Variation in sensitivity to temperature, but not to photoperiod, underlies genetic variation in timing of egg-hatching in the winter moth

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Submitted
Abstract

Phenological synchrony of reproduction and growth with the annual fluctuation in food availability, temperature and precipitation are crucial for survival in most species living in temperate environments. Phytophagous insects can only feed when their host-plant is available. In the winter moth (*Operophtera Brumata*, Lepidoptera, Geometridae) the larvae feed on young oak leaves until they pupate in the soil. In this species a phenological mismatch between egg-hatching and development of oak leaves has been shown to have severe fitness consequences on larval survival and adult fecundity. In order to appropriately time their hatching, winter moth eggs need to integrate environmental cues such as photoperiod and temperature. Here we show that there is large variation in timing of egg-hatching among clutches confirming that there is genetic variation in egg-hatching date. It is this variation natural selection can act on. We tested, in a laboratory experiment, if winter moth eggs that were exposed to different photoperiodic regimes differed in their egg-hatching date and we show that photoperiodic information is not used to time egg-hatching. As all eggs were exposed to the same temperature, we conclude that the differences observed in timing of egg-hatching were due to genetic variation in temperature sensitivity. We thus provide the first experimental evidence that the micro-evolutionary change in egg-hatching is likely to involve a change in the mechanisms underlying winter moth response to temperature, and not to photoperiod. Egg-hatching is likely to involve a change in the mechanisms underlying winter moth response to temperature, and not to photoperiod.
Introduction

Seasonality is a key factor that shapes life-history characteristics of mosorganisms living in temperate regions. In most species, behavioural, physiological and metabolic processes are affected by the seasonal changes in temperature, photoperiod and food availability. Several biological activities such as feeding, growing or reproducing have to be appropriately timed to match favourable environmental conditions or resource availability. To time these activities, organisms integrate biotic and abiotic cues such as temperature, photoperiod, food availability or other species-specific stimuli. Among these, annual photoperiodic regime and fluctuations in temperature are the most important cues in many species.

Nowadays, increasing temperatures are altering phenologies of many organisms (Parmesan et al. 2003; Root et al. 2003; Parmesan 2006). The ecological consequences are complex and diverse. Most species have advanced their phenology; however, species at different trophic levels have shifted their phenology at a different pace (Visser et al. 2005; Thackeray et al. 2010). Especially in ectotherms, increased ambient temperatures have direct consequences for metabolic rates, activity patterns and developmental rates (Bale et al. 2002). As a consequence, increased temperature may have detrimental effects on phenological interactions such as predator-prey or herbivore-host plant. In the case of herbivorous insect species, a phenological mismatch between the insect’s active period and the host-plant availability could have strong negative impact on the insect’s fitness (Feeny 1970; Mattson 1980; van Asch et al. 2007b). As the environment changes, organisms need to change by altering their response to the environmental cues and adapt in this way to the new conditions.

In our study species, the winter moth (Operophtera brumata), the phenological match with the host plant, the oak (Quercus robur), has been extensively studied (Embree 1970; Buse et al. 1996; Buse et al. 1999; van Asch et al. 2007a). The disruption of the phenological synchrony between the timing of egg-hatching and the bud burst of the host-plant has severe fitness consequences for the newly hatched larvae. In recent years, increased temperatures have led to a faster insect development and as a consequence to an increased phenological mismatch with the oak. In response to their changing environment and to the resulting selection pressure for late-hatching, the winter moth has adapted genetically (van Asch et al. 2013). Both field and experimental data show that nowadays the eggs, when kept at the same temperatures, take longer to hatch than ten years ago (van Asch et al. 2013). Changes in temperature sensitivity have been hypothesized, whether the underlying mechanism is based or includes sensitivity to other cues has not yet been elucidated. Winter egg-development is well-known to be highly temperature dependent but it
is not known whether the photoperiodic information is also taken into account to regulate timing of egg-hatching. Thus, with this study we aimed to unravel whether photoperiodic cues are used by winter moth eggs to time egg-hatching and whether there is genetic variation in the sensitivity to photoperiod. We exposed developing winter moth eggs to five different photoperiod treatments that mimicked the natural day-night schedule progressing with the calendar date and we scored egg-hatching date. The five photoperiodic treatments differed with respect to the time of the year at which they were applied: two treatments mimicked early or late photoperiodic schedule, respectively, and one treatment, used as control, mimicked the same photoperiodic schedule as during the time of the year at which the experiment was carried out. We expected eggs exposed to the late photoperiodic schedule to hatch earlier than eggs exposed to the early photoperiodic schedule. We used a split-brood design to also estimate any genetic effect in winter moth sensitivity to photoperiod.

With this study, we contributed to the understanding of the winter moth’s response to the environmental changes and, in particular, to the identification of the “wheels” which natural selection has acted upon in order to restore the phenological synchrony between the two trophic levels. Understanding the environmental cues a species uses, and whether the sensitivity to the cues can evolve is an essential step to be able to predict species’ response to climate change.

