Thermal responses of the embryos and early instar larvae of the Antarctic midge Belgica antarctica (Insecta: Diptera)

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Abstract

The Antarctic midge, *Belgica antarctica*, is the only insect that is endemic to the Antarctic Peninsula and its offshore islands. The final (fourth) instar larvae exhibit high inherent thermal tolerance to short-term temperature exposure, and many studies have focused on the underlying molecular physiological mechanisms of their thermal tolerance. In contrast, the thermal strategy of embryos has received little attention, and the effects of long-term thermal exposure on newly hatched larvae have not been investigated. The present study reports that the average supercooling point of egg masses was −4.7 °C and that the first instar larvae successfully emerged from the frozen egg masses. The embryos also demonstrated significant heat tolerance and the first instar larvae successfully emerged from the egg masses kept at temperatures ranging from 0 to 15 °C. These indicate that embryos have sufficient tolerance to cold and heat during Antarctic summer. Interestingly, the first instar larvae developed into fourth instar larvae at 4 °C, but not at other temperatures. Early instar larvae have a narrow temperature window for their development, suggesting that they are susceptible to environmental changes.

Keywords

Antarctic midge, Developmental rate, Embryo, Hatchability, Stress tolerance, Supercooling point

Introduction

The ability to survive extreme temperatures is critical for insects, especially those living in polar environments. The Antarctic midge, *Belgica antarctica* (Insecta: Diptera), is an iconic species in the Antarctic ecophysiology. This species is endemic to the Antarctic Peninsula and its offshore islands and is the only holometabolous insect living in Antarctica (Usher and Edwards 1984; Wirth and Gressitt 1967). This species has a two-year life cycle (Sugg et al. 1983). Like other midges, it has four larval stages and spends most of its life as a larva (Sugg et al. 1983). The embryonic, pre-pupal, pupal, and adult stages are observed only during the short summer, and only larvae at all stages can overwinter in the field (Sugg et al. 1983).

The larvae and adults have distinct thermal survival strategies. Larvae are more tolerant to heat than adults (Rinehart et al. 2006). During larval development, larvae maintain a high inherent tolerance to temperature stress by constitutively upregulating heat shock proteins (*small hsp*s, *hsp70*, and *hsp90*). No further upregulation of these genes was observed and thermotolerance was not enhanced by high or low temperatures. In contrast, adults exhibit a lower intrinsic tolerance to heat and do not exhibit constitutive upregulation of *hsp*s; however, *hsp*s can be thermally activated, resulting in enhanced thermotolerance. Larvae are also more tolerant to cold than adults (Baust and Edwards 1979). Larvae are freeze-tolerant and also employ cryoprotective dehydration as a strategy for survival at low temperatures (Baust and Edwards 1979; Elnitsky et al. 2008; Teets et al. 2011). In contrast, adults are freeze-susceptible and lack adequate quantities of cryoprotectants (Baust and Edwards 1979). Larvae are also tolerant to other environmental stresses, such as desiccation, high and low pH, and osmotic stress (Lee and Denlinger 2015). This astonishing larval stress tolerance has attracted the attention of many researchers; thus, its underlying molecular and physiological mechanisms

have been studied extensively (Baust and Lee 1987; Hayward et al. 2007; Kawarasaki et al. 2019; Teets et al. 2012; Yi et al. 2011; Yoshida et al. 2021).

In contrast to larvae and adults, the thermal strategy of embryos has received little attention. Adult females deposit egg masses on the ground in spring or early summer in Antarctica (Edwards and Baust 1981) where embryos may be exposed to severe temperature fluctuations, ranging from −3 to 22 °C (Kawarasaki et al. 2014; Rinehart et al. 2006). Immobile embryos are thought to have sufficient heat and cold tolerance for a range of temperatures naturally occurring during summer; however, this has not been verified empirically. In addition, previous studies have predominantly investigated the response to short-term temperature extremes of fourth (final) instar larvae (Baust and Lee 1987; Rinehart et al. 2006). The response of early instar larvae to long-term thermal extremes has received little attention. To address these issues, we investigated the embryonic and larval development of *B. antarctica* at various temperatures. First, we measured the supercooling point (SCP) of the egg mass and observed emergence of the first instar larvae from the frozen egg masses. We also assessed hatchability and calculated embryonic developmental rates at various temperatures. In addition, we observed the development of newly hatched larvae at various temperatures.

