

# Strong differences in egg heat tolerance between closely related lowland and alpine butterfly species

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## Abstract

1. Temperature variation is one of the best-known and studied factor constraining life history along the altitudinal gradients.
2. Ectotherms such as insects are sensitive to temperature across all life stages, resulting in a wide range of adaptations in populations and species living at different elevations. In butterflies, the egg stage is particularly sensitive to temperature variation as eggs cannot move and deploy behavioural responses to overheating.
3. Using a gradient polymerase chain reaction thermocycler to finely control temperature treatments, we tested for differences in egg heat tolerance between five closely related *Coenonympha* (Nymphalidae: Satyrinae) species living at different elevations.
4. We found strong differences in egg heat tolerance ranges between lowland species (*C. arcania* (Linnaeus) and *C. pamphilus* (Linnaeus)) and two high elevation alpine species (*C. gardetta* (Prunner) and *C. cephalidarwiniana* Verity). Surprisingly, the third alpine species (*C. darwiniana* Staudinger) showed a heat tolerance range closer to that of the lowland species than that of alpine species.
5. The sensitivity of eggs to overheating matched the temperature regimes recorded in the microhabitats utilised for oviposition and may explain the oviposition site selection of females observed in the wild.
6. The narrower egg heat tolerance of some of alpine species may partly explain the position of their lower elevational limit but also raises concern about the fate of these species in the face of current climate change.
7. Our results highlight the need to study all life stages, as well as microclimate choice for oviposition, to understand species distribution and the threats to their persistence.

## KEYWORDS

climate change, *Coenonympha*, elevational gradient, Satyrinae

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## INTRODUCTION

The elevational gradient imposes strong selection on populations as many ecological factors vary abruptly with elevation (Hodkinson, 2005), notably temperature, which decreases linearly by 0.5°C per 100 m of ascent (Körner, 2007). In butterflies, the effect of such strong temperature gradient at small geographic scale have been studied at both microevolutionary (e.g. Montejo-Kovacevich et al., 2019; Klockmann & Fischer, 2017; Nève & Després, 2020; Vrba et al., 2022) and macroevolutionary scales (Kleckova et al., 2014; Leingärtner et al., 2014; Nève & Després, 2020). Experimental approaches to study temperature tolerance often focussed on the adult stage, but stressors such as thermal extremes can affect life stages differently. It is therefore important to study insect thermal tolerance in all stages (Kingsolver et al., 2011; Klockmann et al., 2017; Klockmann & Fischer, 2017; Radchuk et al., 2013). The butterfly egg stage is immobile, and the developing caterpillar embryo is unable to respond behaviourally to overheating (Bennett et al., 2014). A few studies have highlighted the importance of egg thermal tolerance in geographic and elevational distributions of butterfly species and their oviposition strategies (Bennett et al., 2014; Davies et al., 2006; Lawson et al., 2012; Merrill et al., 2008). However, none has compared different species distributed along the altitudinal gradient to determine if the egg stage can contribute to interspecific thermal niche and distribution differences.

In this study, we hypothesise that the elevation range of *Coenonympha* (Nymphalidae: Satyrinae) species might be linked with heat tolerance of eggs as they are exposed to higher thermal maxima in lowlands than at high elevations. Closely related species within the genus replace each other along the elevational gradient despite their similar phenotypes, phenology and host plants. Based on the potential role of egg heat tolerance in butterfly distribution and ecology, and considering that females were observed choosing shady microhabitats for oviposition (Pers. Obs.), we hypothesised that the temperature differences experienced by eggs laid at different elevations could influence species distributions. To test this hypothesis, we (i) estimated experimentally the range of heat tolerance of eggs in five *Coenonympha* species and (ii) tested for intraspecific variation in egg heat tolerance linked to elevation (local adaptation) for two of our study species. Last, (iii) we experimentally assessed how temperature variation in microhabitats typical of egg laying sites could affect exposure of eggs to heat and oviposition preferences. We discuss the differences in egg thermal adaptation with regard to known differences in distribution and elevational range between these species.

