



HAL
open science

Day/night variations of feeding and immune activities in larvae of the European grapevine moth, *Lobesia botrana*.

Corentin Iltis, Jérôme Moreau, Guillaume Gamb, Corentin Manière, Céline Boidin-Wichlacz, Aurélie Tasiemski, Denis Thiéry, Philippe Louâpre

► To cite this version:

Corentin Iltis, Jérôme Moreau, Guillaume Gamb, Corentin Manière, Céline Boidin-Wichlacz, et al.. Day/night variations of feeding and immune activities in larvae of the European grapevine moth, *Lobesia botrana*.. *Entomologia Generalis*, 2021, 41 (6), pp.601-614. 10.1127/entomologia/2021/1208 . hal-03475096

HAL Id: hal-03475096

<https://hal.science/hal-03475096>

Submitted on 24 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Day/night variations of feeding and immune activities in larvae of the European**
2 **grapevine moth, *Lobesia botrana***

3 **Short title: Day/night variations of basal immunity in an insect**

4 **Corentin Iltis*^{1,2}, Jérôme Moreau^{1,3}, Guillaume Gamb¹, Corentin Manière¹, Céline**
5 **Boidin-Wichlacz⁴, Aurélie Tasiemski⁴, Denis Thiéry⁵, and Philippe Louâpre¹**

6 ¹ *UMR CNRS 6282 Biogéosciences, Université Bourgogne Franche-Comté,*
7 *6 Boulevard Gabriel, 21000 Dijon, France.*

8 ² *Present address: Earth and Life Institute (ELI), Biodiversity Research Centre, Université*
9 *Catholique de Louvain, 1348 Louvain-la-Neuve, Belgium.*

10 ³ *UMR 7372 Centre d'Etudes Biologiques de Chizé, CNRS & La Rochelle Université, 79360*
11 *Villiers-en-Bois, France.*

12 ⁴ *Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL -*
13 *Center for Infection and Immunity of Lille, F-59000 Lille, France.*

14 ⁵ *UMR INRAe 1065 Santé et Agroécologie du Vignoble, Institut des Sciences de la Vigne et du*
15 *Vin, 71 Avenue Edouard Bourleaux, 33882 Villenave-d'Ornon, France.*

16

17 * Corresponding author. E-mail: corentin.iltis@gmail.com. ORCID: 0000-0002-1379-8029

18 **Abstract**

19 Daily varying intensities of exposure to infectious enemies should select for the evolution of a
20 daily structure of host immunity with a marked peak and trough (i.e. a daily rhythm). Such
21 nycthemeral variations have been documented for insect inducible immunity (responsiveness
22 to microbial challenge), while the existence of similar daily patterns in basal immunity
23 remains unexplored. Basal immunity is defined as the background, readily available
24 protection aimed at preventing infection at any time. Daily rhythmic elevation of basal
25 immunity should bear substantial costs and be performed only when facing highly predictable
26 changes in threat of infection. This could be the case for risk of food-borne infection,
27 presumably fluctuating with daily patterns of host feeding activity. This study investigated the
28 existence of day/night variations in feeding activity and basal immunity, using larvae of the
29 moth *Lobesia botrana* (Lepidoptera: Tortricidae) raised under a realistic daily cycle of light
30 and temperature conditions. At night, larvae (i) spent a greater amount of time feeding, (ii)
31 displayed a higher total phenoloxidase activity and (iii) underwent subtle changes in the
32 balance of some haemocyte types newly described for this species (increase in the relative
33 abundances of prohaemocytes and plasmatocytes, decrease in the one of oenocytoids). These
34 data provide the first evidence of nycthemeral variations of basal immunity in an insect
35 model, with peaks in some immune effectors co-occurring with maximal feeding activity
36 (during nighttime). The ecological implications and the contribution of this work to improve
37 the general understanding of the temporal structure of immunity are discussed.

38 **Keywords**

39 Basal immunity, ecological and evolutionary immunology, nycthemeral variations,
40 haemocyte types, Tortricidae.

41 **Introduction**

42 The fundamental observation that organisms do not constitutively maintain immunity at its
43 maximum levels has given rise to the field of evolutionary and ecological immunology
44 (Schmid-Hempel 2003; Schulenburg et al. 2009; Siva-Jothy et al. 2005). Its underlying theory
45 predicts that the expression of immune traits can be adjusted in a dynamic way, depending on
46 environmental factors like population density (Barnes and Siva-Jothy 2000), food availability
47 and quality (Ponton et al. 2013; Vogelweith et al. 2011), temperature (Raffel et al. 2006) or
48 parasitism threat (Schmid-Hempel and Ebert 2003; Sheldon and Verhulst 1996). Indeed,
49 resource allocation to immunity is predominantly shaped by the intensity of exposure to
50 pathogens and parasitic faunas (parasites, parasitoids), which is spatially and temporally
51 dynamic (Lindström et al. 2004; Martinez-Bakker and Helm 2015). Accordingly, spatial
52 variations in levels of immunity have been found between distinct host populations
53 experiencing geographically heterogeneous pressures exerted by these natural enemies
54 (Corby-Harris and Promislow 2008; Lindström et al. 2004; Vogelweith et al. 2013). Temporal
55 fluctuations in immunity may allow an organism to cope with recurrent variations in threat of
56 infection resulting from seasonality (seasonal scale) or natural alternance of day and night
57 conditions (daily scale) (Martin et al. 2008; Martinez-Bakker and Helm 2015; Westwood et
58 al. 2019).

59 Virtually all host species are confronted with daily rhythmic, predictable changes in
60 their abiotic (e.g. light, temperature) and biotic environment, including exposure to natural
61 enemies (Martinez-Bakker and Helm 2015; Westwood et al. 2019). For instance, most
62 parasitoid species exhibit daily fluctuations in their patterns of foraging and oviposition, such
63 that their hosts are expected to face varying parasitism pressures over the course of a day,
64 culminating during the peak of parasitoid activity (Bertram et al. 2004; Fleury et al. 2000).
65 Similarly, some parasites are able to schedule their burst of emergence in the environment at

66 specific times of the day to maximise transmission rates during a restricted time interval that
67 coincides with host activity (Lu et al. 2009). Daily rhythms in host exposure to natural
68 enemies can also arise from host activity profiles, as convincingly illustrated by food-borne
69 infections. Natural food of both animal and vegetal origins is far from being totally aseptic
70 and, instead, harbours various microbial consortia partly constituted by pathogenic bacteria,
71 fungi and viruses (De Filippis et al. 2018; Turner et al. 2013). Thus, natural food can be
72 considered as a potential source of oral infection for a host through ingestion of intestinal
73 pathogens and parasites (Vogelweith et al. 2015). Given that many host species display a
74 daily structure of food intake with defined peak and trough (rather than feeding evenly
75 throughout 24 h), it is assumed that the risk of food-borne infection should predictably
76 fluctuate over the course of a day and peak during the active phase of host feeding (Westwood
77 et al. 2019).

78 Faced with such fluctuating environmental pressures, insect hosts rely on basal and
79 inducible immunity to defend themselves against infectious enemies (Gillespie et al. 1997;
80 Kanost et al. 2004; Schmid-Hempel 2005). Basal immunity corresponds to the background,
81 readily available protection displayed by the host to prevent potential infection, that is, levels
82 of immune defences prior to a challenge. This immune maintenance is conferred by the
83 presence of effectors constitutively expressed in the insect haemolymph, such as the number
84 of haemocytes (blood immune cells of arthropods and molluscs) recruited during various
85 cellular processes and the melanisation cascade catalysed by the phenoloxidase enzyme (PO)
86 produced from inactive prophenoloxidase (PPO) (Cerenius and Söderhäll, 2004; Lavine and
87 Strand, 2002; Strand, 2008). Conversely, inducible immunity is mobilised upon an actual
88 infection and refers to the insect ability to mount an appropriate immune response to fend off
89 the multiplying intruder. As part of this immune response, the insect can upregulate the
90 expression of the immune effectors mentioned above, and synthesise *de novo* specific immune

91 agents (e.g. anti-microbial peptides) following the recognition of a pathogenic signal (Brown
92 et al. 2003; Bulet et al. 1999; Schmid-Hempel 2005).

93 The existing body of evidence – gathered so far on dipteran insect models including
94 *Drosophila melanogaster* (Drosophilidae) and *Anopheles stephensi* (Culicidae) – suggests
95 time-of-day dependent variations in inducible levels of insect immunity: the strength and
96 efficiency of the immune response expressed to combat a deadly microbial intruder (Lee and
97 Ederly 2008; Murdock et al. 2013; Stone et al. 2012). In the diurnal species *D. melanogaster*
98 for instance, the immune response to an artificial bacterial infection (phagocytic activity,
99 expression of immune-related genes coding for anti-microbial peptides) is more elevated in
100 case of challenge occurring during the resting rather than the active phase (Lee and Ederly
101 2008; Stone et al. 2012). This daily enhancement of inducible immunity is accompanied with
102 faster bacterial clearance and improved host survival upon the few hours after the infection
103 (Lee and Ederly 2008; Stone et al. 2012). Comparatively to this literature on host inducible
104 immunity, little is known about the way daily cycles may shape the insect investment in basal
105 levels of immunity, in anticipation of the risk of infection. Still, a dynamic investment in host
106 basal immunity all along the day could be expected, considering that keeping immune
107 defences at high levels of readiness constitutively should result in severe energetic losses and
108 even collateral damage for the host (immunopathology) (Schmid-Hempel 2003; Schulenburg
109 et al. 2009). For example, daily rhythms in host basal immunity could have evolved to cope
110 with periodic and highly predictable risk of encounter with enemies, as would be the case for
111 food-borne infections (Westwood et al. 2019). In this context, host exposure to enemies
112 should periodically oscillate between maximum and minimum threats of infection occurring
113 during active and resting phases of feeding, respectively (Westwood et al. 2019).