Materials and methods

Insect

Larvae of *B. antarctica* were collected with the substrates, including small rocks, detritus, algae, and moss, from January to February 2017 (2017 cohort) and from December 2017 to March 2018 (2018 cohort). The larvae and substrates were transported to Osaka City

University (Permission No. 24K134, Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries, Japan) through Miami University. In the laboratory, the larvae were maintained in a sealed plastic box (26 × 18.5 × 6 cm) at 4 °C under 18-h light and 6-h dark cycle (LD 18:6 h) with a fluorescent lamp (FL15WF; Panasonic, Kadoma, Japan) to approximate the summer conditions of their habitat. The photon flux density was 3.18 μmol $m^{-2} \cdot s^{-1}$.

Adults emerged in the laboratory from June to December 2017 from the 2017 cohort and from late March to December 2018 from the 2018 cohort. All the adults were collected and allowed to mate in a Petri dish (7.0 cm in diameter, 1.0 cm in height) containing a piece of wet paper towel at 4 °C under LD 18:6 h. The Petri dish was sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, MN, USA) to maintain high humidity. The females laid egg masses on the paper towel within 1–2 days after emergence, and the egg masses were transferred to another Petri dish.

Egg mass supercooling point measurement and egg hatching from the frozen egg mass

Three egg masses collected from the 2017 cohort were maintained at 4 °C in a Petri dish. The number of fertilized eggs was counted 15 days after oviposition. The excess water surrounding the mass was removed using a paper towel. Thereafter, each egg mass was placed in a 0.6-mL plastic tube with a T-type thermocouple (JBS-7115-5M-T, GRAPHTEC, Yokohama, Japan) inserted into the egg mass jelly. The tube was sealed with Parafilm and placed in a freezer to cool at −1.1 °C min^{-1}. The temperature was measured every second using a midi LOGGER GL220 (GRAPHTEC) data logger. The SCP was determined by the temperature at which the release of the latent heat of fusion or exotherm occurred (Carrillo et al. 2004; Sinclair 1999).

After the egg masses reached −11.2 °C, they were removed from the freezer and transferred to a Petri dish at 4 °C under LD 18:6 h to observe hatching.

Embryonic and larval development at various temperatures

One to five egg masses deposited at 4 °C from the 2017 and 2018 cohorts were continuously maintained at 4 °C or transferred to 0 or 10 °C within 24 h after oviposition. The egg masses from the 2017 cohort were also exposed to 15 °C. We used several photoperiodic conditions in the experiment in 2017 due to logistic unavailability (constant darkness at 0 °C, LD 18:6 h at 4 °C, LD 10:14 h at 10 °C, and LD 16:8 h at 15 °C) using the fluorescent lamp. The photon flux densities were 0.00, 3.18, 7.90, and 22.32 μmol $m^{-2} \cdot s^{-1}$ for the 0, 4, 10, and 15 °C experiments, respectively. In the 2018 experiment, the photoperiod was set at LD 18:6 h with a white laser-emitting diode (Zenigata LED, 3 W class, Sharp, Sakai, Japan). The photon flux densities were 1.95, 1.30, and 1.85 μmol $m^{-2} \cdot s^{-1}$ for the 0, 4, and 10 °C experiments, respectively. Each egg mass was kept on a piece of wet paper towel in a Petri dish sealed with Parafilm. The embryos were observed daily.

The proportion of fertilized eggs was calculated by dividing the number of fertilized eggs by the total number of eggs in the mass. The hatchability was calculated by dividing the number of hatched larvae by the number of fertilized eggs. The embryonic developmental rate was calculated as the reciprocal of the embryonic period (days) and regression was calculated from the data at 0, 4, and 10 °C. The total effective temperature (K) was determined by the linear model (Damos and Savopoulou-Soultani 2012):

$$K = y \times (x - a)$$

where y is the embryonic period, x is the ambient temperature, and a is the lower developmental threshold. The lower developmental threshold was estimated by extrapolation

of the regression line and was determined as the point at which the developmental rate became zero.

