built by translating the normal regime according to a constant + 5 °C increment over the six thermal segments (A). Larvae assigned to the control thermal treatment were simply kept within the normal regime during the whole course of the experiments (14 days), while those undergoing the heat wave treatment were first placed in the normal regime, then subjected at one particular larval age to a heat wave extending over 48 h, before returning to the normal regime until the experiments ended (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

L16:D8 to reproduce natural day and night durations during summertime (Fig. 1A).

Individuals assigned to the control thermal treatment were not exposed to the heat wave and therefore left developing in the normal regime until the measurements were taken. Individuals undergoing the heat wave treatment were transferred from incubators running the normal regime to incubators running the heat wave regime at a given standardised time, and maintained in the heat wave regime for 48 h precisely, after which they returned to the normal regime (Fig. 1B). The heat wave regime was built based on the normal regime with a + 5 °C increment in mean temperature (i.e. a constant increase of 5 °C over all temperature segments) (Fig. 1A). From a climatological perspective, our experimental simulation of a heat wave matches the definition provided by Tan et al. (2006), namely a period of at least one day during which the daily maximum temperature exceeds 35 °C. From an ecological perspective, the heat wave regime included temperatures exceeding by more than 5 °C the thermal optimum for development in *L. botrana* larvae (28–30 °C), thus highlighting the stressful nature of these extreme thermal conditions for *L. botrana* larvae (Gutierrez et al., 2012). By comparing larval performance under expected 'normal' and heat wave conditions associated with climate change projections, our study enables to explore the consequences of future heat events on *L. botrana* in the context of anthropogenic climate disturbance, as these heat waves are anticipated to become an increasingly important component of future climates.

Provided that *L. botrana* larvae require 17–18 days on average to complete all five larval instars within the normal regime (Iltis et al., 2019), the duration of exposure to the heat wave regime was set to two days (approximately 10% of the total larval development duration) for two main reasons. The first was to maximise the age difference between 'young' (second instar) and 'old' larvae (fourth instar) at the time of the heat wave application while avoiding temporal overlap in the exposure to the heat wave between these two larval age groups. The second reason was to avoid measurements on larvae that had initiated pupation after the occurrence of the heat wave, because *L. botrana* larvae may respond to an environmental stress by accelerating their development

and reaching earlier pupation (Vogelweith et al., 2013a).

2.3. Larval instar and food treatment: Diet compositions

Larvae were subjected to heat wave conditions at two distinct larval ages: 5 days, corresponding to the second larval instar (L2), or 10 days, corresponding to the fourth larval instar (L4). These two larval ages were selected to minimise variability in developmental stage within each group, based on available information about the relative durations of the different larval instars in *L. botrana* at different temperatures (Moshtaghi Maleki et al., 2016; Thiéry and Moreau, 2005). During the heat wave, larvae were supplied with AM as they had not been changed from rearing tube since the start of the experiments. Once the heat wave ended (i.e. 7 days after the initial deposition for L2 or 12 days after the initial deposition for L4), the larvae were gently manipulated with a fine paintbrush to be extracted from their rearing tube and placed into a new tube filled with either AM as a high nutritional quality diet or depleted medium (DM) as a low nutritional quality diet. The depleted medium recipe was designed based on a previous work (Thiéry and Moreau, 2005) and was defined by a 50% decrease in plant material by comparison with AM (Table 1). This resulted in an alteration of diet nutritional value from both quantitative (i.e. dilution effect, since less plant material was added to the same volume of medium) and qualitative perspectives (i.e. reduction in the carbohydrate-to-protein ratio, since the plant material is the main source of carbohydrates in the diet recipe). Importantly, such an alteration of diet nutritional value is known to severely depress *L. botrana* larval performance, ranking DM as a particularly poorly nutritive food for the insect (Moreau et al., 2006; Thiéry and Moreau, 2005). In order to standardise the stress associated with the manipulation, all larvae were transferred to a new rearing tube – either at 7 or 12 days – including individuals that did not change thermal regime (i.e. individuals maintained in the normal thermal regime all throughout the experiments) and those that did not experience a shift in diet quality (i.e. individuals raised on AM all throughout the experiments).

2.4. Overall experimental design

To summarise, we compared the performance of larvae continuously reared in normal thermal conditions (control thermal treatment) to larvae briefly exposed to a non-lethal heat wave (heat wave treatment), and manipulated both the stage (larval instar: L2 or L4) at which the heat wave occurred during larval development and the nutritional quality of the food available after the heat wave (food treatment: AM or DM). Hence, this work was performed using a full factorial design with eight experimental blocks resulting from the combination of three factors (thermal treatment, larval instar and food treatment) of two levels each (Fig. 2).

2.5. General procedure for measurements

All energetic and immune measurements were taken at a larval age of 14 days after initial deposition in rearing tubes, corresponding to the fifth and last larval instar in this species. For standardisation purposes, individuals displaying a developmental stage that was too advanced (larvae starting to pupate) were discarded for the subsequent measurements. As a measure of development, the pupation rate at 14 days was then calculated for each of the eight experimental blocks. It was expressed as the ratio of the number of individuals removed from the experiments following the above selection procedure to the total number of larvae alive at 14 days. Of note, larval survival up until the measurements was very high (90–96%) across the eight experimental blocks. At the time of the measurements, larvae were haphazardly distributed among two experimental groups, because the energetic and immune traits could not have been measured on the same individuals. In Group 1, larvae were flash-frozen and thereafter their energetic budgets