114 This study proposes to explore the day/night variations in feeding activity and basal
115 immunity (i.e. using non-experimentally challenged individuals) in an insect host. Laboratory

116 work was conducted on larvae of the grapevine moth *Lobesia botrana* (Lepidoptera:
117 Tortricidae), which have evolved a defensive strategy relying on a relatively important
118 investment in basal immunity (i.e. a high basal activity of the PO/PPO system) by comparison
119 with closely related tortricid species (Vogelweith et al. 2014). They can therefore be expected
120 to adjust the expression of these core defensive traits to contend with varying threat of
121 infection over the course of a day. On the one hand, larval behaviour was monitored to
122 quantify the day/night amount of time spent feeding as a surrogate for the intensity of
123 exposure to food-derived enemies in natural conditions. Larvae of this species can be
124 expected to display a day/night structure of feeding activity as a way to cope with
125 nycthemeral variations in the nutritional value (e.g. amounts of defensive compounds) of
126 grape berries they feed upon (Carbonell-Bejerano et al. 2014). On the other hand, this work
127 examined the day/night basal levels of several effectors involved in insect cellular and
128 humoral immunity. Focus was placed on systemic immunity (haemolymph) because immune
129 traits in the circulating compartment can be affected in case of oral infection, with
130 demonstrated consequences for the outcomes of the infection process (Freitak et al. 2014; Wu
131 et al. 2016). For cellular immunity, quantitative and qualitative measurements focused on
132 haemocyte load and the relative abundances of the different haemocyte types, respectively.
133 The haemocyte pool of insects is indeed constituted by several types of immune cells, each
134 fulfilling a specific defensive function (e.g. phagocytosis, encapsulation, PO secretion) as a
135 component of host immunocompetence (Lavigne and Strand 2002; Strand 2008; Vogelweith et
136 al. 2016). For humoral immunity, measures encompassed the activity of the PO/PPO system,
137 which controls the production of melanin and intermediate compounds with cytotoxic
138 properties to kill microscopic and macroscopic intruders (Cerenius and Söderhäll, 2004). As
139 frequently observed in other Lepidopteran species, it may be expected that *L. botrana* larvae
140 behave so they restrict their feeding activity to a specific phase, either daytime or nighttime,

141 rather than foraging at constant rates throughout the day (Goodspeed et al. 2012; Niepoth et
142 al. 2018; Suszczynska et al. 2017). Likewise, they are expected to condense their investment
143 in basal immunity to daytime or nighttime, rather than keeping these immune defences at
144 costly, high levels of readiness all along the day.

145 **Material and methods**

146 *Insect stock and rearing methods*

147 This study was performed with *L. botrana* individuals originating from a large laboratory
148 stock, comprised of several thousand of caged moths (INRAe, Villenave d'Ornon, France).
149 The genetic diversity of the stock was preserved by ensuring a yearly supply with wild
150 individuals picked from nearby vineyards. This strain has been already used in former studies
151 focused on the immunity of *L. botrana* larvae (Iltis et al. 2018; 2019; Vogelweith et al. 2014).
152 Since its establishment a few years ago and until the start of the experiments, the population
153 has been maintained without diapause on an artificial diet (see composition below) under
154 controlled rearing conditions (temperature: $20 \pm 0.5^\circ\text{C}$, relative humidity: $60 \pm 5\%$,
155 photoperiod: L17:D6 and 1 h of dusk, luminosity: 650 lux during photophase and 100 lux at
156 dusk). Such a diapause-free biological cycle is unlikely to have impacted the basal immunity
157 of this laboratory-reared population over the daily scale examined in this study, since immune
158 variations related to diapause strategies should operate on longer timescales (e.g. seasonal
159 scale). Moreover, a previous work has reported that *L. botrana* individuals belonging to the
160 laboratory stock used here display similar patterns of investment in basal and inducible
161 immunity by comparison with insects collected in field conditions during summer period
162 (Vogelweith et al. 2014). A few bands of waxed paper (5 x 10 cm) were hung inside the
163 rearing cages as suitable oviposition supports for mated females. Once covered with eggs
164 (after 2-3 days), the papers were renewed and placed in plastic boxes under a moist surface to
165 minimise egg mortality due to desiccation.

166 Within the 24 h following hatching from the eggs, neonate larvae were collected with
167 a soft paintbrush and deposited individually in microtubes containing 1.5 ml of artificial
168 culture medium (Thiéry and Moreau 2005). In order to focus on basal immunity and avoid
169 immune challenge due to pathogen ingestion, larvae were fed with a rearing medium
170 supplemented with antifungal and antibacterial substances strongly limiting microbial
171 presence in larval food (composition for 1000 ml: 1000 ml permuted water, 15 g agar, 86.6 g
172 maize flour, 41.3 g wheat germ, 45.5 g beer yeast extract, 6 g ascorbic acid, 3.4 g mineral salt,
173 128 mg pyrimethanil, 2.7 g benzoic acid, 2.8 mg methyl 4-hydroxybenzoate and 5 ml 95%
174 ethanol). Tube caps were pierced for air circulation and covered with a piece of fine mesh
175 fabric to prevent larvae from escaping. Individuals were then transferred to climate chambers
176 (ST 2/2 BASIC, Pol-Eko Aparatura, Wodzisław Śląski, Poland) to complete their
177 development until behavioural and immune measurements.

178 Within these devices, larvae were all raised under a realistic cycle of diurnally
179 changing conditions of light and temperature (temperature $\pm 0.1^{\circ}\text{C}$, relative humidity: 50 \pm
180 10%, photoperiod: L18:D6, luminosity: 650 lux during photophase). Rearing incubators were
181 programmed to run a ‘mean summer day’, defined according to recent climatic observations
182 collected between 15 July and 15 August in Burgundy (Eastern France), where *L. botrana*
183 naturally occurs (for more details, see Iltis et al. 2019). Emphasis was placed on summer
184 period because *L. botrana* is mainly active over this date range, as evidenced by one peak of
185 adult emergence observed in field conditions (Barnay et al. 2001; Martín-Vertedor et al.
186 2010). The daily cycle of temperatures met by larvae throughout their development was
187 constituted by six thermal segments of 4 h each: 17.8, 22.3, 24.9, 22.9, 19.0 and 16.4°C. To
188 reflect a natural alternance of diurnal and nocturnal conditions, lights were switched off
189 during the 19.0 and 16.4°C thermal segments. Abiotic conditions inside the incubators were
190 tracked with independent data loggers (Hobo, Onset Computer Corporation, Bourne, USA)

191 and did not deviate from the regime programmed throughout the experiments. To enable
192 simultaneous collection of daytime and nighttime data during each working day (~ 10 h),
193 larvae were randomly distributed among different incubators with shifted phases (i.e. differing
194 in the onset timing of daytime and nighttime).

195 Experiments involved individuals reaching their 4th larval instar (i.e. at a larval age of
196 about 2 weeks after hatching), the penultimate stage of larval development in this species
197 (Thiéry and Moreau 2005). This selection procedure thus enabled to exclude individuals close
198 to metamorphosis (late 5th instar larvae), which may undergo profound behavioural and
199 physiological modifications as they start to pupate and stop feeding. Larvae were randomly
200 divided among two experimental groups because each group required distinct temporal
201 organisation for measurements (see below). Group 1 was reserved for behavioural surveys
202 (time budget allocated to feeding activity). Group 2 was devoted to immune assays
203 (haemocyte load, activity of the PO/PPO system, relative abundances of different haemocyte
204 types).

205 *Group 1: behavioural measurements of larval feeding activity*

206 Temporal organisation

207 The feeding behaviour of each tested individual was measured during four specified time
208 intervals of 1 h, termed as blocks, each being associated with a given thermal segment
209 (daytime: 22.3 and 24.9°C, nighttime: 19.0 and 16.4°C) (Fig. 1). Importantly, each block was
210 disposed in the central part of its respective thermal segment of 4 h, such that measurements
211 started 1 h 30 min after the onset and ended 1 h 30 min before the offset of the thermal
212 segment (Fig. 1). This allowed to avoid any excessive abrupt change in thermal conditions
213 that could disturb larval behaviour during the tests (i.e. through a short-term larval
214 behavioural response to rapidly changing thermal conditions). Of note, this temporal

215 organisation also excluded the confounding effects due to sudden light transition between
216 daytime and nighttime (or inversely), which could also yield similar stress-related effects on
217 the expression of larval feeding behaviour.

218 Behavioural measurements of larval feeding activity

219 Behavioural trials were conducted on 47 individuals ($n = 47$ for daytime, $n = 47$ for
220 nighttime) to quantify the amount of time each spent feeding during daytime and nighttime.
221 Once they reached their 4th larval instar, larvae were gently extracted from their rearing tube
222 and individually placed in standardised compartmentalised Petri dishes (100 mm diameter x
223 15 mm height, 4 compartments of 29.4 cm³ each) covered with a thin layer of fresh rearing
224 medium (~ 10 ml per compartment). The lid was placed on the Petri dish to restrict larva's
225 movements within the boundaries of its assigned compartment and pierced to allow air
226 circulation in the different compartments. Therefore, each larva was confined to a single
227 compartment with accessible food *ad libitum* and impossibility of interacting with other
228 individuals in adjacent compartments. Petri dishes were kept within the incubators from
229 which larvae originated throughout the 24 h of survey. After an acclimation period of 15 min,
230 the 24-h sequence started to be recorded with a camera (AD7013MTL, Dino-Lite, Almere,
231 Netherlands) fixed on a tripod to keep vision on the four compartments simultaneously. All
232 the sequences were initiated during the 24.9°C thermal segment (photophase). Non-heating
233 red light lamps were placed inside the incubators to enable nighttime observations without
234 disturbing larval behaviour. Light and temperature conditions were spatially homogeneous
235 inside the climate chambers and all tested larvae were presumably exposed to the same abiotic
236 conditions during the tests. Behavioural surveys were restricted to 24 h in a way to avoid
237 larval moulting between fourth and fifth instar during the tests, which could have deeply
238 disturbed feeding behaviour. Indeed, *L. botrana* larvae require on average a few days to pass
239 through their fourth instar – especially in males which develop faster than females as larvae

240 (Thiéry and Moreau 2005) – such that extending behavioural tests to several days of
241 continuous monitoring could have resulted in a large proportion of individuals entering
242 premoult or moulting stage during the experiments. Furthermore, behavioural tests were
243 not supposed to be stress-inducing as they were conducted within the rearing incubators,
244 under the same abiotic conditions that larvae encountered throughout their development, and
245 without possibility of antagonistic interactions between larvae in adjacent compartments.
246 Hence, a relatively short acclimation period (15 min) was deemed sufficient by comparison
247 with former studies involving a 24-h adjustment time prior to behavioural measurements of
248 feeding activity as a way to allow larvae to acclimate to a sudden environmental change
249 between rearing and test conditions, which did not occur in the present work (Havko et al.
250 2020; Herden et al. 2016).

251 Once the 24 h elapsed, the videotapes were collected and further analysed with
252 DinoCapture software (version 2.0, Dino-Lite, Almere, Netherlands). The feeding behaviour
253 of each tested individual was examined during daytime (two blocks) and nighttime (two
254 blocks) (i.e. four blocks throughout the 24-h sequence). During each block, larval behaviour
255 was continuously monitored to record the amount of time spent feeding. Feeding was scored
256 in case of rhythmic head movement in a stationary position, with head lowered on the
257 medium surface, indicating biting and food ingestion (Bernays et al. 2004). Locomotion is
258 infrequent in *L. botrana* larvae since they usually complete their development within a silk
259 nest comprised of an aggregate of several floral buds or grape berries. Hence, the simple
260 distinction between feeding and resting (no obvious head movement) remains relevant with
261 regard to the biological features of the species. The larval investment in feeding was
262 calculated as the time budget (in min) allocated to this activity during each block.