Newly hatched larvae were maintained under the same environmental conditions as the embryos. Milk agar (2% milk and 0.5% agar in water) was used as the larval food in 2017. The larvae were fed milk agar with powdered phytophagous fish meal (Hikari Crest Pleco; Kyorin Company, Himeji, Japan) in 2018. The Petri dish and food were replaced every 10 days, and the number of surviving larvae and larval stages were recorded.

Statistical analysis

Hatchability was analyzed using Tukey-type multiple comparisons of proportions.

Results

Egg mass supercooling point measurement and egg hatching from the frozen egg mass

SCP data for the three egg masses are presented in Table 1. The average ± SD of the SCPs was −4.7 ± 1.1 °C. After the egg masses reached −11.2 °C, they were removed from the freezer and maintained at 4 °C under LD 18:6 h to observe egg hatching. The hatchability was high and ranged from 87.5% to 96.0% (Table 1).

Embryonic and larval development at various temperatures

The thermotolerance of embryos exposed to temperatures over the long term was also investigated. In the experiment in 2017, we maintained the egg masses with the proportions of

157 fertilized eggs of 70.1% (n = 211), 77.7% (n = 413), 63.5% (n = 266), and 95.2% (n = 145) at 0, 4, 10,
158 and 15 °C, respectively. Although statistical differences were detected (Tukey-type multiple
159 comparisons of proportions, $P < 0.01$), hatchability higher than 93.9% was observed at all
160 temperatures (Fig. 1a). In 2018, we maintained the egg masses with the proportions of fertilized
161 eggs of 41.7 % (n = 151), 93.8% (n = 48), and 78.8% (n = 52) at 0, 4, and 10 °C, respectively. The
162 hatchability was also high (ranging from 95.6 to 100.0%), irrespective of the temperature
163 conditions (Fig. 1b).

164 The developmental rates were 0.02 ± 0.00, 0.05 ± 0.00, 0.10 ± 0.00, and 0.16 ± 0.03 day^{-1}
165 (average ± SD) at 0, 4, 10, and 15 °C, respectively, in 2017 (Fig. 1c). Because the developmental
166 rates at 15 °C varied due to less frequent hatching observation (once a day) and developmental
167 retardation in some embryos (see Online Resource 1), we performed a regression of the data
168 excluding those at 15 °C. The regression estimated a lower developmental threshold of −2.7 °C.
169 Using this threshold temperature, the total effective temperature necessary for development
170 was estimated to be 123.8 degree-days. The values of the developmental rate in 2018 were 0.02 ±
171 0.00, 0.05 ± 0.00, and 0.11 ± 0.00 day^{-1} (average ± SD) at 0, 4, and 10 °C, respectively (Fig. 1d). The
172 lower developmental threshold was −2.3 °C, and the total effective temperature was 112.6
173 degree-days. These values were nearly identical between 2017 and 2018. The combined data set
174 revealed regression $y = 0.0080x + 0.0211$ ($r^2 = 0.979$). The lower developmental threshold was
175 −2.6 °C and the total effective temperature was 122.4 degree-days.

176 We continuously observed the larval development at these temperatures (Fig. 2).
177 Although the survival rate was low, some larvae successfully developed to the final (fourth)
178 instar stage at 4 °C in 2017. In contrast, no first instar larvae developed to the second instar at
179 0 °C. At 10 and 15 °C, only a small portion of larvae developed to the third instar, but these did
180 not continue developing further to the fourth instar. Similar results were observed in 2018, in

which the larvae performed successfully at 4 °C, but not at 0 or 10 °C. The days when half of the larvae reached the fourth instar stage at 4 °C were 388 and 210 days in the 2017 (only milk agar) and in 2018 (milk agar and the fish meal) experiments, respectively, suggesting that nutritional conditions greatly affect the larval developmental rate.