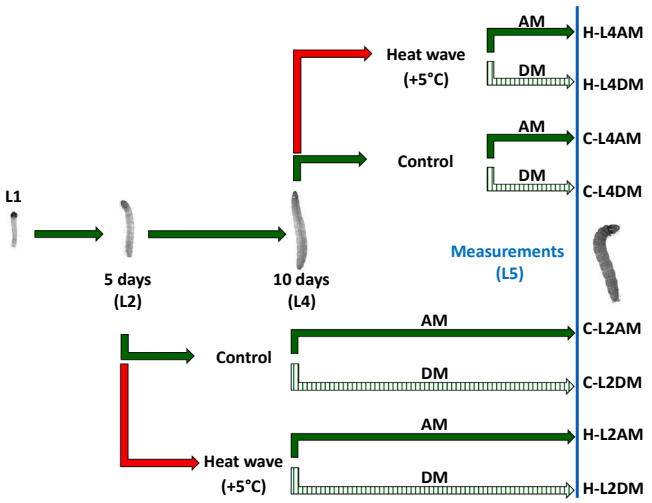

Fig. 2. General design of the experiment with the eight experimental blocks resulting from the combination of thermal treatment (control versus heat wave), larval instar (L2 versus L4) and food treatment (augmented medium versus depleted medium). First instar larvae (L1, age < 1 day) were placed in the normal thermal regime (green) and fed with augmented medium (AM, solid arrow) until they were split between the different food treatments. At a larval age of 5 days (corresponding to the second larval instar, L2) or 10 days (fourth larval instar, L4), they were either kept in the normal regime (control thermal treatment) or briefly exposed to the heat wave regime (red) for two days before returning to the normal regime (heat wave treatment). Subsequently, at a larval age of either 7 days (L2) or 12 days (L4), all individuals were placed in the normal regime, isolated in new rearing tube and either kept supplied with AM or transferred to depleted medium (DM, hatched arrow) until the measurements were taken. All measurements were carried out at a larval age of 14 days (corresponding to the fifth and last larval instar, L5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were quantified through colorimetric assays. In Group 2, haemolymph samples were collected to assess the basal levels of different immune parameters (haemocyte load and activity of the PO/PPO system).

2.6. Group 1: Energetic reserves

The energetic budgets were quantified for a subsample comprised of 327 individuals: 162 larvae assigned to the control thermal treatment ($n = 43$ for L2AM, $n = 45$ for L2DM, $n = 37$ for L4AM, $n = 37$ for L4DM) and 165 larvae assigned to the heat wave treatment ($n = 41$ for L2AM, $n = 42$ for L2DM, $n = 33$ for L4AM, $n = 49$ for L4DM). Fourteen day-old larvae were placed in empty microtubes and instantaneously frozen by immersion in liquid nitrogen. These samples were kept at −80 °C until the energetic assays were run. Larvae were weighed with a microbalance (Quintix 35–15, Sartorius) to measure fresh body mass (±0.1 mg), 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). The energetic budgets of the larvae were estimated through quantification of the body amounts of the four major energetic compartments in insects (proteins, lipids, soluble carbohydrates and glycogen) using spectrophotometric methods (for a detailed description of these methods, see Iltis et al., 2019). This procedure enables to simultaneously evaluate, in the same individual, the body levels of the four nutrients (Iltis et al., 2019). For the proteins, a Bradford approach involving a DC Protein Assay kit (Bio-Rad) was used. For the three other energetic compounds, biochemical measurements were based on the protocol developed by Foray et al. (2012), which has been successfully used to evaluate the energetic state of *L. botrana* larvae in a recent work (Iltis et al., 2019). Absorbance readings were carried out at room

temperature in a microplate reader (Versamax, Molecular Devices), with the SoftMax Pro software (version 4.0, Molecular Devices). The protein and lipid measurements were taken in two replicates, and individuals for whom the coefficient of variation calculated over these two values exceeded 30% were excluded from the experiments (Iltis et al., 2019). The measurements displayed a high repeatability overall, as suggested by the low percentages of individuals removed from the protein dataset (1/327 = 0.3%) and the lipid dataset (17/327 = 5.2%).

2.7. Group 2: Immune parameters

Haemolymph samples were taken on a subset of 314 larvae: 161 individuals assigned to the control thermal treatment ($n = 43$ for L2AM, $n = 45$ for L2DM, $n = 35$ for L4AM, $n = 38$ for L4DM), and 153 individuals assigned to the heat wave treatment ($n = 38$ for L2AM, $n = 37$ for L2DM, $n = 32$ for L4AM, $n = 46$ for L4DM). Larvae were chilled on ice for 15 min to facilitate manipulation, before being photographed using a stereomicroscope at a magnification of 12.5x (Stemi 508, Zeiss). These photographic data were subsequently analysed with the ZEN imaging software (version 2.3, Zeiss) to measure the distance between the most distant lateral sides of the head capsule (precision: ± 1 μm) as a proxy of individual body size and to control for potential allometric effects on larval immunity (Vogelweith et al., 2013b). For this experimental group, larval body size was measured rather than larval body mass because alive larvae – particularly fifth instar ones – are particularly active when manipulated and they are difficult to accurately weigh (Muller et al., 2015). Immediately following the acquisition of the photographic data, larvae were placed on a cold surface and pricked in the dorsal part of the abdomen to collect a 1 μl sample of haemolymph with a cold sterile glass capillary (Hirschmann Laborgeräte). This sample was gently mixed with 20 μl of filtered, cold phosphate-buffered saline (PBS, 10 mM, pH 7.4). A volume of 10 μl of this solution was pipetted into a Neubauer improved haemocytometer placed under a phase contrast microscope at a magnification of 400x (Primo Star, Zeiss) in order to evaluate the haemocyte load. The remaining 11 μl of diluted haemolymph samples were supplemented with 10 μl of filtered PBS, flash-frozen in liquid nitrogen and stored at −80 °C for later measurements of PO/PPO system activity.