## MATERIALS AND METHODS

### Study species and egg collection

The *Coenonympha arcania-gardetta* complex is composed of four, closely related, univoltine species. *Coenonympha arcania* (Linnaeus) is a widespread Western Palearctic species found at low elevation

(0–1600 m), and *C. gardetta* (Prunner) is an alpine grassland specialist found at high elevation (1600–2600 m). *Coenonympha gardetta* is replaced in two alpine regions by two other species, also found at high elevation (1600–2500 m): *C. darwiniana* Staudinger in the Swiss and Italian Ticino regions and *C. cephalidarwiniana* Verity occupying the southern part of the French and Italian Alps (taxonomy following Gallo & Bisi, 2020). *Coenonympha cephalidarwiniana* and *darwiniana* exhibit a different mix of *gardetta* and *arcania* ancestries and have been proposed to represent divergent hybrid species originating from a single hybridization event (Capblancq et al., 2015). These hybrid species exhibit intermediate phenotypes and ecology, occupying a high elevation niche but being often associated with warmer habitats than *gardetta* (Capblancq et al., 2019). *C. pamphilus* (Linnaeus) is a multivoltine, ubiquitous and widespread species in Western Europe, living in all types of open habitats from hot and dry Mediterranean steppes to humid and cold alpine grasslands from sea level to over 2000 m of elevation (Gil-Tapetado et al., 2022).

Females of each species were caught using nets at various locations in June–July 2022 and 2023 (Table 1). Females were placed for 2 days in rearing cages and were fed using a mixture of sugar, honey and water. Females readily laid eggs on the mesh of the cages. After 2 days, eggs were collected using a fine wet paintbrush and pooled by population in petri dishes.

### Egg heat tolerance range estimation

For each species and locality, eggs were individually placed in the 96 wells of polymerase chain reaction (PCR) plates. An Eppendorf Mastercycler Gradient PCR thermocycler was used to expose each plate column (eight wells per column) to temperatures ranging from 40.1°C to 50.4°C for 1 h. The exact temperatures of each column were imposed by the technical constraints of the gradient thermocycler and were distributed as follows: 40.1°C; 40.1°C; 40.7°C; 41.5°C; 42.7°C; 44°C; 45.3°C; 46.7°C; 48°C; 49.1°C; 49.9°C; 50.4°C (Figure S1A). The temperature extremes were chosen following Bennett et al. (2014) who showed a decrease in hatching rate for temperatures above 48°C in another Nymphalid butterfly, *Euphydryas editha* (Boisduval). After exposure to heat, plates were placed at room temperature and egg hatching, coded as 1 for hatched egg or 0 for unhatched (dead) egg, was checked twice a day for 10 days.

### Field temperature measurements

In order to estimate the temperature putatively experienced by eggs in nature and to evaluate to what extent selecting shaded microhabitats could limit the exposure of eggs to extreme temperatures, dataloggers (HOBO Pendant MX Temperature Data Loggers) monitoring temperature every 15 minutes were placed in the field for 3 days during the flight season (4–7 July 2022). Both *arcania* (1500 m) and *cephalidarwiniana* (2000 m) collecting sites (Table 1) were surveyed by placing dataloggers in pairs: one in the shaded grass under a bush,

**TABLE 1** Localities where the gravid females of the different *Coenonympha* species were collected, providing information on locality name, latitude, longitude, elevation and collection date.

Species	Site	Latitude	Longitude	Elevation	Collecting date	Number of females	Number of tested eggs (PCR plates)
<i>C. arcania</i>	Canal de Mal Cros, Saint-Michel-de-Chaillol, France	44.6898	6.1536	1500 m	3 July 2022 and 1 July 2023	40 and 40	384 (4) and 480 (5)
<i>C. arcania</i>	Gorges de la Vis, Vissec, France	43.8994	3.4490	500 m	6 June 2023	20	672 (7)
<i>C. cephalidarwiniana</i>	Bois des Oures, Saint-Michel-de-Chaillol, France	44.6984	6.1669	2000 m	4 July 2022 and 2023	40 and 40	384 (4) and 480 (5)
<i>C. gardetta</i>	Roche noire valley, Le Monétier-les-Bains, France	45.0546	6.3864	2300 m	11 July 2022	40	288 (3)
<i>C. gardetta</i>	Plateau de l'Arselle, Chamrousse, France	45.0994	5.8811	1700 m	3 July 2023	30	576 (6)
<i>C. darwiniana</i>	Bochetta di Muino, Toceno, Italy	46.1712	8.4915	1850 m	9 July 2023	50	864 (9)
<i>C. pamphilus</i>	Gorges de la Vis, Vissec, France	43.8994	3.4490	500 m	6 June 2023	10	288 (3)

Note: The number of females and the number of eggs used in the experiments per species and site are also provided. Abbreviation: PCR, polymerase chain reaction.

resembling a site where females were observed ovipositing, and one 1–2 m away under a tuft of grass in an unshaded open meadow, for comparison (positioning example in Figure S1B).