263 *Group 2: immunological parameters*

264 Temporal organisation

265 By contrast with long-lasting behavioural trials, collections of immune data were
266 instantaneous, and thus required a different scheduling (Fig. 1). Immune samplings were
267 dispersed throughout daytime and nighttime to obtain an integrative picture of larval
268 immunity, representative of changes occurring in both light and temperature conditions.
269 Sampling periods excluded the first 25% of each phase (daytime or nighttime), that is, the first
270 4 h of daytime (which lasted 16 h) and the first 2 h of nighttime (which extended over 8 h)
271 (Fig. 1). As mentioned in case of behavioural trials (Group 1), this procedure enabled to avoid
272 confounding stress physiological effects possibly arising from steep changes in light
273 conditions during the transition from daytime to nighttime, or inversely. Similarly, samplings
274 were never conducted during the first 20 min of a thermal segment to ensure temperature
275 stabilisation and larval acclimation to the shift in thermal conditions occurring between two
276 successive thermal segments. For haemocyte load and activity of the PO/PPO system,
277 immune samplings were dispersed over three thermal segments of daytime (22.3, 24.9 and
278 22.9°C) and two thermal segments of nighttime (19.0 and 16.4°C). For the relative
279 abundances of haemocyte types, immune samplings occurred during one thermal segment of
280 daytime (24.9°C) and one thermal segment of nighttime (16.4°C) (Fig. 1).

281 Haemocyte load and activity of the PO/PPO system

282 Basal levels of haemocyte load and activity of the PO/PPO system were assessed on a total of
283 211 individuals ($n = 102$ for daytime, $n = 109$ for nighttime), following standardised methods
284 formerly developed (Vogelweith et al. 2011). Immune measurements were run at room
285 conditions (temperature: $20 \pm 0.5^\circ\text{C}$, relative humidity: $60 \pm 5\%$, luminosity: 650 lux). Prior
286 to the haemolymph samplings, larvae were transferred to sterile microtubes and dipped in an

287 ice-filled box for 15 min to facilitate handling, because *L. botrana* larvae are particularly
288 active when manipulated. Individuals to be tested during nighttime were maintained in
289 constant darkness until haemolymph collection by covering the box with an opaque lid.
290 Larvae were placed on a cold surface and wounded in the dorsal part of their abdomen (using
291 a sterile entomological pin) to collect a droplet of haemolymph (~ 1 µl) with a cold sterile
292 glass capillary (Hirschmann Laborgeräte, Eberstadt, Germany). Haemolymph sampling
293 usually requires no more than 10 sec, such that the abrupt shift from darkness to light
294 experienced by some individuals (in case of nighttime measurements) was unlikely to affect
295 their immunity, even on the very short term. The 1 µl volume of haemolymph was mixed with
296 20 µl of filtered, cold phosphate-buffered saline (hereafter PBS, 10 mM, pH 7.4). Tubes were
297 homogenised before 10 µl of the solution were immediately pipetted into a Neubauer
298 haemocytometer under a phase contrast microscope (Primo Star, Zeiss, Oberkochen,
299 Germany) at a magnification of 400x for a standardised estimation of the haemocyte load. The
300 remaining 11 µl of diluted haemolymph were supplemented with 10 µl of filtered PBS and
301 instantly snap-frozen in liquid nitrogen, before being stored at – 27°C for subsequent
302 measurements of PO/PPO system activity.

303 The activity of the PO/PPO system was extrapolated spectrophotometrically,
304 distinguishing the activity of the functional PO extract naturally present in the sample (i.e. PO
305 activity) from the potential activity of the whole PO/PPO system (i.e. total PO activity). This
306 latter immune trait can be defined as the activity of both spontaneously functional PO and
307 proenzyme stock, and its estimation required the cleavage of PPO into PO by means of
308 chymotrypsin addition. Frozen haemolymph samples were gently thawed on ice for 15 min
309 and then centrifuged (4000 g, 15 min, 4°C) before two supernatant aliquots of 5 µl each were
310 pipetted into microplate wells. The first aliquot was mixed with 160 µl of diluted PBS
311 solution (35 ml ultrapure water, 5 ml filtered PBS) and was reserved for measurement of PO

312 activity. The second aliquot was supplemented with 160 μ l of chymotrypsin solution (35 ml
313 ultrapure water, 5 ml filtered PBS, 2.45 mg trypsin) to quantify total PO activity. The reaction
314 started after the addition of a colorimetric substrate comprised of 20 μ l of L-Dopa solution
315 (40 ml ultrapure water, 160 mg L-Dopamine). This enzymatic reaction proceeded for 40 min
316 at 30°C in a microplate reader (Versamax, Molecular Devices, San Jose, USA). Absorbance
317 curves were fitted to regular absorbance recordings (every 15 s) at 490 nm. These curves were
318 then analysed with SOFT-Max Pro software (version 4.0, Versamax, Molecular Devices, San
319 Jose, USA) to extract the maximum enzymatic activity V_{\max} (the maximum speed of
320 conversion of L-Dopamine into dopachrome by PO), which can be approximated by the
321 maximum slope of the absorbance curve during its starting linear phase. All immune
322 measurements were finally reported to 1 μ l of pure haemolymph to enable comparisons
323 between experimental groups.

324 Description and relative abundances of different haemocyte types

325 Flow-cytometric analyses were run on 30 individuals ($n = 15$ for daytime, $n = 15$ for
326 nighttime) to discriminate the different haemocyte types present in the haemolymph and
327 assess their relative abundances. The method used to collect one droplet of haemolymph was
328 identical to the one described for measurements of haemocyte load, PO and total PO activities
329 (see above). For fixation, the haemolymph samples were flushed into 0.5 ml microtubes
330 containing 9 μ l of a 4% paraformaldehyde solution diluted with sterile filtered PBS. The
331 mixture was then gently homogenised and tubes were conserved at 4°C before analytical
332 experiments. Haemolymph samples were processed through a flow cytometer (LSR Fortessa
333 X20, Becton Dickinson, San Jose, USA). Cells were discriminated based on their
334 complexity/granularity (side scatter) and size (forward scatter), with 10,000 threshold events
335 counted per sample. Results were analysed using Kaluza software (version 2.1, Beckman

336 Coulter, Brea, USA). Relative abundances were expressed in proportions of the total cell
337 count assessed in the haemolymph sample.

338 *Statistical analyses*

339 The aim of this study was to investigate the day/night fluctuations of larval feeding activity
340 and basal immunity while accounting for the possible variability related with realistic changes
341 in temperature conditions occurring within daytime and nighttime. Hence, statistical models
342 for almost all traits (feeding activity, haemocyte load, PO and total PO activities) incorporated
343 a fixed-effect factor termed as ‘phase’ (daytime *versus* nighttime) alongside with a random-
344 effect factor termed as ‘timepoint’ and represented by the timing at which measurements were
345 taken within each of these two phases (thermal segment sampled). Data for the relative
346 abundances of haemocyte types were collected during one single thermal segment for each
347 phase and, therefore, the statistical models only encompassed the fixed effect driven by the
348 phase.

349 Data for time budget allocated to feeding activity met the assumptions required for
350 parametric tests (normality and homoscedasticity), and were therefore analysed through a
351 univariate analysis of variance (ANOVA) with mixed effects, also integrating the identity of
352 the tested individual as an additional random effect. As overdispersed count data, haemocyte
353 load was processed using a Generalised Linear Mixed Model (GLMM) with a negative
354 binomial error structure. Data for PO activity required a log-transformation to be normalised,
355 while those for total PO activity naturally complied with the assumptions of parametric tests.
356 Consequently, PO and total PO activities were studied by means of ANOVAs with mixed
357 effects. The relative abundances of the different haemocyte types were compared between
358 daytime and nighttime with Student’s *t*-tests for independent samples. Normality and
359 homoscedasticity were assessed with a Shapiro-Wilk and a Levene test, respectively,

360 conducted on the residuals of the fitted model. All statistical analyses were run using R
361 software (version 4.0.3, R Core Team).

362 **Results**

363 *Group 1: behavioural measurements of larval feeding activity*

364 The time budget devoted to feeding was significantly impacted by phase (i.e. day/night cycle)
365 (ANOVA with mixed effects, $\chi_1^2 = 5.61$, $P = 0.02$). Larvae spent a greater amount of time
366 feeding (+16%) during nighttime than daytime (Fig. 2). As a random effect, the timepoint of
367 data collection during the different phases did not affect larval feeding activity ($\chi_1^2 = 3.61$, P
368 = 0.06) (Fig. 2).

369 *Group 2: immunological parameters*

370 Haemocyte load and PO activity were not significantly modulated by phase (haemocyte load:
371 GLMM negative binomial error, $\chi_1^2 = 2.34$, $P = 0.12$; PO activity: ANOVA with mixed
372 effects, $\chi_1^2 = 1.23$, $P = 0.27$) (Fig. 3(A), 3(B)). Total PO activity was significantly impacted
373 by phase (ANOVA with mixed effects, $\chi_1^2 = 7.16$, $P = 0.007$): individuals sampled during
374 nighttime exhibited higher basal levels of total PO activity (+26%) comparatively to those
375 sampled during daytime (Fig. 3(C)). As a random effect, the timepoint of data collection
376 during the different phases had no effect on any of the three immune traits inspected
377 (haemocyte load: $\chi_1^2 = 0.03$, $P = 0.87$; PO activity: $\chi_1^2 = 0$, $P = 1$; total PO activity: $\chi_1^2 = 0$,
378 $P = 1$) (Fig. 3(A), 3(B), 3(C)).

379 Flow-cytometric analyses combined with microscopic observations led to the
380 discrimination of four haemocyte types circulating in the haemolymph of 4th instar *L. botrana*
381 larvae: prohaemocytes, oenocytoids, granulocytes and plasmatocytes (Fig. 4). Prohaemocytes
382 are the smallest cells, displaying a round shape, a low cytoplasmic granularity and a high

383 nuclear cytoplasmic ratio (N:C, i.e. ratio of the size of the nucleus to the size of the
384 cytoplasm). Oenocytoids are intermediate in size and granularity, with a centred nucleus and a
385 high N:C (4:1). Granulocytes are relatively large cells with high granularity, easily identified
386 by the inclusion of numerous granules and vesicles in the cytoplasm. Plasmatocytes are
387 among the biggest immune cells with an off-centred nucleus and a low N:C (between 1:2 and
388 1:1).