The enzymatic activity of PO and PPO was extrapolated spectrophotometrically following the method described by Vogelweith et al. (2011). A clear distinction was made between the activity of the functional PO naturally present in the haemolymph sample (PO activity), and the potential activity of the whole PO/PPO system, i.e. the activity of both functional PO and proenzyme stock (total PO activity). This distinction allowed to assess the levels of PO activity naturally expressed by larvae at a given time alongside with the overall maximal enzymatic activity that can be potentially mobilised upon an actual infection. In addition, PO and total PO activities in *L. botrana* can exhibit different responses to a change occurring in thermal conditions, thereby providing different information about the effects of temperature on the immune function of this species (Iltis et al., 2019, 2018). The determination of the total PO activity required the transformation of PPO into PO by means of chymotrypsin addition. Frozen samples were put on ice until completely thawed and were centrifuged (4000 *g*, 15 min, 4 °C) before 2 × 5 μl of supernatant were pipetted into microplate wells. One of these two aliquots was mixed with 160 μl of diluted PBS solution (35 ml ultrapure water, 5 ml filtered PBS) to measure the PO activity, while the other was supplemented with 160 μl of chymotrypsin solution (35 ml ultrapure water, 5 ml filtered PBS, 2.45 mg trypsin) to measure the total PO activity. Finally, 20 μl of L-Dopa solution (40 ml ultrapure water, 160 mg L-Dopamine) was deposited in each well as a colorimetric substrate. The enzymatic reaction was allowed to proceed at 30 °C for 40 min in a microplate reader (Versamax, Molecular Devices). Absorbance readings were taken every 15 s at 490 nm. Absorbance curves were subsequently analysed with the SoftMax Pro software (version 4.0, Molecular Devices) to extract the maximum enzymatic activity V_{max} (the maximum speed of conversion of L-Dopamine into dopachrome). This value was calculated as the maximum slope of the absorbance curve during the starting linear phase of the reaction. All immune measurements were recalculated in order to refer to 1 μl of pure haemolymph.

2.8. Statistical analyses

Since our aim was to quantify the impacts of the heat wave relative to the control thermal treatment taking into consideration both the time left to recover from heat damage (determined by the timing of heat wave application during ontogeny) and the nutritional value of the food provided post-stress, we merged the larval instar and food treatment into one variable with four modalities (L2AM, L2DM, L4AM, or L4DM) for the statistical analyses. This allowed us to test whether the duration of exposure to the different diets after the heat stress is a key determinant of the larval ability to stand the heat, as initially postulated. Besides, larval stage and food treatment are interrelated as they both

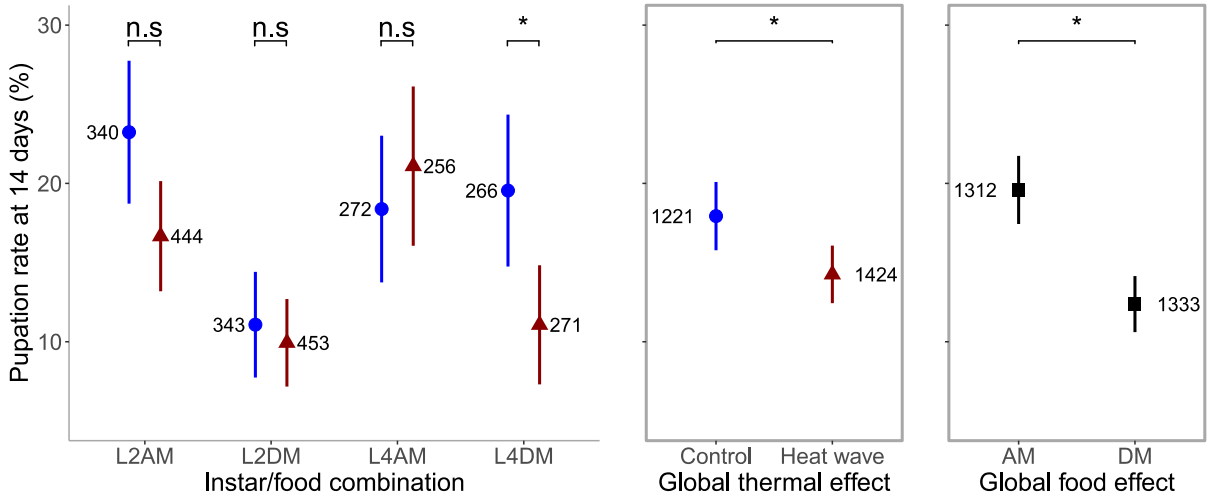

Fig. 3. Responses of the pupation rate at 14 days to the thermal treatment (blue circles: control, red triangles: heat wave) and the instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change), to the global effect of the thermal treatment alone, and to the global effect of the food treatment alone. The represented values are the means ± 95% c.i. Asterisks indicate a significant difference (Cohen's *h* significantly different from zero), n.s. stands for non-significant and the numbers refer to the sample sizes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

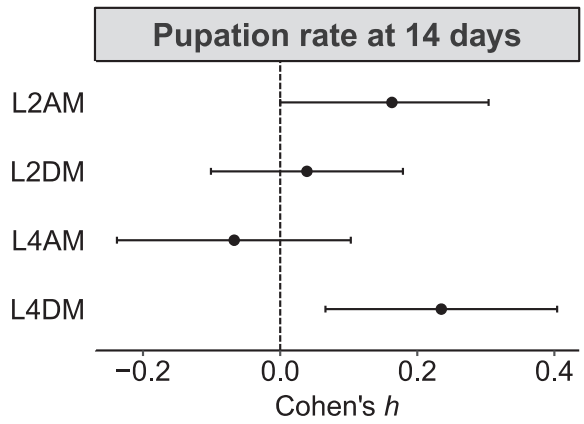

Fig. 4. Cohen's *h* and their 95% bootstrapped confidence intervals (10,000 iterations) reflecting the effect size of the thermal treatment (control versus heat wave) on pupation rate at 14 days for each of the four modalities of instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change). Effect size is considered to be significantly different from zero when the associated confidence interval does not bracket the zero value.

define the different experimental conditions (i.e. duration of exposure to the different diets) faced by larvae after the heat event. Therefore, in all the statistical tests carried out, the models involved the simple effects of thermal treatment (control or heat wave), the combination of larval instar and food treatment, and the interaction between thermal treatment and instar/food combination. In addition, one covariate was included in the models to control for potential allometric effects on energetic reserves (larval body mass) or immune parameters (larval body size).

