Genotypic and Environmental Effects on Flight Activity and Oviposition in the Glanville Fritillary Butterfly

Marjo Saastamoinen* **and Ilkka Hanski†**

Department of Biological and Environmental Sciences, University of Helsinki, P.O. Box 65 (Viikinkaari 1), Helsinki FI-00014, Finland

Submitted July 9, 2007; Accepted January 4, 2008; Electronically published April 15, 2008

abstract: Adverse environmental conditions constrain active flight and thereby limit reproduction in most insects. Butterflies have evolved various adaptations in order to thermoregulate, allowing females to search for nectar and to oviposit under unfavorable thermal conditions. We studied experimentally and with observational data the effect of low ambient temperatures experienced in the morning on the timing of oviposition and clutch size in the Glanville fritillary butterfly (*Melitaea cinxia*). Comparisons were made between individuals with different forms of the gene *Pgi*, encoding the glycolytic enzyme phosphoglucose isomerase, since naturally segregating variation at *Pgi* is known to be correlated with flight metabolic rate, flight performance, and fecundity. Experiencing low temperature in the morning delayed the initiation of oviposition and decreased clutch size. We used a thermal image camera to measure the thoracic surface temperature of butterflies immediately after voluntary flight. Single nucleotide polymorphism at *Pgi* was associated with thoracic temperature at low ambient temperatures. This has consequences for reproduction because females that are able to fly at lower ambient temperatures generally initiate oviposition earlier in the afternoon, when the environmental conditions are most favorable and the average egg clutch size is generally largest. These results suggest that variation in physiological and molecular capacity to sustain active flight at low ambient temperature has significant fitness-related consequences in insects.

Keywords: body temperature, clutch size, flight performance, phosphoglucose isomerase, single nucleotide polymorphism (SNP).

Temperature is a key environmental factor that affects development, growth, and survival of individuals and the dynamics of populations (Sinclair et al. 2003) especially in ectotherms, which lack effective intrinsic mechanisms to control body temperature. In terrestrial ectothermic vertebrates such as reptiles, behavioral thermoregulation is causally related to reproduction and patterns of life-history evolution (reviewed in Shine 2005). In butterflies and many other insects, active flight is essential for foraging, mate location, and oviposition; hence, time available for flight may constrain the ability of an individual to utilize resources and to reproduce (Kingsolver 1983). Insect flight is energetically very expensive (Suarez 2000) and requires the maintenance of high muscle temperature, around 30°–35°C in many butterflies (Watt 1973; Dennis 1993). To attain high body temperature, butterflies often use solar radiation (Shreeve 1992), and many species have evolved various behavioral, physiological, and morphological adaptations in order to thermoregulate (Van Dyck 2003). Thermoregulation is of great importance in temperate species in particular because they need to increase their body temperature well above the ambient air temperature to initiate flight. At the same time, small butterflies cool rapidly by convection during flight, especially in low ambient temperatures (Gilchrist 1990).

Comprehensive studies on *Colias* butterflies (reviewed in Watt 1992, 2003) and more recently on the Glanville fritillary (*Melitaea cinxia*; Haag et al. 2005; Saastamoinen 2007*a*) have demonstrated a significant correlation between allelic variation in the glycolytic enzyme phosphoglucose isomerase (PGI) and variation in flight metabolic rate, flight performance, and activity. In *Colias*, individuals with different PGI genotypes (allozymes) differ in their metabolic capacity, which leads to differences in flight performance (Watt et al. 1983). Both female fecundity and male mating success are directly influenced by flight performance and thereby also by the PGI genotype (Watt 1992). Female *Colias* with a particular PGI genotype (3/4 heterozygotes) are able to fly at lower ambient temperatures, which increases time available for flight (Kingsolver 1983) and gives these females more time for oviposition (Watt 1992), increasing their reproductive performance. For a comparable reason, in male *Colias* but-

* E-mail: marjo.saastamoinen@helsinki.fi.

† E-mail: ilkka.hanski@helsinki.fi.

Am. Nat. 2008. Vol. 171, pp. 701–712.
DOI: 10.1086/587531

terflies the kinetically favored genotypes have significant advantage in mating success compared with other males (Watt et al. 1985).