**Materials and methods**

*Origin of the animals and experimental design*

Winter moth eggs were obtained from wild-caught adult moths collected with funnel traps on oak trees (*Quercus robur*) in a forest near Oosterhout (51°52’22.30”N, 5°50’21.77”E) from 25th November to 29th November 2013. On the day of capture, moths were brought to the laboratory, weighed and placed in transparent tubes. Each tube contained a female and if available a male caught on the same date in the same trap. If the male was not available the female was place in the tube alone and the unfertilized eggs were discarded. All females were provided with tissue paper to lay their eggs on and were kept in an outdoor insectarium. After death, adult moths were removed and the tissue paper with the eggs was transferred to a Petri dish. The developing eggs were kept in the insectarium until the start of the experiment. On the 15th January, thirty-one egg clutches (size of clutch ranging from 200 to 300 eggs) were used for the experiment. All the egg clutches (here we refer to clutch as the total number of eggs laid by one female) were split in sub-clutches of approximately fifteen eggs. Each sub-clutch was randomly allocated to one of the five experimental treatments. As a result of this split brood design, each treatment group had the same
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genetic composition, thus we ensured that the differences observed were due to the photoperiodic treatments.

The photoperiodic treatments resembled the change in day length as under natural conditions at the same latitude. Eggs were exposed to photoperiodic treatments that mimicked the change in day length starting both on earlier and later calendar dates than the control treatment: 15\textsuperscript{th} December (very early photoperiod= EEP); 1\textsuperscript{st} January (early photoperiod= EP); 1\textsuperscript{st} February (late photoperiod= LP); 15\textsuperscript{th} February (very late photoperiod= LLP). The starting calendar date of the control treatment corresponded to the date at the start of the experiment: 15\textsuperscript{th} January (control photoperiod= CP). Each of the five photoperiodic treatments was replicated three times giving a total of fifteen groups. All experimental groups were kept in the same climate room at 10°C in independent boxes equipped with a light bulb (Goodbay LED 2W/6200K, white) and a ventilation system throughout the entire duration of the experiment. Egg developmental time was calculated for each sub-clutch as the total number of days from the capture date of the adult female moths (considered a proxy for the actual egg-laying date) till median egg-hatching date. To determine median egg-hatching date eggs were monitored for hatching three times a week and median egg-hatching date was calculated as the date at which 50% of the sub-clutch had hatched. Hatching success was high with almost all eggs hatching.

**Statistical analysis**

To analyse the effect of photoperiodic treatment on egg developmental time we performed a linear mixed model analysis (lmer function, lme4 package, R software version. 3.0.2.). Egg developmental time was normally distributed (visual inspection of the histogram). In the initial mixed model we fitted photoperiodic treatment as a fixed effect, family (i.e. mother identity) as random effect and we included the interaction term (treatment*family).

To perform the model selection procedure we first tested the significance of the interaction term using an analysis of variance (ANOVA) between the model with and without the interaction term. After removing the interaction (see result section), we tested the significance of the fixed effect using Kenward-Roger approximation (KRmodcomp function in the R package pbkrtest (Halekoh et al. 2014)). This statistical method for linear mixed models takes the model of interest (with the focal effect) and compares it with a reduced model (without the focal effect) by adjusting both the $F$ statistic and the degrees of freedom (df) (Kenward et al. 1997). Based on the result obtained from the Kenward-Roger approximation test, we used an ordered heterogeneity test (hereafter referred to as OH test) to test if the differences in egg developmental time followed the expected direction (Rice et al. 1994). The OH test
uses the \( p \)-value obtained from the survival analysis (non-directional test) and the Spearman’s correlation coefficient (\( \rho \)) to test the probability for a trend across the treatments in the predicted direction. All OH tests are one-tailed as we had a directional prediction of the effect of the photoperiodic treatments. Egg developmental time was expected to be the longest when eggs were exposed to the very early photoperiodic treatment (EEP) and shortest when eggs were exposed to the very late photoperiodic treatment (LLP). Thus the expected order was EEP≥EP≥CP≥LP≥LLP. Furthermore, we explored the importance of the random effect using a Restricted Likelihood Ratio Test for linear mixed models (exactRLRT function, RLRsim package)(Crainiceanu et al. 2004). In the latter test, we compared the reduced model with the random effect against the same model without random effect. The test is based on simulated values from the finite sample distribution and it tests whether the variance of a random effect is zero in a linear mixed model with known correlation structure of the tested random effect and independent and identically distributed errors.

## Results

Winter moth egg developmental time was not affected by photoperiodic treatments (OH test \( p>0.1 \); Fig. 1). The full model including the interaction term (photoperiodic treatment *family) was reduced to a model including only the fixed effect (photoperiodic treatment) and the random effect (family) as the interaction term was not significant (ANOVA \( p=0.9873 \)).