The pupation rate at 14 days was compared among groups using a Generalised Linear Model (GLM) with a binomial error structure coupled with a logit link function. The body amounts of the four energetic compounds (proteins, lipids, soluble carbohydrates and glycogen) were normally distributed and satisfied the assumption of homoscedasticity (homogeneity of variances) among thermal treatments and pairs of larval instar and food treatment. Hence, all these data were studied with analyses of covariance (ANCOVAs). As overdispersed count data, haemocyte load was processed using a GLM with negative binomial error structure and a log link function. Data for PO and total PO activities were not normally distributed and were best fitted by GLMs with Gamma error structure and a log link function. To tease out significant differences among the groups, Cohen's *d* (or Cohen's *h* in case of data expressed in percentages: pupation rate at 14 days) with their bootstrapped confidence intervals ($CI_{95\%}$, 10,000 iterations) were calculated and reported (Nakagawa and Cuthill, 2007). Congruently with our initial goal, these standardised measures of effect size enabled us (i) to quantify the overall effect of the heat wave on larval performance and (ii) to assess how the timing of when this hot event occurred (larval instar) combined with the quality of the food available for recovery (nutritional diet) might influence the importance of the heat wave effect. All statistical analyses were carried out using the R software (version 4.0.3), implemented with the following packages: *ggplot2*, *ggsignif*, *cowplot* (for drawing figures), *stats* and *CRAN* (for model analyses).

3. Results

3.1. Pupation rate at 14 days

The pupation rate at 14 days was impacted by the thermal treatment (GLM binomial error structure: $\chi^2_1 = 6.62$, $P = 0.01$) and the combination of larval instar and food treatment ($\chi^2_3 = 31.9$, $P < 0.001$), but not by the interaction between thermal treatment and instar/food combination ($\chi^2_3 = 7.74$, $P = 0.05$). The simple effect of thermal treatment indicates that the heat wave delayed the development of *L. botrana* larvae: the pupation rate at 14 days after egg hatching was higher overall for individuals constantly held in the normal regime than for individuals that experienced the heat wave (Fig. 3) (Cohen's $h = 0.101$, $CI_{95\%} = [0.024; 0.178]$). Interestingly, this detrimental effect of the heat wave on larval development was only apparent for one modality of instar/food combination: L4DM (Figs. 3, 4). This indicates that the heat wave delayed development mostly if it occurred late during larval development and if individuals were subsequently supplied with low-quality food. The simple effect of the instar/food combination on pupation rate at 14 days was mainly driven by food treatment: individuals kept on high-quality food developed faster (displayed a higher pupation rate at 14 days after egg hatching) than those transferred to low-quality food at any larval instar (Fig. 3) (Cohen's $h = 0.197$, $CI_{95\%} = [0.121; 0.274]$).

3.2. Group 1: Energetic reserves

The body levels of the four energetic compounds displayed similar patterns of response to the different variables included in the models: they were affected by the combination of larval instar and food treatment, but not by thermal treatment or by the interaction between thermal treatment and instar/food combination (Table 2). The effect of instar/food combination on the reserves was explained by food treatment: individuals provided with high-quality medium all throughout their development possessed higher body amounts of proteins, lipids,

Table 2
Effects of thermal treatment (control or 48 h exposure to a heat wave), instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change), the interaction between these two variables and larval body mass (energetic reserves) or body size (immune parameters) on the different larval traits measured.

	Thermal treatment		Instar/food combination		Interaction		Covariate (mass or size)	
	Test value	*P*	*Test value*	*P*	*Test value*	*P*	*Test value*	*P*
Energetic reserves								
Proteins[a]	$F_{1,317} = 0.23$	0.63	**$F_{3,317} = 17.6$**	**< 0.001**	$F_{3,317} = 2.47$	0.06	**$F_{1,317} = 342$**	**< 0.001**
Lipids[a]	$F_{1,301} = 2.00$	0.16	**$F_{3,301} = 9.29$**	**< 0.001**	$F_{3,301} = 1.08$	0.36	**$F_{1,301} = 197$**	**< 0.001**
Soluble carbohydrates[a]	$F_{1,318} = 1.83$	0.18	**$F_{3,318} = 7.75$**	**< 0.001**	$F_{3,318} = 0.63$	0.60	**$F_{1,318} = 78.6$**	**< 0.001**
Glycogen[a]	$F_{1,318} = 0.01$	0.93	**$F_{3,318} = 21.8$**	**< 0.001**	$F_{3,318} = 2.66$	0.05	**$F_{1,318} = 307$**	**< 0.001**
Immune parameters								
Haemocyte load[b]	**$\chi^2_1 = 4.24$**	**0.04**	$\chi^2_3 = 4.49$	0.21	$\chi^2_3 = 4.65$	0.20	$\chi^2_1 = 1.76$	0.18
PO activity[c]	**$\chi^2_1 = 4.82$**	**0.01**	$\chi^2_3 = 0.69$	0.81	$\chi^2_3 = 1.46$	0.57	$\chi^2_1 = 1.13$	0.21
Total PO activity[c]	**$\chi^2_1 = 4.37$**	**< 0.001**	$\chi^2_3 = 2.28$	0.13	$\chi^2_3 = 0.38$	0.81	**$\chi^2_1 = 2.99$**	**0.01**

[a] ANCOVA.
[b] GLM with negative binomial error structure.
[c] GLM with Gamma error structure.