## Statistical analysis

Egg survival rate was analysed with a Generalised Linear Mixed-Effects Model (GLMM) using a binomial link function, temperature and sampling site elevation as fixed effects and PCR plate, plate column and catching year as random effects using the *lme4* R package (Bates et al., 2015). To test for intraspecific variation in egg heat tolerance, which might result from local adaptation, egg batches obtained from females of *arcania* and *gardetta* caught from populations at two different elevations were considered separately. As no intraspecific variation in egg heat tolerance was detected, all five species were analysed with sites pooled by species and in the same way but using temperature and species as fixed effects and PCR plate, plate column and sampling year as random effects.

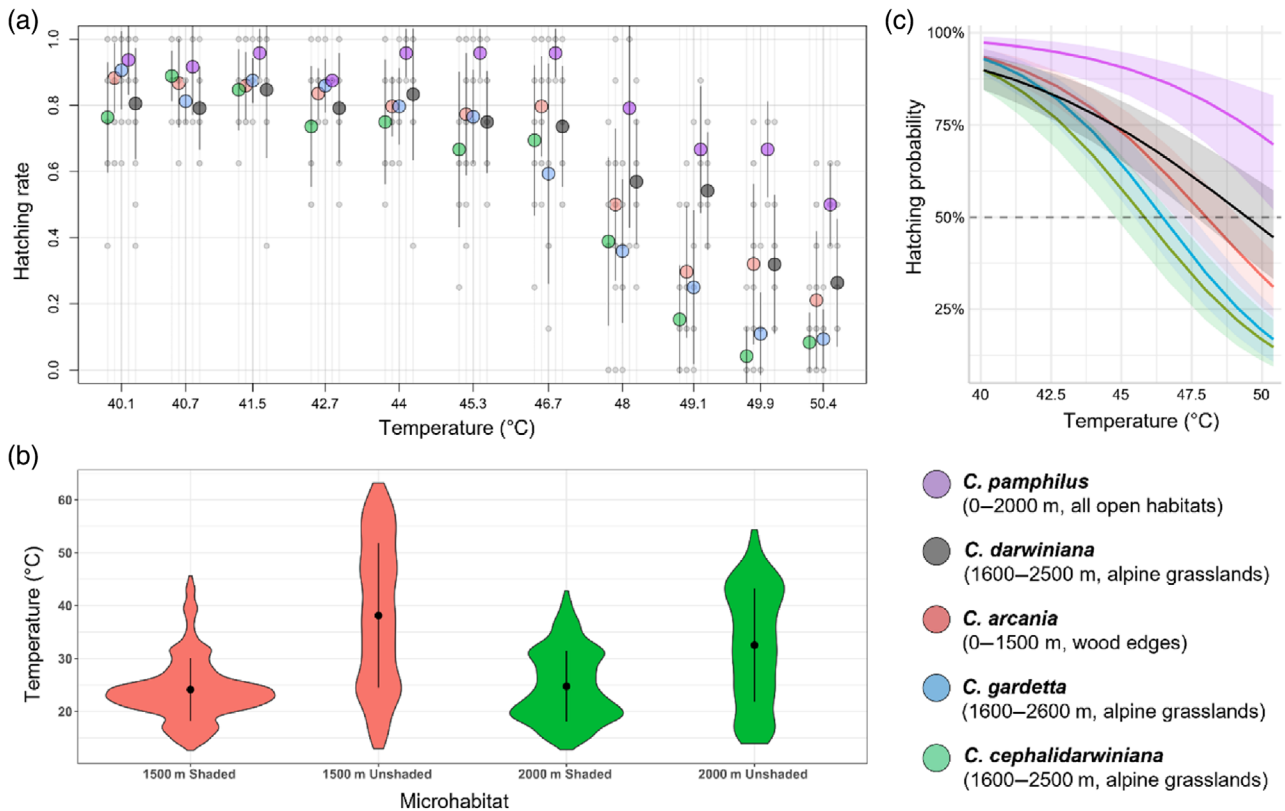
## RESULTS AND DISCUSSION

In order to determine the importance of egg thermal tolerance in the elevational distribution of *Coenonympha* butterflies, we developed an innovative yet simple protocol to assess the heat tolerance of eggs. This protocol allowed us to subject a large number of eggs simultaneously and quickly to precise temperature values encompassing a

wide temperature range (Table 1). The high hatching rate observed for lower temperatures (>85% between 40.1°C and 45.3°C) coupled with the sharp decline at higher temperatures indicates a clear sensitivity of eggs to heat in the five *Coenonympha* species (Figure 1a; Data S2). Field temperature measurements produced a total of more than 6800 measures over the 3 days of recording and validated the relevance of the chosen temperature range: At 1500 m, temperature only rarely and briefly exceeded 40°C and almost never exceeded 45°C in shaded sites, whereas it exceeded 45°C for large parts of the day and often exceeded 50°C in unshaded sites. At 2000 m, temperature almost never exceeded 40°C in shaded sites and rarely and briefly exceeded 45°C in unshaded sites (Figure 1b; Data S2).