**Table 1** Statistical analysis of egg developmental time. Linear mixed model estimates (without intercept), standard error and T values are given for the five photoperiodic treatments. Very early (EEP), early (EP), control (CP), late (LP) and very late (LLP) photoperiodic treatments mimicked natural change in daylength starting on earlier and later calendar dates than the control treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Estimate</th>
<th>SE</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEP</td>
<td>102.86</td>
<td>0.9</td>
<td>113.7</td>
</tr>
<tr>
<td>EP</td>
<td>101.39</td>
<td>0.9</td>
<td>112.1</td>
</tr>
<tr>
<td>CP</td>
<td>101.38</td>
<td>0.9</td>
<td>112.0</td>
</tr>
<tr>
<td>LP</td>
<td>102.26</td>
<td>0.9</td>
<td>113.0</td>
</tr>
<tr>
<td>LLP</td>
<td>101.63</td>
<td>0.9</td>
<td>112.4</td>
</tr>
</tbody>
</table>
In the final model, the differences in egg developmental time between photoperiodic treatments \((p=0.0007; F_{\text{value}}=4.95; df=4; \text{Table 1 for model estimates})\) were not ordered along the expected treatment effects (OH test \(p>0.1\); expected order tested: \(\text{EEP} \geq \text{EP} \geq \text{CP} \geq \text{LP} \geq \text{LLP}\)). The restricted Likelihood Ratio Test to test for importance of the random effect was highly significant (RLRT=50703; \(p<0.0001\)). Overall, the variation in mean egg developmental time per family across the five photoperiodic treatments (Fig. 2) was much larger than the variation in mean egg developmental time found among treatments (Fig. 1).

**Figure 1** Winter moth egg developmental time for photoperiodic treatment. Data points represent linear mixed model estimates (±SE; see table 1 for statistics and model description). Photoperiodic treatments mimicked natural photoperiodic schedule starting on earlier or later calendar date than the control date (EEP=very early; EP=early; CP=control; LP=late; LLP=very late). Egg developmental time was not affected by the photoperiodic treatment \((p>0.01)\).
Our results experimentally show that winter moth egg developmental time is not affected by the photoperiodic information as eggs exposed to different photoperiodic treatments did not show differences in egg developmental time. We found, however, large variation in egg developmental time across clutches laid by different females (family). Difference in egg developmental time between early and late families ranged from 93 to 110 days. Previous studies by van Asch and colleagues (van Asch et al. 2007a) already provided evidence for genetic (co)variation in the egg-hatching date reaction norm, which means that egg developmental time varies depending on the genetic background.

Figure 2 Winter moth egg developmental time (mean±SE) per family. Point represents mean egg developmental time per family across all the five photoperiodic treatments. 31 families were used in the experiment. The variation in egg developmental time among families (93-100 days) was larger than the variation in egg developmental time among treatments.
With our experiment, we confirm that genetic variation in developmental time exists. Moreover, as we exposed the eggs to different photoperiodic treatments while keeping all the eggs at the same temperature, we could infer which cues winter moth eggs are sensitive to. Eggs from different families, kept at the same temperature but different photoperiods, had different egg developmental times. This result provide evidence that egg developmental time is affected by temperature and not to photoperiod. Although with this experimental design we cannot distinguish between the genetic background per se and epigenetic or maternal effects, it is likely that the latter do not play a role as the animals used in the experiment did not differ in catching date and location and were exposed to the same conditions during the process of egg-laying. Thus, our findings support the hypothesis that (genetic) differences in sensitivity to temperature rather than to photoperiod cause the variation in egg developmental time.

A possible mechanism explaining how differences in temperature sensitivity arises could be that, depending on their genetic background, the eggs have a slower or a faster developmental rate throughout the entire development. Another explanation is that the eggs take longer to complete development, i.e. eggs from late families, because they slow down or arrest their development at a given point of development. However, further physiological studies, in combination with modelling approaches, are needed to formally test these hypotheses and unravel the differences in the mechanisms underlying the egg’s (developmental) response to temperature. In the field, winter moth eggs have to hatch in synchrony with oak bud burst to successfully grow as larvae, pupate and reproduce as adults. Timing of egg-hatching is therefore a life-history trait under strong selection pressure. In previous studies, it has been shown that timing of egg-hatching is heritable and genetic variation in egg-hatching date is present in Dutch populations of winter moths (van Asch et al. 2007a). Further studies have also shown that, under selection pressures for longer developmental time, these populations have adapted to the new climatic conditions and reduced the phenological mismatch with the host plant. Therefore, this study, in combination with the previous findings, provides the first experimental evidence that the micro-evolutionary change observed in timing of egg-hatching in the winter moth is likely to involve a genetic change in the mechanisms underlying the phenological response to temperature, and not to photoperiod.

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