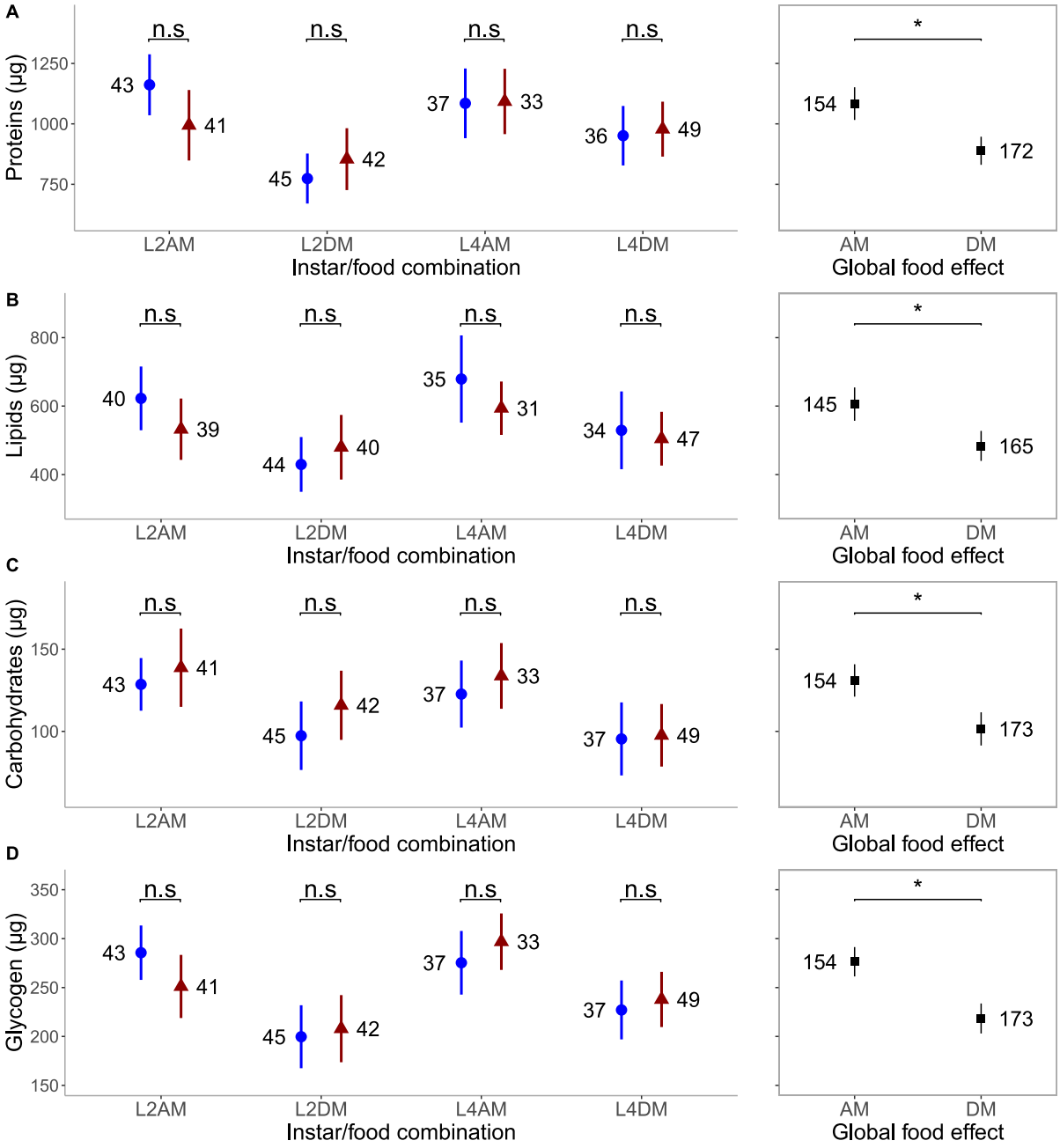

Fig. 5. Responses of the total body amounts of proteins (A), lipids (B), soluble carbohydrates (C) and glycogen (D) to the thermal treatment (blue circles: control, red triangles: heat wave) and instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change), and to the global effect of the food treatment alone. The represented values are the means ± 95% c.i. Asterisks indicate a significant difference (Cohen's *d* significantly different from zero), n.s. stands for non-significant and the numbers refer to the sample sizes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

soluble carbohydrates and glycogen than individuals fed with low-quality food at any larval instar (Fig. 5A–D). Cohen's *d* values remain in accordance with these statistical conclusions drawn when comparing high-quality versus low-quality food treatments (proteins: $d = 0.484$, $CI_{95\%} = [0.267; 0.713]$; lipids: $d = 0.421$, $CI_{95\%} = [0.203; 0.649]$; soluble carbohydrates: $d = 0.456$, $CI_{95\%} = [0.234; 0.692]$; glycogen: $d = 0.590$, $CI_{95\%} = [0.370; 0.820]$). Measures of effect size did not indicate any difference in energetic reserves between the control and heat wave treatments for any of the four modalities of instar/food combination (Fig. 6). For all the energetic compounds examined, the body amounts were positively influenced by larval body mass: heavier larvae possessed higher reserves than lighter ones (Table 2) (slope values ± s.e.m., proteins: 67.2 ± 3.6; lipids: 42.2 ± 3.0; soluble carbohydrates: 6.8 ± 0.8; glycogen: 16.0 ± 0.9).

3.3. Group 2: Immune parameters

The three immune parameters inspected were all impacted by thermal treatment in the same way, but not by the combination of larval instar and food treatment or by the interaction between thermal treatment and instar/food combination (Table 2). Overall, the heat wave decreased larval basal immunity, as shown by the higher haemocyte load, PO and total PO activities for individuals continuously raised in the normal regime compared with individuals that experienced the heat

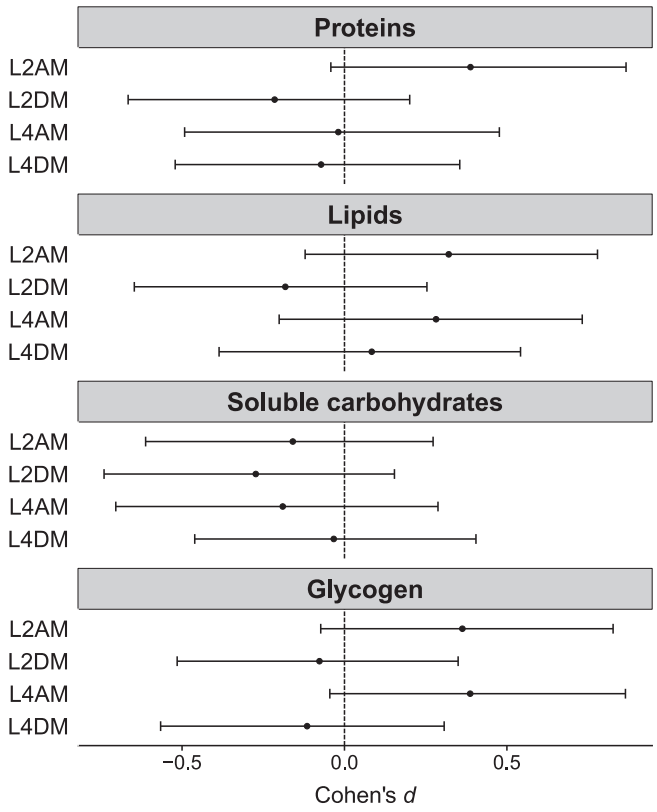

Fig. 6. Cohen's *d* and their 95% bootstrapped confidence intervals (10,000 iterations) reflecting the effect size of the thermal treatment (control versus heat wave) on the energetic reserves for each of the four modalities of instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change). Effect size is considered to be significantly different from zero when the associated confidence interval does not bracket the zero value.