389 Interestingly, three haemocyte types were found to be significantly influenced by
390 phase: prohaemocytes (Student's *t*-test, $t_{28} = -2.26$, $P = 0.03$), oenocytoids ($t_{28} = 4.76$, $P <$
391 0.001) and plasmatocytes ($t_{28} = -4.03$, $P < 0.001$). The relative abundances of prohaemocytes
392 and plasmatocytes were higher for individuals sampled during nighttime by comparison with
393 those sampled during daytime (Fig. 5). The inverse pattern was observed for the relative
394 abundance of oenocytoids, which was higher for individuals sampled during daytime relative
395 to those sampled during nighttime (Fig. 5). By contrast with these three haemocyte types, the
396 relative abundance of granulocytes did not significantly differ between daytime and nighttime
397 ($t_{28} = -0.76$, $P = 0.45$) (Fig. 5).

398 Discussion

399 This study explored the existence of day/night fluctuations of feeding behaviour (presumably
400 influencing the dynamic of exposure to food-borne enemies in natural conditions) and basal
401 immunity in the larval stage of a non-experimentally challenged insect. In terms of behaviour,
402 results outline that *L. botrana* larvae spent a greater amount of time feeding at night, which
403 could imply that the highest risk of food-borne infection would occur during this time
404 interval. In terms of immunity, both the humoral and cellular branches were impacted by the
405 day/night cycle. Collectively, data gathered on PO and total PO activities indicate that (i) *L.*
406 *botrana* larvae maintained an amount of active enzyme (PO activity) at constant basal level
407 throughout 24 h, and (ii) they increased the inactive proenzyme stock (PPO) involved in total

408 PO activity during nighttime. Although haemocyte load remained unaffected by the day/night
409 cycle, the balance of the different types constituting the haemocyte pool was highly dynamic
410 all along the day. Out of the four haemocyte types identified through flow-cytometric
411 analyses (prohaemocytes, oenocytoids, granulocytes, plasmacytes), the relative abundances
412 of three types were found to vary between daytime and nighttime. At night, the relative
413 abundances of prohaemocytes and plasmacytes increased, whereas the one of oenocytoids
414 decreased. These data provide the first evidence of nycthemeral variations of basal immunity
415 in an insect model, leading to possible implications for the general understanding of the
416 temporal structure of insect immunity.

417 This work identified four out of the five haemocyte types recovered in Lepidoptera:
418 only spherulocytes were not discriminated owing to their rarity and morphological/cytological
419 similarity with prohaemocytes (Ribeiro and Brehélin 2006; Strand 2008; Vogelweith et al.
420 2016). However, their relative abundance can be deemed negligible comparatively to
421 prohaemocytes, which were the most abundant haemocyte type in the haemolymph samples.
422 Such a predominance remains unsurprising for immature larvae used in this study, given that
423 prohaemocyte abundance progressively declines with larval age due to differentiation in other
424 types (Ghoneim et al. 2015). The nighttime increase in the relative abundances of
425 prohaemocytes and plasmacytes could compensate for the concurrent decrease in the one of
426 oenocytoids. Indeed, the circulating stock of oenocytoids is more likely renewed through
427 differentiation of already circulating haemocytes as progenitor cells (prohaemocytes and
428 plasmacytes), rather than mitosis or release of oenocytoids from the hematopoietic organs
429 (Liu et al. 2013; Nakahara et al. 2010; Strand 2008). The nighttime drop in the relative
430 abundance of oenocytoids could be related with the concomitant increase in total PO activity,
431 since oenocytoids are labile cells functioning as the site of PPO synthesis and releasing this
432 proenzyme in the haemolymph through lysis (González-Santoyo and Córdoba-Aguilar 2012;

433 Jiang et al. 2010). This study is among the first ones to demonstrate that the composition of
434 the insect haemocyte pool in different cell types can display nycthemeral variations, in the
435 absence of immune challenge (Islam and Roy 1982). In that respect, it suggests that the
436 mechanisms causing these variations (e.g. haemocyte differentiation, migration between
437 compartments) can operate rather quickly (on a daily scale).

438 In an ecological context, this nighttime elevation of several immune parameters could
439 translate into a higher ability of *L. botrana* larvae to combat a potential infection during
440 nocturnal time. The nighttime production of PPO (increase in total PO activity) might lead to
441 a more efficient melanisation response to an infection. Indeed, this immune process relies on
442 the activation of the available PPO stock rather than an additional synthesis of this proenzyme
443 following challenge (Adamo 2004). For instance, studies conducted on several insect models
444 (mosquitoes, bumblebees) reported no significant upregulation of immune-related genes
445 encoding PPO production as an immediate response to the infection (Cui et al. 2000; Erler et
446 al. 2011). However, there is no broad empirical support to a positive relationship between
447 basal levels of PO/PPO system activity and successful host defence against pathogens and
448 parasites (Adamo 2004; González-Santoyo and Córdoba-Aguilar 2012; Mucklow et al. 2004).
449 This suggests that host immunocompetence is a multifactorial trait that cannot be assessed
450 through measurements of PO/PPO system activity alone (Mucklow et al. 2004). Besides this
451 humoral aspect of immunity, the present study appears to indicate an increased ability of *L.*
452 *botrana* larvae to encapsulate an infectious enemy throughout nighttime, because
453 plasmatocytes act as the main capsule-forming agents in Lepidoptera (Lavine and Strand
454 2002; Strand 2008). Of note, variations in the abundance of haemocyte types mediating
455 encapsulation response were shown to correlate positively with the degree of host resistance
456 to an infection involving multicellular organisms (Fors et al. 2014; Gerritsma et al. 2013). As
457 a next step, it would be worth investigating whether the nighttime upregulation of immune

458 traits highlighted here can translate into an actual elevation of larval resistance (decreased
459 infection rates) or tolerance (improved survival rates) in face of an experimental oral
460 infection, using antibiotic-free food allowing for the development of potentially pathogenic
461 microorganisms. In the same vein, further investigations should also examine the relation
462 between daily patterns of host immunity and both the intensity (e.g. bacterial load present in
463 the larval food) and the nature (infectious enemy involved) of the infection threat (Vogelweith
464 et al. 2015).

465 In natural environments, the day/night contrast in feeding activity reported here could
466 reflect the daily dynamic of food-borne infection of *L. botrana* larvae, likely to ingest
467 microbes (yeasts, bacteria and fungi) developing in consortium on the grape surface (Barata et
468 al. 2012; Vogelweith et al. 2015). Many Lepidopteran larvae (in both moths and butterflies)
469 have been observed feeding predominantly at night, and this behaviour is thought to have
470 evolved as a strategy of predator and parasitoid avoidance, or a way to take advantage of a
471 nighttime reduction in the amount of toxins contained in plant tissues (Goodspeed et al. 2012;
472 Suszczynska et al. 2017). In vineyards, mortality of *L. botrana* larvae is chiefly driven by
473 parasitoids belonging to the Hymenoptera and Diptera orders, but the daily activity profiles of
474 these natural enemies remain fairly undocumented (Thiéry et al. 2018). Focusing on the plant-
475 insect interaction, some transcripts related with the synthesis of defensive compounds in the
476 flesh of grape berries were found to be upregulated during daytime (e.g. terpenoid
477 biosynthetic genes) (Carbonell-Bejerano et al. 2014). Such nycthemeral variations of plant
478 nutritional value could have possibly driven the evolution of a nocturnal pattern of foraging in
479 carpophagous *L. botrana* larvae.

480 This work has implications with regard to the evolutionary dynamic of host-pathogen,
481 host-parasite or host-parasitoid interactions. Indeed, its results imply that the susceptibility of
482 a host species to an infection should fluctuate over the course of a day, and a similar

483 phenomenon has been described regarding the virulence and transmission of the infectious
484 enemy (reviewed by Westwood et al. 2019). It thus suggests that the daily rhythms inherent to
485 host resistance (immunity) and enemy virulence could be a major determinant of the
486 outcomes of the infection and the evolutionary trajectory of the interaction (Martinez-Bakker
487 and Helm 2015; Prior et al. 2020; Westwood et al. 2019). Extrapolating the results and
488 conclusions of this study to natural settings would require accounting for the bottom-up
489 regulation of plant traits (largely influenced by grape variety) on the nycthemeral variations
490 of larval immunity. This is especially true given that the abundance and composition of
491 microbial communities developing on the berry surface is influenced by grape cultivar, and so
492 should be the selective pressures exerted on the day/night structure of larval immunity (Barata
493 et al. 2012; Bokulich et al. 2014).

494 Additionally, there should be value in uncovering the role (if any) played by light and
495 temperature conditions in shaping the immune response patterns to day/night conditions here
496 highlighted. This work was not designed to quantify specifically the importance of these
497 abiotic variables for the fluctuations of insect immune function. This would require, for
498 instance, comparing different cycles of light and temperature conditions, uncoupling these
499 two environmental parameters, or more thoroughly controlling for the effect of the timing of
500 data acquisition (e.g. by evenly distributing timepoints of data collection and standardising
501 their number and duration for both day and night). From a mechanistic perspective, several
502 processes can be proposed to explain the possible influence of fluctuating light and
503 temperature conditions on the insect immunity. On the one hand, the insect could plastically
504 adjust its immunity to short-term changes in light and/or temperature occurring throughout the
505 day. For instance, temperature is known to be a critical determinant of immune function in *L.*
506 *botrana* larvae, such that cooler conditions during nighttime may explain the concomitant rise
507 in some immune traits like total PO activity, pending that such thermally-induced plasticity in

508 immune traits can occur on a daily scale (Iltis et al. 2018; 2019). On the other hand, another
509 plausible explanation to these results would lie in the chronobiological route, involving an
510 endogenous biological rhythm (orchestrated by a circadian clock) to account for the
511 nycthemeral variations observed. This cycle could be entrained by daily recurrent
512 fluctuations of light and temperature, since they may both act as environmental cues (i.e.
513 ‘Zeitgebers’) driving the circadian machinery and regulating its outputs *in natura* (Bell-
514 Pedersen et al. 2005; Vanin et al. 2012). Identifying the nature of these mechanisms should
515 help to examine the importance of day/night transitions (i.e. dusk and dawn in natural
516 conditions) in shaping the daily profiles here reported, considering that environmental
517 changes occurring during these transitions could serve as external cues enabling the insect to
518 adaptively anticipate the upcoming environment. In the present investigation, it appears
519 unlikely that transitions between the two phases would have driven the daily pattern responses
520 of immunity observed, provided that data collection was evenly distributed over each of the
521 different timepoints. Therefore, individuals sampled at the very end of the last timepoint for
522 daytime and nighttime (approximating dusk and dawn conditions, respectively) only
523 represented a minor proportion of the overall dataset, but more mechanistic investigations are
524 clearly needed at this point to ascertain this statement.