In the Glanville fritillary, previous studies have identified seven allozyme alleles at the PGI locus in the Åland Island metapopulation in southwestern Finland (Saccheri et al. 1998; Haag et al. 2005). Two alleles, PGI-*d* and PGI-*f*, are present in high frequencies (0.49 and 0.29, respectively; Saccheri et al. 1998; Haag et al. 2005). Haag et al. (2005) showed that the frequency of PGI-*f* is highest in newly established populations, in which females tend to be more dispersive than in older populations (Hanski et al. 2004, 2006). Just as in *Colias*, genetic variation at PGI is associated with reproductive performance in the Glanville fritillary, as females with the PGI-*f* allele lay larger egg clutches than females without this allele (Saastamoinen 2007*a*).

Apart from the genotypic effect, many environmental factors affect clutch size in the Glanville fritillary (Saastamoinen 2007*a*), and there may be interactions among the genetic and environmental effects. Thus, females who initiate oviposition earlier in the afternoon, when environmental conditions tend to be most favorable, lay significantly larger clutches than females ovipositing later in the day (Saastamoinen 2007*a*). As PGI-*f* females are able to initiate oviposition earlier in the day than PGI-non-*f* females (Saastamoinen 2007*a*), the genetic effect may be the ultimate cause of the environmental effect. Alternatively, or in addition, PGI-*f* females may lay larger clutches independently of the prevailing environmental conditions if, for example, they have increased egg maturation rate due to their generally higher metabolic rate compared with females with other genotypes (Haag et al. 2005).

This study was designed to test the genetic and environmental effects on flight activity, timing of oviposition, and clutch size. We investigated experimentally and with observational data whether cool morning hours would delay the initiation of oviposition later in the same day, whether such a delay would have consequences for clutch size, and whether these effects would differ between the PGI genotypes. We hypothesized that if the generally larger clutches laid by PGI-*f* females are due to some inherent genotypic effect unrelated to environmental condition, then PGI-*f* females should lay larger clutches under all circumstances, even if their oviposition is delayed to late afternoon. In addition, the larger clutches in PGI-*f* females could at least partly be due to the earlier flight activity and oviposition during the day, possibly allowed by their ability to fly in lower temperatures than PGI-non-*f* females. To test this, we measured the body temperatures of butterflies with different PGI genotypes at flight in the morning, when the ambient temperature was low and it constrained activity. Higher body temperature would indicate less severe constraint.

Material and Methods

Study System

The Glanville fritillary butterfly (*Melitaea cinxia*) occurs at its northern range limit in Finland, in the Åland Islands, where it inhabits dry meadows with at least one of the two larval host plant species, *Plantago lanceolata* and *Veronica spicata* (Nieminen et al. 2004). The habitat is highly fragmented, and the butterfly has a classic metapopulation structure with a high rate of population turnover (local extinctions and recolonizations; Hanski 1999). Females eclose with the full number of oocytes in their ovarioles (Boggs and Nieminen 2004). Eggs are matured in clutches, and females may lay several clutches of usually 130–190 eggs during their lifetime (Saastamoinen 2007*a*, 2007*b*). Increased clutch size in the Glanville fritillary leads to a clear fitness benefit since the offspring group size has a consistent positive effect on survival throughout development from the egg stage to the last larval stage (Kuussaari et al. 2004).

In the spring of 2005, postdiapause larvae were collected from 60 local populations across the Åland Islands and reared in common garden conditions in the laboratory (12L : 12D, 25° and 20°C, respectively). The pupae were weighed at the age of 1 day on a Scaltec SBC 33 electrobalance (accuracy 0.1 μg). Following eclosion, butterflies were individually marked with a permanent marker pen on the underside of the hind wing, and a small piece (2 mm diameter) of hind wing was removed with a biopsy punch (Tamro, Vantaa, Finland) and stored in alcohol (99.6%) at −20°C for subsequent genotyping.

Following marking on the day of eclosion, butterflies ($n = 242$: 126 females and 116 males originating from 60 different local populations) were released into a large outdoor population cage (32 m × 26 m × 3 m; Hanski et al. 2006; a few butterflies eclosed in the evening and were released on the following morning). The cage was covered with mesh that prevented butterflies from escaping but allowed experiments to be conducted under practically natural environmental conditions (Hanski et al. 2006). The density of butterflies in the cage changed in the course of the season in the same manner as in natural populations, with peak density in the second week of June, and slightly earlier in males than in females. Wild-collected host plants of *P. lanceolata* and *V. spicata* (200 plants) were potted and provided for oviposition in the center of the cage. Large numbers of naturally occurring flowering plants in the cage provided nectar for adult butterflies.