wave (Fig. 7A–C). These conclusions are supported by measures of effect size calculated when comparing control versus heat wave treatments (haemocyte load: $d = 0.213$, $CI_{95\%} = [0.002; 0.435]$; PO activity: $d = 0.290$, $CI_{95\%} = [0.068; 0.511]$; total PO activity: $d = 0.366$, $CI_{95\%} = [0.144; 0.594]$). Among the different modalities of instar/food combination, measures of effect size for the thermal treatment reveal complex patterns of immunological response to the heat wave (Fig. 8). For haemocyte load, the heat wave decreased basal immunity only if it occurred early during larval development and if individuals were fed with low-quality food after the heat stress (L2DM) (Fig. 8). For PO activity, the negative effect of the heat wave was detectible only if the heat wave happened early during larval development and if individuals were maintained on high-quality food after the heat stress (L2AM) (Fig. 8). For total PO activity, the heat wave exerted adverse effects on immunity only if individuals were subsequently provided with low-quality food, irrespective of the timing of the heat event during larval development (L2DM and L4DM) (Fig. 8). Finally, total PO activity was the only immune effector affected (positively) by larval body size: the larger the larvae, the higher the basal levels of total PO activity (slope ± s.e.m.: 0.002 ± 0.0005).

4. Discussion

Three major hierarchical conclusions can be drawn from this work, from the broadest to the most specific. First, the non-lethal heat wave applied was detrimental overall to *L. botrana* larvae, as witnessed by its pervasive negative effects on development and immunity. Second, for almost all fitness correlates impacted by the heat wave (except PO activity), the negative effect of the hot event on larval performance was only detected when larvae were exposed to low-quality food (DM) following the heat wave. However, no similar decline in performance was observed for individuals kept on high-quality food (AM), probably due to a complete recovery from the heat stress. This indicates that food quality played an important role in determining the consequences of the heat wave for larval performance (mostly development and immunity), thereby providing experimental support to the hypothesis that larval nutrition could mediate the insect response to heat wave episodes encountered during development, but with no consistent pattern across life stages. Third, clear evidence was indeed found that larval instars differed in their sensitivity to the combined thermal and food stresses above mentioned, as the negative fitness impacts of the heat wave followed by low-quality food were in most cases restricted to one larval instar only. Interestingly, conclusions regarding the larval instar being the most susceptible were highly trait-dependent: the heat wave delayed development only if it affected old larvae, while for immunity, the impacts of the heat wave were mostly detected for young larvae (haemocyte load and PO activity). Hence, contrary to our initial expectations, we did not find obvious experimental support for the assumption that larval nutrition should be more significant in the event of an early heat wave, affecting larvae at the very start of their development. In a rapidly changing climatic context, this study should contribute to increase our mechanistic understanding of how individuals and species could cope with temporary extreme heat events from physiological, ecological and evolutionary perspectives.

Climate projections for the coming century suggest that living organisms will face the challenge of more and more frequent, intense and increasingly longer periods of abnormally high temperatures (IPCC, 2014; Meehl and Tebaldi, 2004). In our study, we found that a 48-h exposure to a realistic heat wave elicited extensive and detrimental effects on several aspects of larval performance in the moth *L. botrana*. On one hand, the heat wave delayed larval development overall, as evidenced by a lower pupation rate at 14 days for individuals briefly subjected to a heat wave relative to those continuously reared in the normal regime. In insect species, an extended development appears to be a common pattern of individual response to a heat wave, and presumably results from the physiological damage sustained during periods of exposure to temperatures above the thermal optimum for developmental function (i.e. the temperature at which development is the fastest) (Chiu et al., 2015; Gillespie et al., 2012; Jeffs and Leather, 2014). Here, *L. botrana* larvae that experienced the heat wave regime spent 12 h every day at supraoptimal temperatures, provided that the thermal optimum for development in this species lies between 28 and 30 °C, at least for laboratory-reared strains (Briere and Pracros, 1998). This temporary exposure to stressful thermal conditions for two consecutive days could account for the differences in pupation rate at 14 days observed between the heat wave and control treatments. On the other hand, we found that larval immunity, including all three immune parameters examined, was depressed by the heat wave. This may arise from direct effects of temperature, whereby transient exposure to high temperature spikes might have compromised immune function in this species, as previously evidenced in case of a rise in mean temperature within rearing conditions (Iltis et al., 2019, 2018). These immune differences might also be explained by indirect effects of temperature, mediated by the development rates of individuals. As stated above, individuals facing the heat wave displayed a delayed development, which may have affected their immunity at time of measurements because immune parameters are known to vary throughout a given larval instar in lepidopteran larvae (Beetz et al., 2008; Booth et al., 2015). Our results suggest that heat waves might have persistent implications for the defensive abilities of a host's vulnerable stage against its natural enemies. Such extreme thermal events could therefore predispose natural populations of *L. botrana* to infection by larval parasitoids, although the response of these natural enemies to high temperatures would also need to be considered for a deeper understanding of the consequences of heat waves at the level of