Discussion

The mean SCP of the *B. antarctica* egg mass (eggs with jelly) was −4.7 °C. The SCPs of the field-collected larvae were relatively constant between −6 and −8 °C throughout the year (Baust and Lee 1987), and the SCP of adults was −5.3°C (Baust and Edwards 1979). Similar values have been reported in other studies (Hayward et al. 2007; Kawarasaki et al. 2014). Thus, the SCP of the egg mass was comparable to those at other stages. The first instar larvae successfully emerged from the egg masses exposed to −11.2 °C. However, the ground temperature during summer typically never reaches such a low value, which ranges from approximately −3 to 2 °C (Kawarasaki et al. 2014). Thus, the embryos would show sufficient tolerance to withstand the cold during the Antarctic summer.

The long-term effects of environmental temperatures on embryonic development were examined. High hatchability at 0, 4, 10, and 15 °C indicates that *B. antarctica* embryos exhibit sufficient thermotolerance to withstand hot Antarctic summer days. The total effective temperature indicate that egg hatching occurs in spring or early summer in the Antarctic habitat, which corresponds to the field observations (Sugg et al. 1983). Interestingly, the embryonic period of *B. antarctica* is shorter than that of *Eretmoptera murphyi* (Bartlett et al. 2019), which is a sister species to *B. antarctica* (Allegrucci et al. 2012). *E. murphyi* is a non-native maritime Antarctic species that was introduced to the Signy Islands from sub-Antarctic South Georgia in the 1960s (Block et al. 1984; Convey and Block 1996). *E. murphyi* requires 29–41 days

to complete embryonic development at 5 °C (Bartlett et al. 2019), whereas *B. antarctica* requires approximately 16 days at the same temperature (the present study). This indicates that *B. antarctica* is well-adapted to the low-temperature environment in Antarctica.

The present study revealed that the first instar larvae can develop to the fourth instar at 4 °C, which approximates the ground temperatures of their habitat in summer. However, at other temperatures (0, 10, and 15 °C), the larvae performed poorly and failed to develop. Thus, the early instar larvae have a narrow temperature window for development and may be susceptible to environmental changes. Previous studies have revealed remarkable thermotolerance of *B. antarctica* larvae, but these studies focused on the tolerance of fourth instar larvae to short-term temperature stresses and did not consider the tolerance of the early instar larvae to long-term stresses (Baust and Lee 1987; Elnitsky et al. 2008; Lee et al. 2006; Rinehart et al. 2006). Recently, Devlin et al. (2022) reported the negative effects of long-term temperature increments in overwintering fourth instar larvae; the survival, energy stores, and locomotor activity were significantly lower following the warm overwintering environment than at normal and lower temperatures. These results, together with those of the present study, suggest that extremophile larvae are still susceptible to environmental changes, such as global warming.

One area of future work is to clarify the performance of embryos and larvae under more ecologically relevant conditions, such as fluctuating temperature regimes (Colinet et al. 2015). Because we investigated only two cohorts, it is also critical to investigate the ability of various populations inhabiting various microenvironments (Spacht et al. 2021) to determine whether our findings are characteristic of this species or certain populations. Another area for future work is the physiological analysis of embryonic and larval thermotolerance. The constitutive expression of *hsp*s contributes to larval tolerance to thermal extremes (Rinehart et

al. 2006). It is of interest to investigate when *hsp*s begin to be upregulated during embryonic or larval development.

Author Contributions

MY and SGG conceived and designed the study. MY conducted the experiments. MY analyzed data. MY and SGG wrote the manuscript. Both authors have read and approved the manuscript.

Data availability

All the data obtained during this study are included in this article and its online resource.

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Statements and Declarations

We have no declarations.

253 **Competing Interest**

254 We declare we have no competing interests.

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256

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359

360 **Table 1** Supercooling point of egg mass and hatchability

361

Egg mass	The total number of eggs	The number of fertilized eggs	Supercooling point (°C)	Hatchability (%)*
#1	35	15	−4.0	93.3
#2	106	16	−3.8	87.5
#3	50	48	−6.2	96.0
			−4.7 ± 1.1 (average ± SD)	93.8§

362 * Number of hatched larvae divided by the number of fertilized eggs. The hatching was

363 observed at 4 °C in the egg masses exposed to −11.2 °C.

364 § Total number of hatched larvae divided by total number of fertilized eggs.