525 In conclusion, this study evidences a marked day/night contrast in the basal levels of
526 expression of several immune traits in an insect model. It could be proposed that the increase
527 in basal immunity at night has evolved with a predominantly nocturnal pattern of feeding in *L.*
528 *botrana* larvae (i.e. an endogenous immune cycle). This feeding activity profile has been
529 supposedly shaped by daily dynamics in the strength of bottom-up and top-down forces
530 mediated by plant and natural enemies, respectively, on the behaviour of the phytophagous
531 insect. However, further investigations are required to test the hypothesis postulating that *L.*
532 *botrana* larvae adjust their investment in basal immunity as a specific response pattern to

533 day/night variations in feeding activity and intensity of exposure to food-borne enemies. This
534 work was conducted with almost aseptic and artificial food to investigate day/night variations
535 in insect basal immunity. Hence, as a next step, the functional consequences of these immune
536 fluctuations have to be carefully examined at the level of biotic interactions, in the event of
537 more ecologically relevant risks of oral infection (e.g. larvae feeding upon antibiotic-free
538 food). This procedure would help ascertaining the role of daily variations of immunity in
539 preventing and/or dealing with infection, and estimate the specificity of these processes in
540 face of attacks from a wide range of potential enemies (Stone et al. 2012; Westwood et al.
541 2019). Besides, other biotic (plant) and abiotic parameters (light, temperature) could have
542 shaped the immune pattern observed, which could also evolve in response to the need of
543 temporally segregating incompatible physiological processes or align immunity with other
544 physiological rhythms (Westwood et al. 2019). Thus, assessing the contribution of such
545 environmental variables to this day/night structure of host immunity would be of high
546 relevance to identify the *in situ* environmental drivers of rhythms in traits underpinning host
547 resistance and to extrapolate the results of this study to a broader ecological context. Such
548 investigations should hold promise for improving the general understanding of the temporal
549 variations of insect immunity through the lens of evolutionary and ecological immunology.

550 **Acknowledgements**

551 We thank Lionel Delbac and Samuel Ribeiro for their technical help in maintaining the insect
552 stock and performing the experiments, respectively. We also want to thank Sébastien Zito and
553 Benjamin Bois for providing the climatic data used in this study. We are grateful to Nathalie
554 Jouy for her contribution to flow-cytometric analyses. This study was financially supported by
555 the *Conseil Régional de Bourgogne Franche-Comté* through the *Plan d'Actions Régional*
556 *pour l'Innovation* (PARI) and two other funding sources (FABER LOUAPRE AGREE-BGS,

557 VALEACLIM-BOIS), and the European Union through the PO FEDER-FSE Bourgogne
558 2014/2020 programs.

559 **References**

- 560 Adamo, S. A. (2004). Estimating disease resistance in insects: phenoloxidase and lysozyme-
561 like activity and disease resistance in the cricket *Gryllus texensis*. *Journal of Insect*
562 *Physiology*, 50(2–3), 209–216. <https://doi.org/10.1016/j.jinsphys.2003.11.011>
- 563 Barata, A., Malfeito-Ferreira, M., & Loureiro, V. (2012). The microbial ecology of wine
564 grape berries. *International Journal of Food Microbiology*, 153(3), 243–259. Elsevier.
565 <https://doi.org/10.1016/j.ijfoodmicro.2011.11.025>
- 566 Barnay, O., Hommay, G., Gertz, C., Kienlen, J. C., Schubert, G., Marro, J. P., Pizzol, J., &
567 Chavigny, P. (2001). Survey of natural populations of *Trichogramma* (Hym.,
568 Trichogrammatidae) in the vineyards of Alsace (France). *Journal of Applied*
569 *Entomology*, 125(8), 469–477. <https://doi.org/10.1046/j.1439-0418.2001.00575.x>
- 570 Barnes, A. I., & Siva-Jothy, M. T. (2000). Density-dependent prophylaxis in the mealworm
571 beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an
572 indicator of investment in immunity. *Proceedings of the Royal Society of London. Series*
573 *B: Biological Sciences*, 267(1439), 177–182. <https://doi.org/10.1098/rspb.2000.0984>
- 574 Bell-Pedersen, D., Cassone, V. M., Earnest, D. J., Golden, S. S., Hardin, P. E., Thomas, T. L.,
575 & Zoran, M. J. (2005). Circadian rhythms from multiple oscillators: lessons from diverse
576 organisms. *Nature Reviews Genetics*, 6, 554–556. <https://doi.org/10.1038/nrg1633>
- 577 Bernays, E. A., Singer, M. S., & Rodrigues, D. (2004). Foraging in nature: foraging efficiency
578 and attentiveness in caterpillars with different diet breadths. *Ecological Entomology*,
579 29(4), 389–397. <https://doi.org/10.1111/j.0307-6946.2004.00615.x>
- 580 Bertram, S. M., Orozco, S. X., & Bellani, R. (2004). Temporal shifts in conspicuousness:
581 mate attraction displays of the Texas field cricket, *Gryllus texensis*. *Ethology*, 110(12),

582 963–975. <https://doi.org/10.1111/j.1439-0310.2004.01031.x>

583 Bokulich, N. A., Thorngate, J. H., Richardson, P. M., & Mills, D. A. (2014). Microbial
584 biogeography of wine grapes is conditioned by cultivar, vintage, and climate.
585 *Proceedings of the National Academy of Sciences of the United States of America*,
586 *111*(1), E139–E148. <https://doi.org/10.1073/pnas.1317377110>

587 Brown, M. J. F., Moret, Y., & Schmid-Hempel, P. (2003). Activation of host constitutive
588 immune defence by an intestinal trypanosome parasite of bumble bees. *Parasitology*,
589 *126*(3), 253–260. <https://doi.org/10.1017/S0031182002002755>

590 Bulet, P., Hetru, C., Dimarcq, J. L., & Hoffmann, D. (1999). Antimicrobial peptides in
591 insects: structure and function. *Developmental and Comparative Immunology*, *23*(4–5),
592 329–344. [https://doi.org/10.1016/S0145-305X\(99\)00015-4](https://doi.org/10.1016/S0145-305X(99)00015-4)

593 Carbonell-Bejerano, P., Rodríguez, V., Royo, C., Hernáiz, S., Moro-González, L. C., Torres-
594 Viñals, M., & Martínez-Zapater, J. M. (2014). Circadian oscillatory transcriptional
595 programs in grapevine ripening fruits. *BMC Plant Biology*, *14*(1), 78.
596 <https://doi.org/10.1186/1471-2229-14-78>

597 Cerenius, L., & Söderhäll, K. (2004). The prophenoloxidase-activating system in
598 invertebrates. *Immunological Reviews*, *198*(1), 116–126. [https://doi.org/10.1111/j.0105-](https://doi.org/10.1111/j.0105-2896.2004.00116.x)
599 [2896.2004.00116.x](https://doi.org/10.1111/j.0105-2896.2004.00116.x)

600 Corby-Harris, V., & Promislow, D. E. L. (2008). Host ecology shapes geographical variation
601 for resistance to bacterial infection in *Drosophila melanogaster*. *Journal of Animal*
602 *Ecology*, *77*(4), 768–776. <https://doi.org/10.1111/j.1365-2656.2008.01399.x>

603 Cui, L., Luckhart, S., & Rosenberg, R. (2000). Molecular characterization of a
604 prophenoloxidase cDNA from the malaria mosquito *Anopheles stephensi*. *Insect*

605 *Molecular Biology*, 9(2), 127–137. <https://doi.org/10.1046/j.1365-2583.2000.00169.x>

606 De Filippis, F., Parente, E., & Ercolini, D. (2018). Recent past, present, and future of the food
607 microbiome. *Annual Review of Food Science and Technology*, 9(1), 589–608.
608 <https://doi.org/10.1146/annurev-food-030117-012312>

609 Erler, S., Popp, M., & Lattorff, H. M. G. (2011). Dynamics of immune system gene
610 expression upon bacterial challenge and wounding in a social insect (*Bombus terrestris*).
611 *PLoS ONE*, 6(3), e18126. <https://doi.org/10.1371/journal.pone.0018126>

612 Fleury, F., Allemand, R., Vavre, F., Fouillet, P., & Boulétreau, M. (2000). Adaptive
613 significance of a circadian clock: temporal segregation of activities reduces intrinsic
614 competitive inferiority in *Drosophila* parasitoids. *Proceedings of the Royal Society of*
615 *London. Series B: Biological Sciences*, 267(1447), 1005–1010.
616 <https://doi.org/10.1098/rspb.2000.1103>

617 Fors, L., Markus, R., Theopold, U., & Hambäck, P. A. (2014). Differences in cellular immune
618 competence explain parasitoid resistance for two coleopteran species. *PLoS ONE*, 9(9),
619 e108795. <https://doi.org/10.1371/journal.pone.0108795>

620 Freitag, D., Schmidtberg, H., Dickel, F., Lochnit, G., Vogel, H., & Vilcinskis, A. (2014). The
621 maternal transfer of bacteria can mediate trans-generational immune priming in insects.
622 *Virulence*, 5(4), 547–554. <https://doi.org/10.4161/viru.28367>

623 Gerritsma, S., de Haan, A., van de Zande, L., & Wertheim, B. (2013). Natural variation in
624 differentiated hemocytes is related to parasitoid resistance in *Drosophila melanogaster*.
625 *Journal of Insect Physiology*, 59(2), 148–158.
626 <https://doi.org/10.1016/J.JINSPHYS.2012.09.017>

627 Ghoneim, K., Tanani, M., Hamadah, K., Basiouny, A., & Waheeb, H. (2015). Effects of

628 Novaluron and Cyromazine, chitin synthesis inhibitors, on the larval hemogram of
629 *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *International Journal of*
630 *Advanced Research*, 3(1), 554–576.