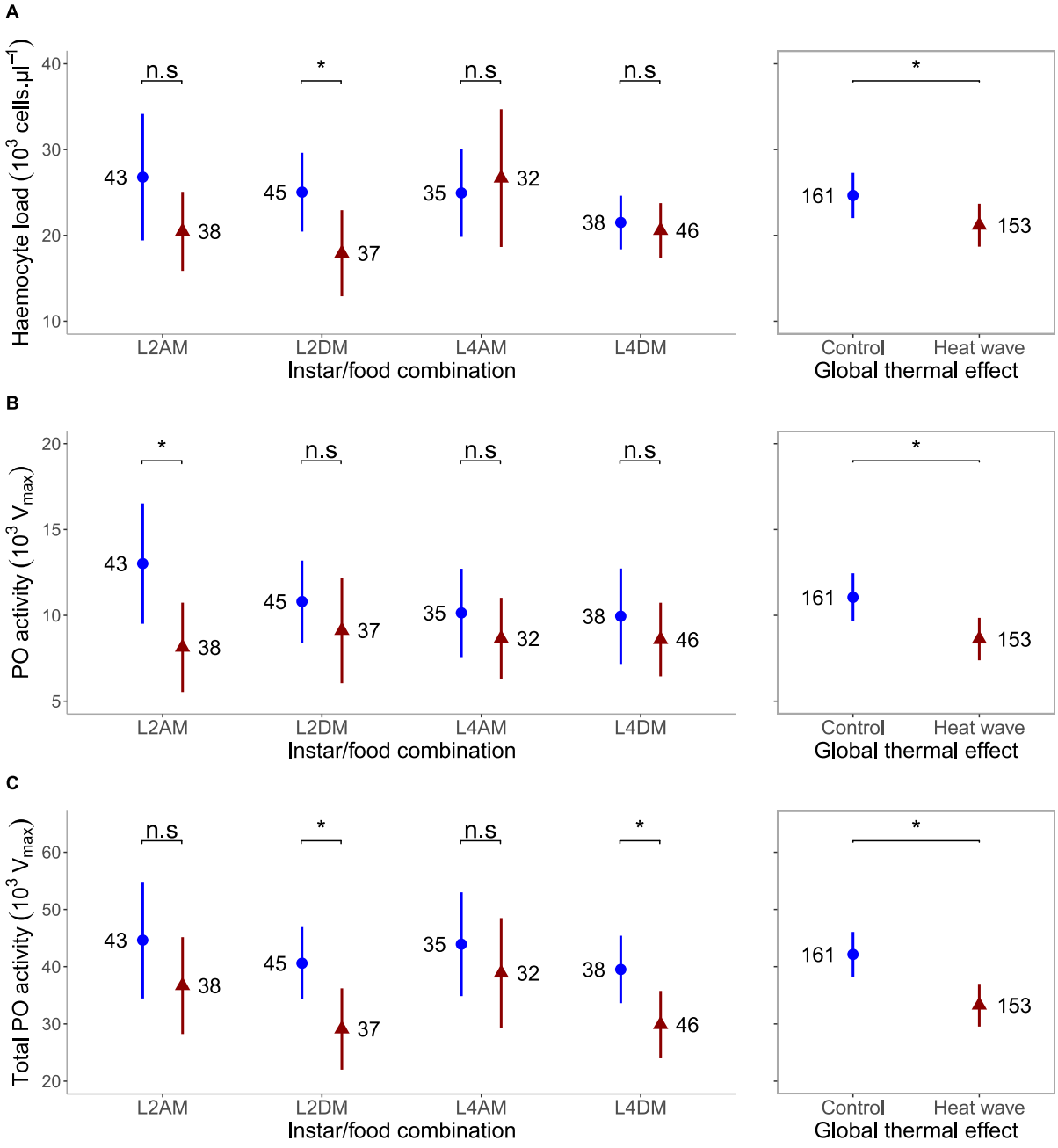

Fig. 7. Responses of haemocyte load (A), phenoloxidase activity (B) and total phenoloxidase activity (C) to the thermal treatment (blue circles: control, red triangles: heat wave) and instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change), and to the global effect of the thermal treatment alone. The represented values are the means ± 95% c.i. Asterisks indicate a significant difference (Cohen's *d* significantly different from zero), n.s. stands for non-significant and the numbers refer to the sample sizes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

interspecific interaction.

The negative effects of the heat wave on fitness-related traits were largely restricted to individuals with access to low-quality food after the heat exposure, while those maintained on high-quality medium did not exhibit a similar decline in larval performance. The importance of nutrition in modulating the thermal response of phytophagous insects is increasingly acknowledged and is thought to result from the impacts of temperature on metabolic rates and energy requirements (Clissold and Simpson, 2015; Diamond and Kingsolver, 2010; Lemoine et al., 2014; Triggs and Knell, 2012). In this regard, previous works have reported that individuals subjected to food deprivation concomitantly with a heat stress were more susceptible to the heat-related reduction of performance (Adamo et al., 2012; Karl et al., 2011). Our study is the first one to emphasise that ability of insects to cope with a temporary heat stress also hinges on the nutritional value of the food available after the heat wave. The synergism between temperature and food stresses here highlighted could result from quantitative (i.e. a lower nutrient intake when feeding upon DM because of dilution effects) and/or qualitative nutritional effects (i.e. an unbalanced nutrient intake because of the low carbohydrate-to-protein ratio of DM) (Andersen et al., 2010; Clissold and Simpson, 2015; Lee and Roh, 2010). Exploring the nutritional basis for the fitness costs incurred by heat-stressed individuals when subsequently raised on low-quality food (for instance by using several diets with different degrees of nutrient dilution and imbalance) would merit

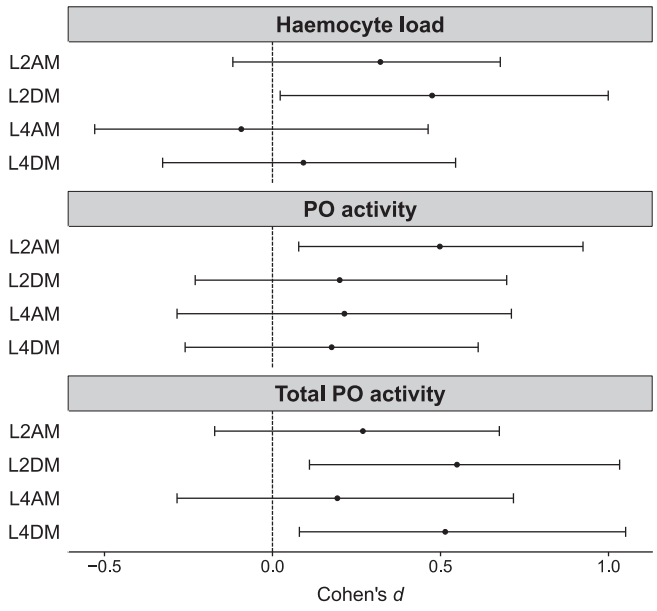

Fig. 8. Cohen's *d* and their 95% bootstrapped confidence intervals (10,000 iterations) reflecting the effect size of thermal treatment (control versus heat wave) on the immune parameters for each of the four modalities of instar/food combination (defined by the larval instar at which larvae were transferred to a new rearing tube and the nutritional quality of the food provided after the tube change). Effect size is considered to be significantly different from zero when the associated confidence interval does not bracket the zero value.

further investigations to clarify whether these effects were driven by an insufficient supply with a particular nutrient (carbohydrates, proteins, or both).