365

366

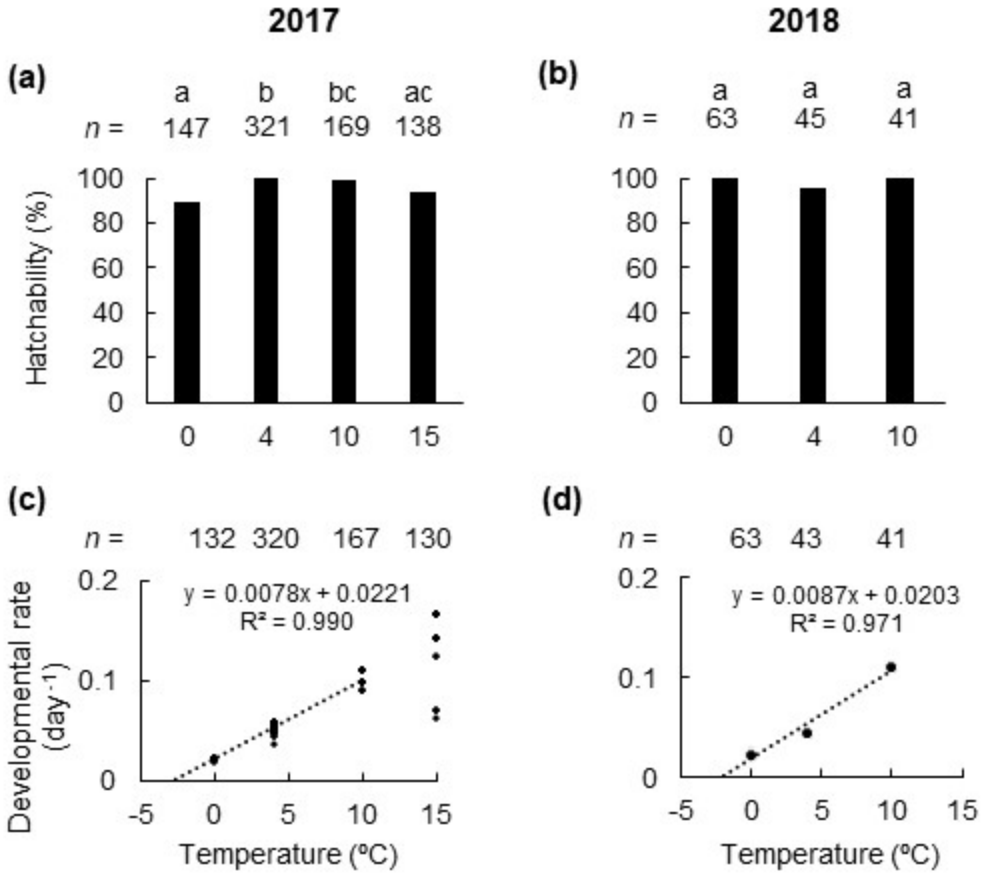
367 **Figure legends**

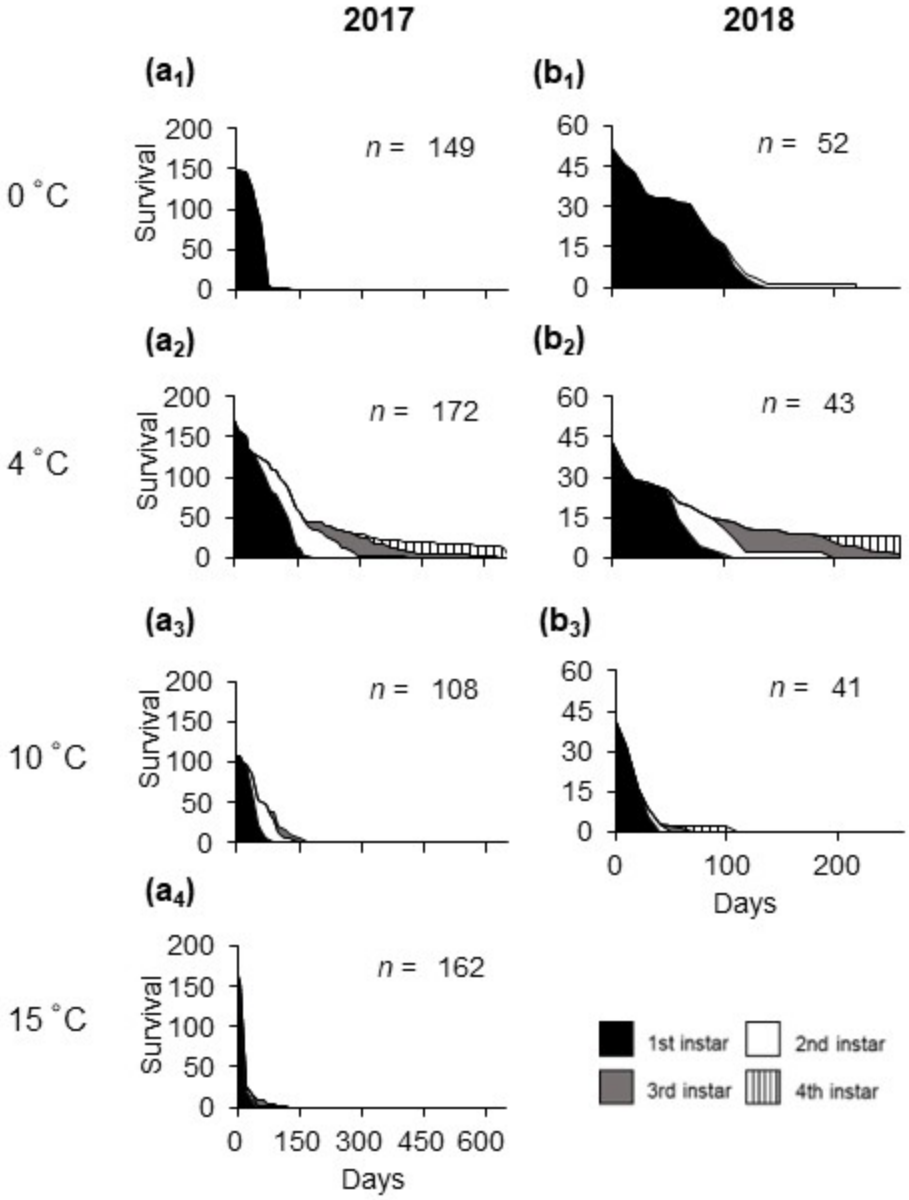
368

369 **Fig. 1** Hatchability and developmental rates at various temperatures. Hatchability (*a*, *b*) and
370 developmental rate (*c*, *d*) of *Belgica antarctica* embryos were measured at 0, 4, 10, and 15 °C. Eggs
371 collected in 2017 (*a*, *c*) and 2018 (*b*, *d*) were used in this study. Different letters above the bar in *a*
372 and *b* indicate statistically significant differences (Tukey-type multiple comparisons of
373 proportions, $P < 0.01$). The dotted lines in *c* and *d* are simple regressions calculated from the
374 developmental rates at 0, 4, and 10 °C. The equation and r^2 value of the regression are also
375 shown. *n* is the number of fertilized eggs (*a* and *b*) or the number of hatched eggs (*c* and *d*).

376

377 **Fig. 2** Survival of larvae at various temperatures. Larval survival of *Belgica antarctica* was
378 observed at 0 (a_1 and b_1), 4 (a_2 and b_2), 10 (a_3 and b_3), and 15 °C (a_4) in 2017 (a_1–a_4) and 2018 (b_1–
379 b_3). Larval instars are shown. *n* is the number of larvae.


2017
2018
(a)
(b)
(c)
(d)
a b bc ac
n = 147 321 169 138
a a a
n = 63 45 41
Hatchability (%)
100
80
60
40
20
0
0 4 10 15
0 4 10
n = 132 320 167 130
n = 63 43 41
Developmental rate (day-1)
0.2
0.1
0
y = 0.0078x + 0.0221
R² = 0.990
y = 0.0087x + 0.0203
R² = 0.971
-5 0 5 10 15
Temperature (ºC)


2017
2018
(a1)
(b1)
(a2)
(b2)
(a3)
(b3)
(a4)
0 °C
4 °C
10 °C
15 °C
Survival
n = 149
n = 52
n = 172
n = 43
n = 108
n = 41
n = 162
Days
1st instar
2nd instar
3rd instar
4th instar