### Differences in egg heat tolerance among *Coenonympha* species

Intraspecific variation in egg heat tolerance was evaluated in relation to site elevation (i.e. local adaptation) in the two so-called “parental” species of the *arcania*–*gardetta* complex. In both *arcania* and *gardetta*, the fitted GLMMs retained a strong negative effect of temperature on hatching probability but no significant effect of elevation (Details in Data S2). Egg thermal tolerance was therefore stable across study sites despite elevational differences and therefore exposure to different temperature regimes. Local adaptation to climatic differences does not seem to be mediated by egg heat tolerance variation in these species. Although the flight periods of *arcania* and *gardetta* are broadly similar (late June to late July in the Alps), there are



**FIGURE 1** (a) Final egg hatching rate of the five *Coenonympha* species tested after being exposed for 1 h to temperatures ranging from 40.1°C to 50.4°C. Coloured dots represent mean hatching rates, error bars represent standard deviation intervals, and grey dots represent replicates containing eight eggs (polymerase chain reaction [PCR] plate columns). (b) Field temperature distributions measured in different microhabitats and elevations. Colours represent the species present at the measuring sites, dots represent means and error bars represent standard deviation intervals. (c) Projection of the best fitting Generalised Linear Mixed-Effects Model (GLMM) [Hatching success ~ Temperature × Species + (1| PCR plate) + (1| plate column) + (1| Year)] fitted on the five species. Estimated temperature associated with a 50% hatching rate (T50): *C. gardetta*: 45.8°C; *C. cephalidarwiniana*: 46.4°C; *C. arcania*: 48°C; *C. darwiniana*: 48.2°C; *C. pamphilus*: 53.5°C (above the tested temperatures). Note that *C. cephalidarwiniana* and *C. darwiniana* are considered to be hybrid species originating from hybridization between *C. gardetta* and *C. arcania*.

phenological differences between populations at different elevations. Indeed, lowland *arcania* females were sampled in early June, whereas those from higher elevation were sampled in early July. Similarly, *gardetta* females from lower elevation were captured in early July, 2 weeks before those from higher elevation (mid-July). Given the strong temperature gradient across elevation, the tracking of the optimal thermal niche by each species may involve intraspecific phenological shifts rather than changes in egg heat tolerance.