631 Gillespie, J. P., Kanost, M. R., & Trenczek, T. (1997). Biological mediators of insect
632 immunity. *Annual Review of Entomology*, 42(1), 611–643.
633 <https://doi.org/10.1146/annurev.ento.42.1.611>

634 González-Santoyo, I., & Córdoba-Aguilar, A. (2012). Phenoloxidase: a key component of the
635 insect immune system. *Entomologia Experimentalis et Applicata*, 142(1), 1–16.
636 <https://doi.org/10.1111/j.1570-7458.2011.01187.x>

637 Goodspeed, D., Chehab, E. W., Min-Venditti, A., Braam, J., & Covington, M. F. (2012).
638 *Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior.
639 *Proceedings of the National Academy of Sciences of the United States of America*,
640 109(12), 4674–4677. <https://doi.org/10.1073/pnas.1116368109>

641 Havko, N. E., Kapali, G., Das, M. R., & Howe, G. A. (2020). Stimulation of insect herbivory
642 by elevated temperature outweighs protection by the jasmonate pathway. *Plants*, 9(2),
643 172. <https://doi.org/10.3390/plants9020172>

644 Herden, J., Meldau, S., Kim, S.-G., Kunert, G., Joo, Y., Baldwin, I. T., & Schuman, M. C.
645 (2016). Shifting *Nicotiana attenuata*'s diurnal rhythm does not alter its resistance to the
646 specialist herbivore *Manduca sexta*. *Journal of Integrative Plant Biology*, 58(7), 656–
647 668. <https://doi.org/10.1111/jipb.12458>

648 Iltis, C., Martel, G., Thiéry, D., Moreau, J., & Louâpre, P. (2018). When warmer means
649 weaker: high temperatures reduce behavioural and immune defences of the larvae of a
650 major grapevine pest. *Journal of Pest Science*, 91, 1315–1326.
651 <https://doi.org/10.1007/s10340-018-0992-y>

652 Iltis, C., Louâpre, P., Pecharová, K., Thiéry, D., Zito, S., Bois, B., & Moreau, J. (2019). Are
653 life-history traits equally affected by global warming? A case study combining a multi-
654 trait approach with fine-grain climate modeling. *Journal of Insect Physiology*, *117*,
655 103916. <https://doi.org/10.1016/J.JINSPHYS.2019.103916>

656 Islam, A., & Roy, S. (1982). Diurnal rhythm of hemocyte population in an insect,
657 *Schizodactylus monstrosus* Drury. *Experientia*, *38*(5), 567–569.
658 <https://doi.org/10.1007/BF02327052>

659 Jiang, H., Vilcinskis, A., & Kanost, M. R. (2010). Immunity in Lepidopteran Insects. In
660 Söderhäll, K. (ed.), *Invertebrate Immunity*. Springer Science+Business Media, New
661 York, pp. 181–204. https://doi.org/10.1007/978-1-4419-8059-5_10

662 Kanost, M. R., Jiang, H., & Yu, X.-Q. (2004). Innate immune responses of a lepidopteran
663 insect, *Manduca sexta*. *Immunological Reviews*, *198*(1), 97–105.
664 <https://doi.org/10.1111/j.0105-2896.2004.0121.x>

665 Lavine, M. D., & Strand, M. R. (2002). Insect hemocytes and their role in immunity. *Insect*
666 *Biochemistry and Molecular Biology*, *32*(10), 1295–1309.
667 [https://doi.org/10.1016/S0965-1748\(02\)00092-9](https://doi.org/10.1016/S0965-1748(02)00092-9)

668 Lee, J. E., & Edery, I. (2008). Circadian regulation in the ability of *Drosophila* to combat
669 pathogenic infections. *Current Biology*, *18*(3), 195–199.
670 <https://doi.org/10.1016/j.cub.2007.12.054>

671 Lindström, K. M., Fofopoulos, J., Pärn, H., & Wikelski, M. (2004). Immunological
672 investments reflect parasite abundance in island populations of Darwin's finches.
673 *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *271*(1547),
674 1513–1519. <https://doi.org/10.1098/rspb.2004.2752>

- 675 Liu, F., Xu, Q., Zhang, Q., Lu, A., Beerntsen, B. T., & Ling, E. (2013). Hemocytes and
676 hematopoiesis in the silkworm, *Bombyx mori*. *Invertebrate Survival Journal*, 10(1), 102–
677 109.
- 678 Lu, D. B., Wang, T. P., Rudge, J. W., Donnelly, C. A., Fang, G. R., & Webster, J. P. (2009).
679 Evolution in a multi-host parasite: chronobiological circadian rhythm and population
680 genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host
681 reservoirs by habitat. *International Journal for Parasitology*, 39(14), 1581–1588.
682 <https://doi.org/10.1016/j.ijpara.2009.06.003>
- 683 Martín-Vertedor, D., Ferrero-García, J. J., & Torres-Vila, L. M. (2010). Global warming
684 affects phenology and voltinism of *Lobesia botrana* in Spain. *Agricultural and Forest*
685 *Entomology*, 12(2), 169–176. <https://doi.org/10.1111/j.1461-9563.2009.00465.x>
- 686 Martin, L. B., Weil, Z. M., & Nelson, R. J. (2008). Seasonal changes in vertebrate immune
687 activity: mediation by physiological trade-offs. *Philosophical Transactions of the Royal*
688 *Society B: Biological Sciences*, 363(1490), 321–339.
689 <https://doi.org/10.1098/rstb.2007.2142>
- 690 Martinez-Bakker, M., & Helm, B. (2015). The influence of biological rhythms on host-
691 parasite interactions. *Trends in Ecology and Evolution*, 30(6), 314–326.
692 <https://doi.org/10.1016/j.tree.2015.03.012>
- 693 Mucklow, P. T., Vizoso, D. B., Jensen, K. H., Refardt, D., & Ebert, D. (2004). Variation in
694 phenoloxidase activity and its relation to parasite resistance within and between
695 populations of *Daphnia magna*. *Proceedings of the Royal Society of London. Series B:*
696 *Biological Sciences*, 271(1544), 1175–1183. <https://doi.org/10.1098/rspb.2004.2707>
- 697 Murdock, C. C., Moller-Jacobs, L. L., & Thomas, M. B. (2013). Complex environmental
698 drivers of immunity and resistance in malaria mosquitoes. *Proceedings of the Royal*

699 *Society. Series B: Biological Sciences*, 280(1770), 20132030.
700 <https://doi.org/10.1098/rspb.2013.2030>

701 Nakahara, Y., Kanamori, Y., Kiuchi, M., & Kamimura, M. (2010). Two hemocyte lineages
702 exist in silkworm larval hematopoietic organ. *PLoS ONE*, 5(7), e11816.
703 <https://doi.org/10.1371/journal.pone.0011816>

704 Niepoth, N., Ke, G., de Roode, J. C., & Groot, A. T. (2018). Comparing behavior and clock
705 gene expression between caterpillars, butterflies, and moths. *Journal of Biological*
706 *Rhythms*, 33(1), 52–64. <https://doi.org/10.1177/0748730417746458>

707 Ponton, F., Wilson, K., Holmes, A. J., Cotter, S. C., Raubenheimer, D., & Simpson, S. J.
708 (2013). Integrating nutrition and immunology: a new frontier. *Journal of Insect*
709 *Physiology*, 59(2), 130–137. <https://doi.org/10.1016/J.JINSPHYS.2012.10.011>

710 Prior, K. F., Rijo-Ferreira, F., Assis, P. A., Hirako, I. C., Weaver, D. R., Gazzinelli, R. T., &
711 Reece, S. E. (2020). Periodic parasites and daily host rhythms. *Cell Host and Microbe*,
712 27(2), 176–187. <https://doi.org/10.1016/j.chom.2020.01.005>

713 Raffel, T. R., Rohr, J. R., Kiesecker, J. M., & Hudson, P. J. (2006). Negative effects of
714 changing temperature on amphibian immunity under field conditions. *Functional*
715 *Ecology*, 20(5), 819–828. <https://doi.org/10.1111/j.1365-2435.2006.01159.x>

716 Ribeiro, C., & Brehélin, M. (2006). Insect haemocytes: what type of cell is that? *Journal of*
717 *Insect Physiology*, 52(5), 417–429. <https://doi.org/10.1016/j.jinsphys.2006.01.005>

718 Schmid-Hempel, P. (2003). Variation in immune defence as a question of evolutionary
719 ecology. *Proceedings of the Royal Society of London. Series B: Biological Sciences*,
720 270(1513), 357–366. <https://doi.org/10.1098/rspb.2002.2265>

721 Schmid-Hempel, P. (2005). Evolutionary ecology of insect immune defenses. *Annual Review*

722 of *Entomology*, 50(1), 529–551. <https://doi.org/10.1146/annurev.ento.50.071803.130420>

723 Schmid-Hempel, P., & Ebert, D. (2003). On the evolutionary ecology of specific immune
724 defence. *Trends in Ecology and Evolution*, 18(1), 27–32. [https://doi.org/10.1016/S0169-](https://doi.org/10.1016/S0169-5347(02)00013-7)
725 5347(02)00013-7

726 Schulenburg, H., Kurtz, J., Moret, Y., & Siva-Jothy, M. T. (2009). Introduction. Ecological
727 immunology. *Philosophical Transactions of the Royal Society B: Biological Sciences*,
728 364(1513), 3–14. <https://doi.org/10.1098/rstb.2008.0249>

729 Sheldon, B. C., & Verhulst, S. (1996). Ecological immunology: costly parasite defences and
730 trade-offs in evolutionary ecology. *Trends in Ecology and Evolution*, 11(8), 317–321.
731 [https://doi.org/10.1016/0169-5347\(96\)10039-2](https://doi.org/10.1016/0169-5347(96)10039-2)

732 Siva-Jothy, M. T., Moret, Y., & Rolff, J. (2005). Insect immunity: an evolutionary ecology
733 perspective. *Advances in Insect Physiology*, 32, 1–48. [https://doi.org/10.1016/S0065-](https://doi.org/10.1016/S0065-2806(05)32001-7)
734 2806(05)32001-7

735 Stone, E. F., Fulton, B. O., Ayres, J. S., Pham, L. N., Ziauddin, J., & Shirasu-Hiza, M. M.
736 (2012). The circadian clock protein timeless regulates phagocytosis of bacteria in
737 *Drosophila*. *PLoS Pathogens*, 8(1), e1002445.
738 <https://doi.org/10.1371/journal.ppat.1002445>

739 Strand, M. R. (2008). The insect cellular immune response. *Insect Science*, 15(1), 1–14.
740 <https://doi.org/10.1111/j.1744-7917.2008.00183.x>

741 Suszczynska, A., Kaniewska, M. M., Bebas, P., Giebultowicz, J. M., & Kotwica-Rolinska, J.
742 (2017). Circadian regulation of caterpillar feeding and growth. *Journal of Insect*
743 *Physiology*, 101, 113–122. <https://doi.org/10.1016/j.jinsphys.2017.07.009>

744 Thiéry, D., & Moreau, J. (2005). Relative performance of European grapevine moth (*Lobesia*

745 *botrana*) on grapes and other hosts. *Oecologia*, 143(4), 548–557.
746 <https://doi.org/10.1007/s00442-005-0022-7>

747 Thiéry, D., Louâpre, P., Muneret, L., Rusch, A., Sentenac, G., Vogelweith, F., Iltis, C., &
748 Moreau, J. (2018). Biological protection against grape berry moths. A review. *Agronomy*
749 *for Sustainable Development*, 38(2), 15. <https://doi.org/10.1007/s13593-018-0493-7>

750 Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome Biology*,
751 14(6), 209. <https://doi.org/10.1186/gb-2013-14-6-209>

752 Vanin, S., Bhutani, S., Montelli, S., Menegazzi, P., Green, E. W., Pegoraro, M., Sandrelli, F.,
753 Costa, R., & Kyriacou, C. P. (2012). Unexpected features of *Drosophila* circadian
754 behavioural rhythms under natural conditions. *Nature*, 484(7394), 371–375.
755 <https://doi.org/10.1038/nature10991>

756 Vogelweith, F., Thiéry, D., Quaglietti, B., Moret, Y., & Moreau, J. (2011). Host plant
757 variation plastically impacts different traits of the immune system of a phytophagous
758 insect. *Functional Ecology*, 25(6), 1241–1247. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-2435.2011.01911.x)
759 [2435.2011.01911.x](https://doi.org/10.1111/j.1365-2435.2011.01911.x)

760 Vogelweith, F., Dourneau, M., Thiéry, D., Moret, Y., & Moreau, J. (2013). Geographical
761 variation in parasitism shapes larval immune function in a phytophagous insect.
762 *Naturwissenschaften*, 100(12), 1149–61. <https://doi.org/10.1007/s00114-013-1119-1>

763 Vogelweith, F., Thiéry, D., Moret, Y., Colin, E., Motreuil, S., & Moreau, J. (2014). Defense
764 strategies used by two sympatric vineyard moth pests. *Journal of Insect Physiology*, 64,
765 54–61. <https://doi.org/10.1016/j.jinsphys.2014.03.009>

766 Vogelweith, F., Moreau, J., Thiéry, D., & Moret, Y. (2015). Food-mediated modulation of
767 immunity in a phytophagous insect: an effect of nutrition rather than parasitic

768 contamination. *Journal of Insect Physiology*, 77, 55–61.