Interestingly, individuals raised on low-quality and high-quality food after the heat wave differed in their energetic budgets, the former having lower reserves compared with the latter. This means that the negative effects of the heat wave on larval immunity were mostly detectible for individuals with relatively low energetic reserves due to poor food quality, a finding that could be attributable to the supposed existence of resource-based trade-offs among immune function and stress response (Karl et al., 2011). Exposure to stressful heat conditions is known to trigger a widespread intracellular response consisting of the expression of a set of heat shock proteins, which act as protective agents actively contributing to enhance the insect's ability to withstand a thermal stress and to recover from the stress injuries (Feder and Hofmann, 1999; King and MacRae, 2015). Similar to maintaining high levels of immunity, it is assumed that the activation of a heat stress response based on the production of heat shock proteins is energy-demanding, such that trade-offs between these two biological functions should be expected in an energy shortage context (Karl et al., 2011; Krebs and Loeschcke, 1994). Under harsh environmental conditions such as those related to heat wave application, larvae constrained by a low-quality food may thus favour allocating resources to an appropriate heat shock response to enhance heat tolerance and maintain high survival rates, like those recorded in this study, at the expense of immune function. In this regard, it is worth-mentioning that PO activity did not follow a pattern of response consistent with this hypothesis of resource-based trade-offs, because it was reduced only for heat-stressed individuals fed with the most nutritive diet (AM) afterwards. We postulate that this could arise from the relatively high carbohydrate-to-protein ratio of this medium by comparison with DM, while several studies evidenced that investment in spontaneous functional PO is often rather limited by protein intake in lepidopteran larvae, especially for basal levels expressed in the absence of an immune challenge (Cotter et al., 2011; Lee et al., 2006; Ponton et al., 2013).

We also found that the heat wave impacts varied substantially among the ontogenetic stages, in a trait-specific manner. In terms of development, the adverse impacts of the heat wave were specifically detected in the case of a late exposure (involving fourth instar larvae), whilst the heat wave applied to larvae at an earlier ontogenetic stage (second instar larvae) did not significantly influence this physiological process. These findings suggest that *L. botrana* larvae may be able to fully recover from the physiological costs associated with exposure to supraoptimal temperatures if enough time has elapsed to allow this recovery to occur (even with low-quality food). Congruently with previous experimental investigations, our results for development seem to indicate that the biological impacts of heat waves can be interpreted through the lens of a subtle balance between the damage sustained during hot, stressful days and the duration of the repair periods acting as buffer against the negative consequences of heat exposure for organisms (Ma et al., 2018; Zhang et al., 2015a). With regards to immunity, the heat wave elicited extensive negative effects on the three traits examined when it occurred relatively early during larval development, whilst the heat wave applied to older larvae only reduced total PO activity. Hence, in contrast with development, the time left to express recovery after the heat wave does not appear to be a good predictor of the consequences of the heat stress in terms of immunity. Rather, these results might more likely arise from differences in susceptibility to high temperatures between the two ontogenetic stages studied (Briere and Pracros, 1998). In this regard, it could be hypothesised that larval investment in a heat stress response might vary ontogenetically, as shown by a marked variation in heat tolerance within and between developmental stages in other butterfly models (Klockmann et al., 2017; Zhang et al., 2015a, 2015b). As a result, the above mentioned trade-off between heat tolerance and immunity could be age-dependent, such that young larvae may invest the most in physiological buffering mechanisms (and, consequently, the least in immunity) to endure prevailing heat conditions because of their intrinsic vulnerability (e.g. limited ability to escape from thermal stress, small body size) to high temperatures and desiccation (Klockmann et al., 2017).

5. Conclusion

To conclude, this present work provides the first experimental evidence that post-stress nutrition (manipulated through variation in food quality after the heat wave) can mediate the effects of a heat wave for individual performance, in a stage- and trait-dependent manner. More direct measurements of larval nutrition, such as food intake and gain in body mass before and after the heat wave application, would help further support the existence of stage-dependent feeding responses potentially involved in recovery process. By stressing the importance of food quality with regards to the ability of individuals to cope with short periods of extreme temperatures, we suggest that insect pests may not necessarily benefit from global warming and a variability in the response of pest populations to climate change can be expected depending on the nutritional value of the plant tissues ingested (Adamo et al., 2012). To a certain extent, the abrupt drop in diet nutritional value experienced by larvae fed with poorly nutritive food after the heat wave could be deemed ecologically realistic. Indeed, grapevine undergoes profound physiological modifications when exposed to a heat stress, with immediate and often long-lasting consequences for berry quality including interruption of fruit maturation, impediment of sugar accumulation, and alteration of plant secondary metabolism (Greer and Weston, 2010; Liu et al., 2012). Further investigations are needed to clarify (i) the magnitude of the presumed decrease in plant nutritional value following heat wave application, (ii) whether this magnitude is strong enough to shape the insect's response to heat and (iii) the role played by local viticultural practices, especially grapevine cultivars, in the response of the plant-insect complex to heat. Lastly, another worth-investigating research avenue could lie in testing for the generality about the importance of developmental stage for the insect response to heat. This raises the question as to whether ontogenetic variation in

insect response to heat might also exist for species with overlapping development stages (e.g. heterometabolous), or if adults in non-capital breeders could further repair through feeding the heat injuries sustained during different immature stages.

CRediT authorship contribution statement

Corentin Iltis: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Philippe Louâpre:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Visualization, Funding acquisition. **Fanny Vogelweith:** Methodology, Validation, Writing - review & editing. **Denis Thiéry:** Resources, Writing - review & editing. **Jérôme Moreau:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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