Across *Coenonympha* species, the best fitting GLMM retained a strong negative effect of temperature on hatching probability (Figure 1a,c; Data S2). Model estimates show a severe reduction in egg hatching probability above 45°C in the *C. arcania*–*gardetta* species complex, whereas *C. pamphilus* exhibits a clear although shallower decline in hatching probability (Figure 1c), highlighting a diversity of egg heat tolerance among *Coenonympha* species. Of the species tested, *pamphilus* has the widest ecological niche and distribution, thriving in conditions that are significantly warmer than those of the species of the *arcania*–*gardetta* complex (Gil-Tapetado et al., 2022). *Coenonympha pamphilus* maintains high egg hatching rates when

subject to much higher temperature extremes than the other species tested. The ability of *pamphilus* to persist in hotter habitats than the other tested species could therefore reflect a higher egg heat tolerance. Within the *arcania*–*gardetta* complex, *arcania* and *darwiniana* maintained high hatching rates at higher temperatures than both *gardetta* and *cephalidarwiniana* (Figure 1a,c). *Coenonympha arcania* is indeed occupying a lower elevational niche, from sea level to ca. 1600 m, than the alpine species, and selection may act on the tolerance of its eggs to higher temperatures. By contrast, the lower egg heat tolerance ranges of *gardetta* and *cephalidarwiniana* associates with their higher and colder elevational niche and may participate in determining their lower elevational limit (approx. 1500 m) by excluding them from lower elevations (Franzén & Molander, 2012). Species exclusion from lower elevations could be expected via a direct effect of temperature on egg mortality; however, thermal stress at the egg stage may also negatively impact subsequent life stages such as larval survival, developmental rate, body mass and heat stress tolerance, pupal development rate or adult body mass (Bennett et al., 2014; Davies et al., 2006; Klockmann et al., 2017; Lawson et al., 2012;

Merrill et al., 2008). However, the relatively high egg heat tolerance of *darwiniana* compared with that of *gardetta* and *cephalidarwiniana*, despite their similar elevation ranges is more surprising. Indeed, niche modelling in the species complex suggests that *darwiniana* uses a cold and humid climatic niche similar to *gardetta*'s (Capblancq et al., 2015). The higher egg heat tolerance found here in *darwiniana* could be an adaptation to exposure to higher temperature in its range, to local adaptation of the south facing population sampled for this experiment or to other differences in the egg ecology or egg-laying behaviour of *darwiniana* that might incur selection on higher temperature tolerance.

### Potential interaction between egg thermal tolerance and climate change

The overall higher sensitivity of alpine species to temperature is of concern in the context of climate change as alpine species may experience temperatures rising over their thermal tolerance. This may cause their lower altitudinal limit to shift upwards, with possible consequences on local extinction probability (Franzén & Molander, 2012). In nature, our observations suggest that egg-laying females spend significant time when selecting egg-laying sites. Oviposition behaviour consists in a slow geotactic walk down the stem of a bush or a tuft of grass, reaching dry material near the litter where a single egg is laid (duration approx. 15 minutes/egg; Pers. Obs.). The vulnerability of eggs to high temperatures observed in our experiments and the field temperatures measured in unshaded places suggest that egg-laying behaviour might respond to selection on egg hatching rates. In a group of butterflies found in relatively open habitats, and whose caterpillars eat grass, ovipositing in shaded places may contribute to avoid egg overheating (Bennett et al., 2014). Evolution of egg thermal tolerance and egg laying strategies could, like the phenological advance observed in lowland *arcania* populations, be responses to the risk of overheating and, more generally, may be predicted to contribute to adaptive responses in alpine species in face of climate change.

### AUTHOR CONTRIBUTIONS

**Paul Doniol-Valcroze:** Conceptualization; investigation; writing – original draft; methodology; validation; visualization; writing – review and editing; formal analysis; data curation. **Laurence Després:** Conceptualization; investigation; funding acquisition; validation; writing – review and editing; project administration; supervision; resources. **Mathieu Joron:** Supervision; resources; project administration; conceptualization; investigation; funding acquisition; methodology; validation; writing – review and editing.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

### DATA AVAILABILITY STATEMENT

Raw data are supplied in Data S2.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1.** (A) Scheme of PCR plate experiment with the temperature applied to eggs using a gradient PCR thermocycler. Each black dot represents one egg. (B) Hobo datalogger positioning examples. Red arrows show unshaded positions and blue arrows shaded positions (Pictures Mathieu Joron).

**Data S2.** Page 1: Summaries of the best fitted GLMMs. Page 2: Hatching success for each of the tested species and plates. Page 3: Measured field temperatures depending on elevation and shadowed status. Time is given in UTC +1 time (Paris legal time).

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