769 <https://doi.org/10.1016/j.jinsphys.2015.04.003>

770 Vogelweith, F., Moret, Y., Monceau, K., Thiéry, D., & Moreau, J. (2016). The relative
771 abundance of hemocyte types in a polyphagous moth larva depends on diet. *Journal of*
772 *Insect Physiology*, 88, 33–39. <https://doi.org/10.1016/J.JINSPHYS.2016.02.010>

773 Westwood, M. L., O'Donnell, A. J., de Bekker, C., Lively, C. M., Zuk, M., & Reece, S. E.
774 (2019). The evolutionary ecology of circadian rhythms in infection. *Nature Ecology and*
775 *Evolution*, 3, 552–560. <https://doi.org/10.1038/s41559-019-0831-4>

776 Wu, K., Yang, B., Huang, W., Dobens, L., Song, H., & Ling, E. (2016). Gut immunity in
777 lepidopteran insects. *Developmental and Comparative Immunology*, 64, 65–74.
778 <https://doi.org/10.1016/j.dci.2016.02.010>

779 **Figure legends**

780 **Fig. 1.** Temporal design of the experiments and scheduling of the different behavioural
781 (Group 1) and immune measurements taken (Group 2). Larvae were exposed to a L16:D8
782 regime with six thermal segments of 4 h each, designed to emulate a mean summer day in
783 Eastern France. In Group 1, tests were conducted to quantify the time budget each individual
784 spent feeding during four timepoints of measurements (green) distributed over the course of a
785 day (A, B, C, D). In Group 2, immunological assays were focused on haemocyte load and
786 activity of PO/PPO system on one side (red) – involving five timepoints of measurements (A',
787 B', C', D', E') – and relative abundances of different haemocyte types on the other (blue). No
788 measurements occurred over hatched areas.

789 **Fig. 2.** Response of the time budget allocated to feeding (during an observation of 1 h) to the
790 random effect represented by the timing of data collection (timepoint of measurements) and
791 the fixed global effect of phase (white = daytime, grey = nighttime). Represented values are

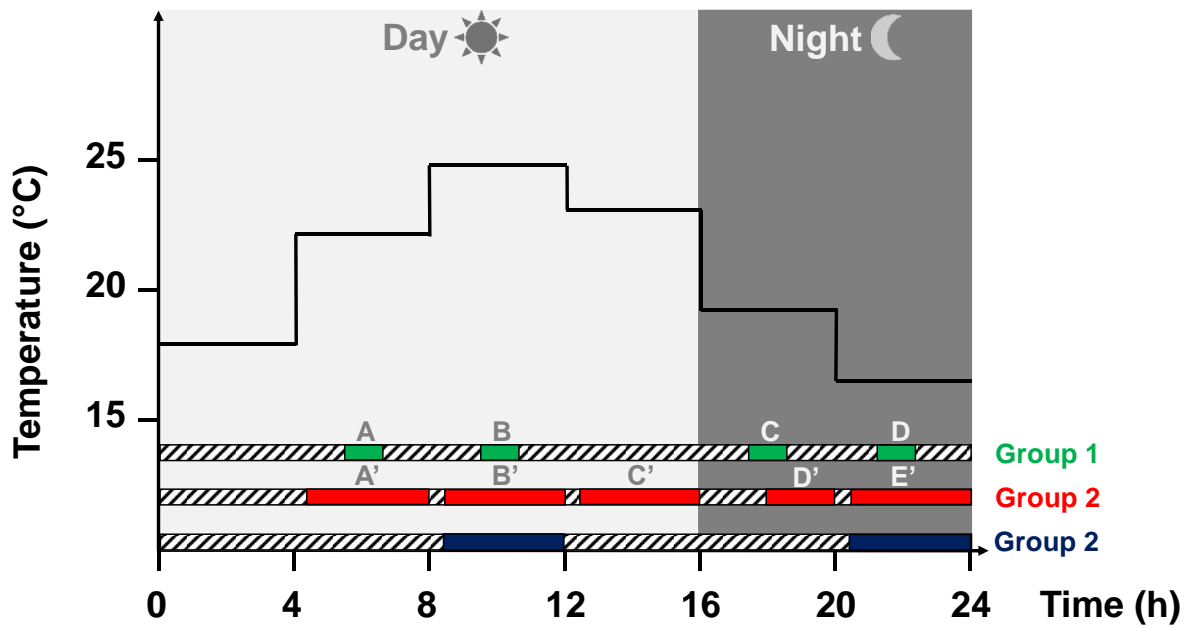
792 means (\pm 95% c.i.). Significant differences are shown by asterisks (* $P < 0.05$). Numbers
793 within the bars indicate sample sizes. Data for the different timepoints and phases were
794 collected on the same individuals.

795 **Fig. 3.** Responses of haemocyte load (A), PO activity (B) and total PO activity (C) to the
796 random effect represented by the timing of data collection (timepoint of measurements) and
797 the fixed global effect of phase (white = daytime, grey = nighttime). Represented values are
798 means (\pm 95% c.i.). Significant differences are shown by asterisks (** $P < 0.01$), and n.s.
799 stands for non-significant. Numbers within the bars indicate sample sizes.

800 **Fig. 4.** *Lobesia botrana* larvae were fixed in 4% paraformaldehyde solution and then
801 dehydrated in a series of ethanol solutions. Specimens were next embedded in a Paraplast
802 Plus[®] and cut in paraffin longitudinal sections with a thickness down to 5 μ m (A). After being
803 dewaxed and rehydrated, paraffin sections were coloured using Papanicolaou stain or
804 toluidine blue (0.1%) for 4 min. Nucleic acids were then labelled in blue using a 4',6-
805 diamidino-2-phenylindole solution (DAPI) diluted in PBS (0.5 μ g/ml). The samples were
806 stained for 20 min with this solution in the dark, at room temperature. They were examined
807 using a fluorescence microscope (Axio Imager 2, Zeiss, Oberkochen, Germany) (B).
808 According to flow-cytometric analyses, four haemocyte types were identified in the
809 haemolymph of 4th instar *L. botrana* larvae: prohaemocytes, oenocytoids, granulocytes
810 (yellow arrow indicates a granule included in the cytoplasm) and plasmatocytes. Photographic
811 data of haemocyte types were obtained using a phase contrast microscope (Primo Star, Zeiss,
812 Oberkochen, Germany) (C).

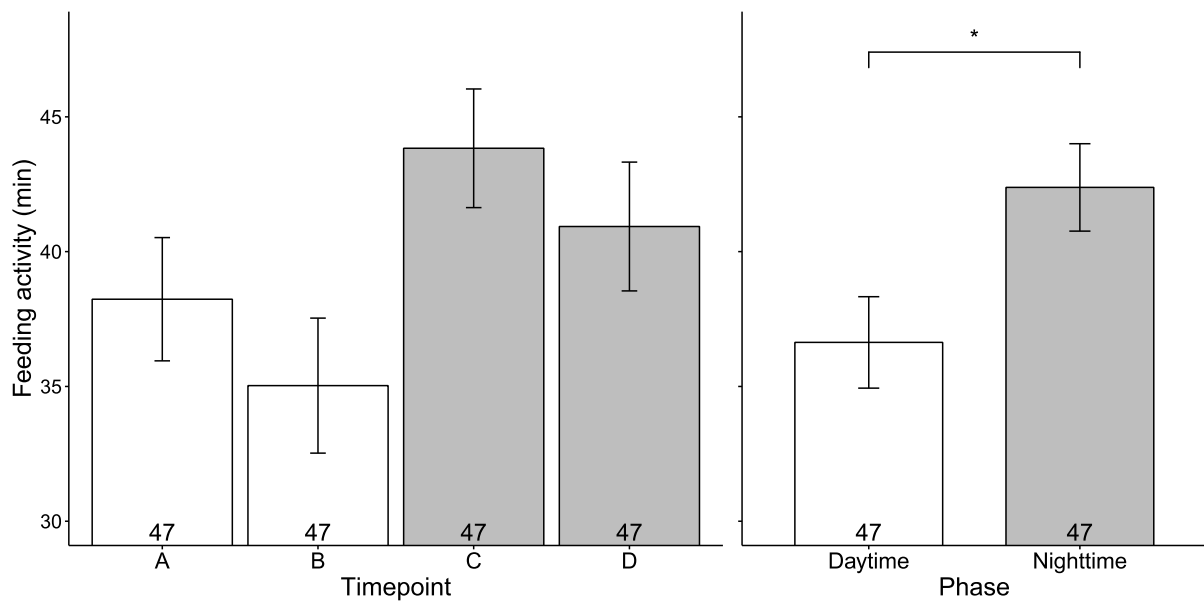
813 **Fig. 5.** Responses of the relative abundances of the four haemocyte types found in
814 haemolymph of 4th instar larvae to the phase (white = daytime, grey = nighttime).
815 Represented values are means (\pm 95% c.i.). Significant differences are shown by asterisks

816 (** $P < 0.001$, * $P < 0.05$), and n.s. stands for non-significant. Numbers within the bars
817 indicate sample sizes.



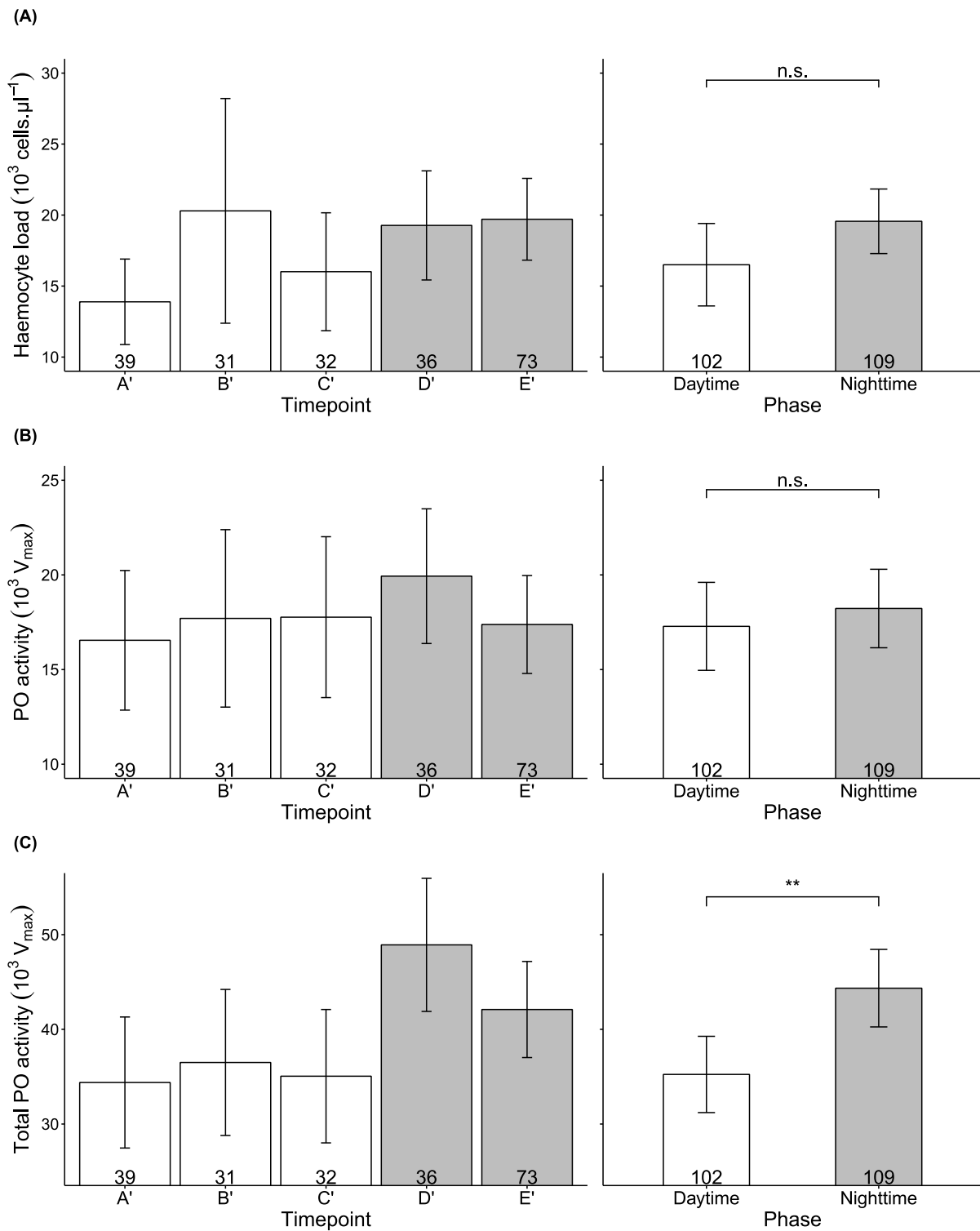
819

820 **Fig. 1.** Temporal design of the experiments and scheduling of the different behavioural
 821 (Group 1) and immune measurements taken (Group 2).



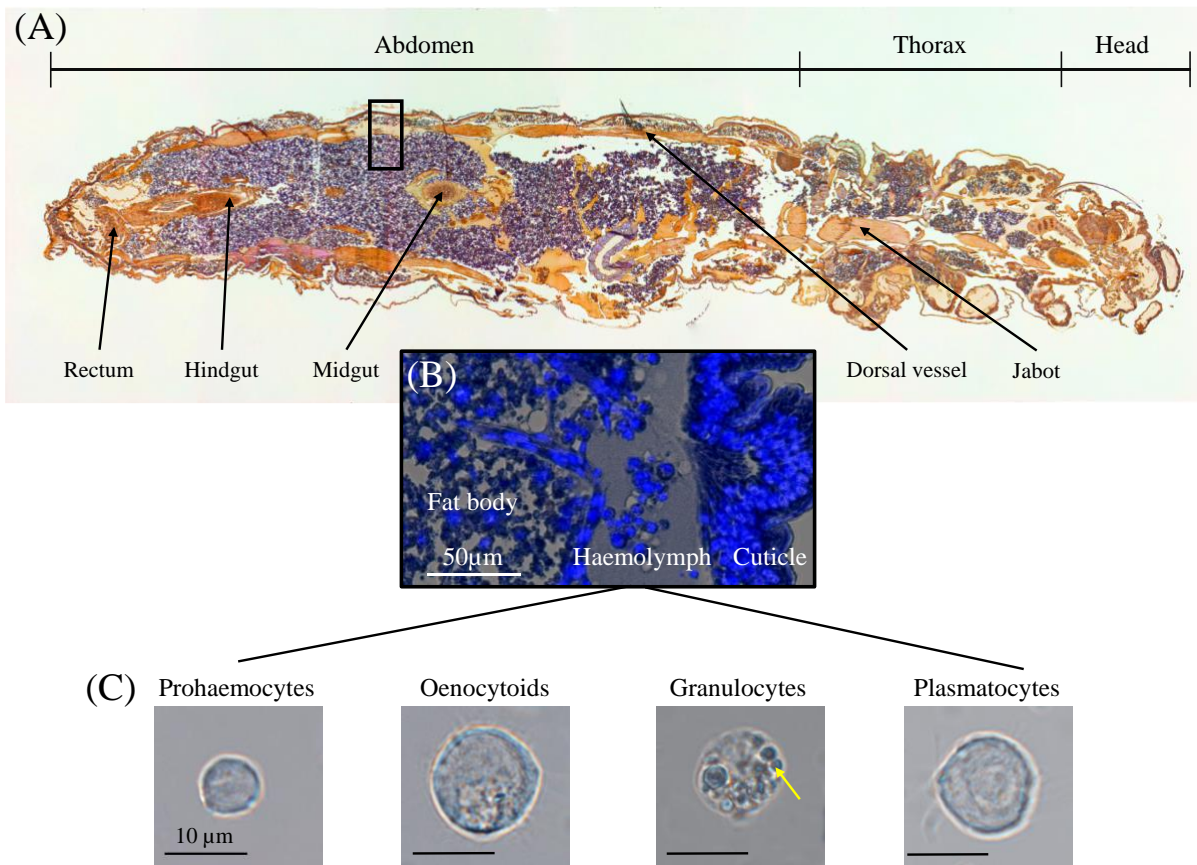
822

823 **Fig. 2.** Response of the time budget allocated to feeding (during an observation of 1 h) to the
 824 random effect represented by the timing of data collection (timepoint of measurements) and
 825 the fixed global effect of phase (white = daytime, grey = nighttime).



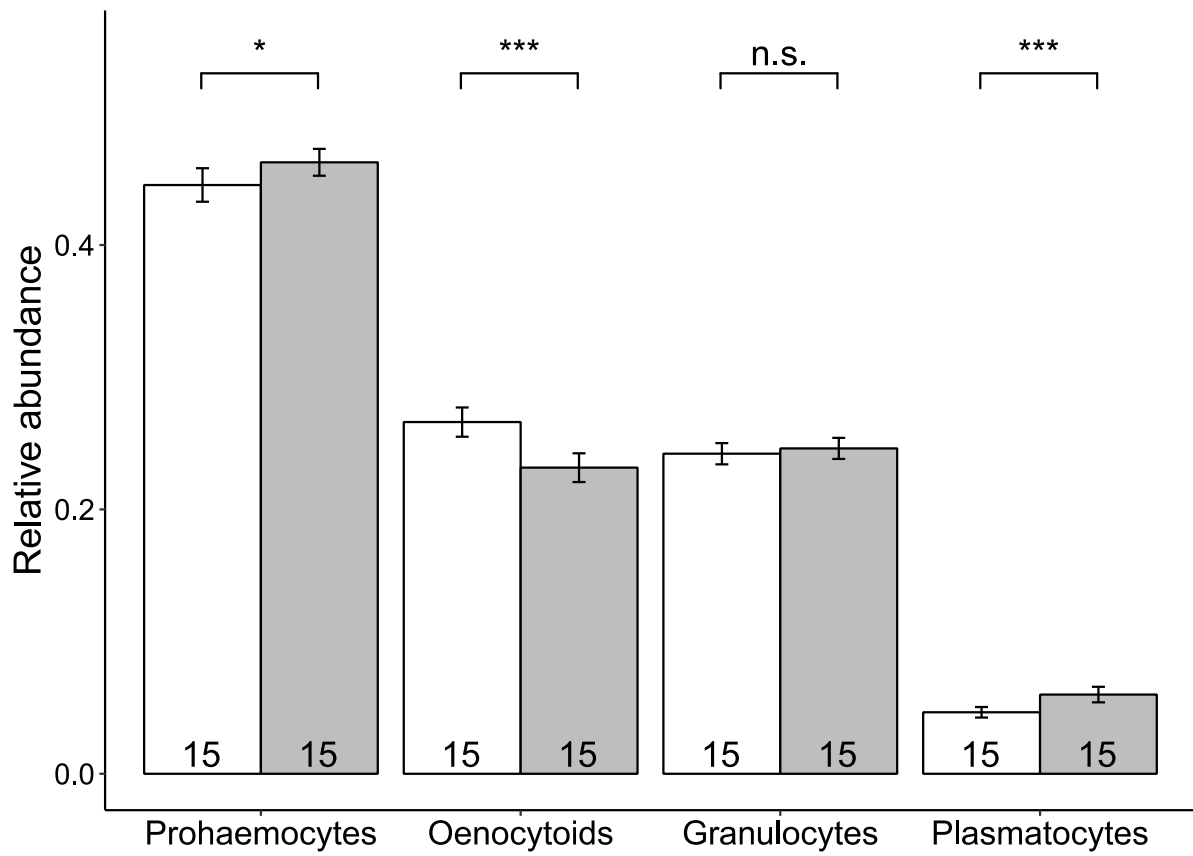
826

827 **Fig. 3.** Responses of haemocyte load (A), PO activity (B) and total PO activity (C) to the
 828 random effect represented by the timing of data collection (timepoint of measurements) and
 829 the fixed global effect of phase (white = daytime, grey = nighttime).



830

831 **Fig. 4.** According to flow-cytometric analyses, four haemocyte types were identified in the
 832 haemolymph of 4th instar *L. botrana* larvae: prohaemocytes, oenocytoids, granulocytes
 833 (yellow arrow indicates a granule included in the cytoplasm) and plasmatocytes.



834

835 **Fig. 5.** Responses of the relative abundances of the four haemocyte types found in

836 haemolymph of 4th instar larvae to the phase (white = daytime, grey = nighttime).