Effect of Low Morning Temperatures

For an experiment on the effects of low morning temperatures on the initiation of oviposition and clutch size,

female butterflies were collected from the cage in the evening (at 6:00). Previous studies have shown that Glanville fritillary females rarely lay eggs on consecutive days, and therefore we included in the experiment only females that had not laid eggs during the same day (we knew this because all ovipositions in the cage were constantly monitored). Furthermore, in the analyses of the results we included the number of clutches laid before the experiment as an additional explanatory variable. At most, six butterflies were tested per day. Butterflies were randomly assigned to either the control or the treatment group, ensuring that no butterfly was assigned to the treatment group more than once. Butterflies were kept overnight and until release in a cool box at 15°C. Butterflies in the control group were released back to the cage the following morning at 9:00, while butterflies in the treatment group were released at noon, 3 h later than the control butterflies. All butterflies were released in the same location in the cage.

The initiation of oviposition was determined by constantly monitoring the host plants in the cage from 10:00 a.m. until 7:00 p.m., as previous studies have shown that females do not initiate oviposition outside this time frame (Saastamoinen 2007*a*). The butterflies were individually marked so it was possible to record ovipositions for all females in the cage. After a female had finished oviposition, the leaf with the egg clutch was removed so we could count the number of eggs at the age of 3 days.

Data on daily oviposition times and ambient temperatures were collected in the same cage in four consecutive years (2003–2006) in experiments recording lifetime egg production (Saastamoinen 2007*a*, 2007*b*). These data were used to compare results for different years with naturally varying environmental conditions. We examined whether naturally occurring low temperatures in the morning affected the probability of oviposition in that day and, if oviposition occurred, whether low morning temperatures caused a delay in the initiation of oviposition. As a measure of morning conditions we used the ambient air temperature at 11:00, which was available for all years and correlates well with temperatures at other morning hours (fig. 5).

Body Temperature during Flight

Body surface temperature immediately after active flight was measured with a thermal image camera (Thermo Tracer TH9100MV/Wv, Sintrol, Helsinki). The thermal image camera is essentially a thermometer with high accuracy (0.6°C accuracy at 30°C) and automatic functions, making recording fast. Butterflies were caught from voluntary flight between 9:00 a.m. and 1:00 p.m. on June 8–20, 2005. Thermal images were recorded within 15 s after capturing the butterfly. The butterfly was held by the wings with forceps to eliminate any heat transfer and was photographed against the ground under a shade to prevent reflection and emission (fig. 1). Butterflies were released at the location of capture immediately after photographing.

The thermal image camera saved visual images that were analyzed with the MicroView program. Thoracic surface temperature was obtained as the average of five random data points within the outline of the thorax (fig. 1). It is important to note that the measurement obtained with the thermal image camera is the thoracic surface temperature, which underestimates actual body temperature in flight for the following reasons. First, the surface temperature is necessarily lower than the temperature of the flight muscles due to heat loss at the surface. Second, as Glanville fritillary butterflies have insulating "fur" that reduces heat loss, the thermal camera measures the surface temperature at the exterior of this insulating layer. Finally, following capture the body temperature starts to decline toward the temperature of the surrounding air. Though the thermal image was obtained within 15 s of capture, some decrease in body temperature must have occurred. However, the great advantage of using the thermal image camera for measuring body temperature is that no harm is done to the butterfly. Our purpose was to compare temperature measurements between different genotypes. The underestimation of the actual body temperature for the previous reasons makes this comparison conservative.

Ambient air temperature was measured with a HOBO weather station data logger (Onset, Bourne, MA) located inside the population cage. The weather station recorded ambient temperature at 30-s intervals at a height of 1.5 m.

DNA Extraction and Pgi *Genotyping*

Underlying variation at the allozyme level in PGI is extensive variation at the DNA sequence level (C. W. Wheat, C. R. Haag, J. H. Marden, I. Hanski, and M. Frilander, unpublished manuscript). L. Orsini, C. W. Wheat, J. Kvist, M. Frilander, and I. Hanski (unpublished manuscript) have identified a set of three single nucleotide polymorphisms (SNPs) that discriminate among the numerically dominant allozymes, including the PGI-*f* allele. We used these SNPs to genotype individuals in this study.

Genomic DNA was isolated using a Nucleo spin tissue extraction kit (Macherey-Nagel, Düren, Germany) with overnight incubation at 56°C. The three SNPs described by L. Orsini, C. W. Wheat, J. Kvist, M. Frilander, and I. Hanski (unpublished manuscript) were genotyped by primer extension reactions (Sokolov 1990) in which the screening primers, designed with a 3′ end immediately adjacent to the SNP, undergo a single nucleotide extension by a fluorescent-labeled ddNTP corresponding to the SNP allele. Each polymerase chain reaction (PCR; 20 μL) con-

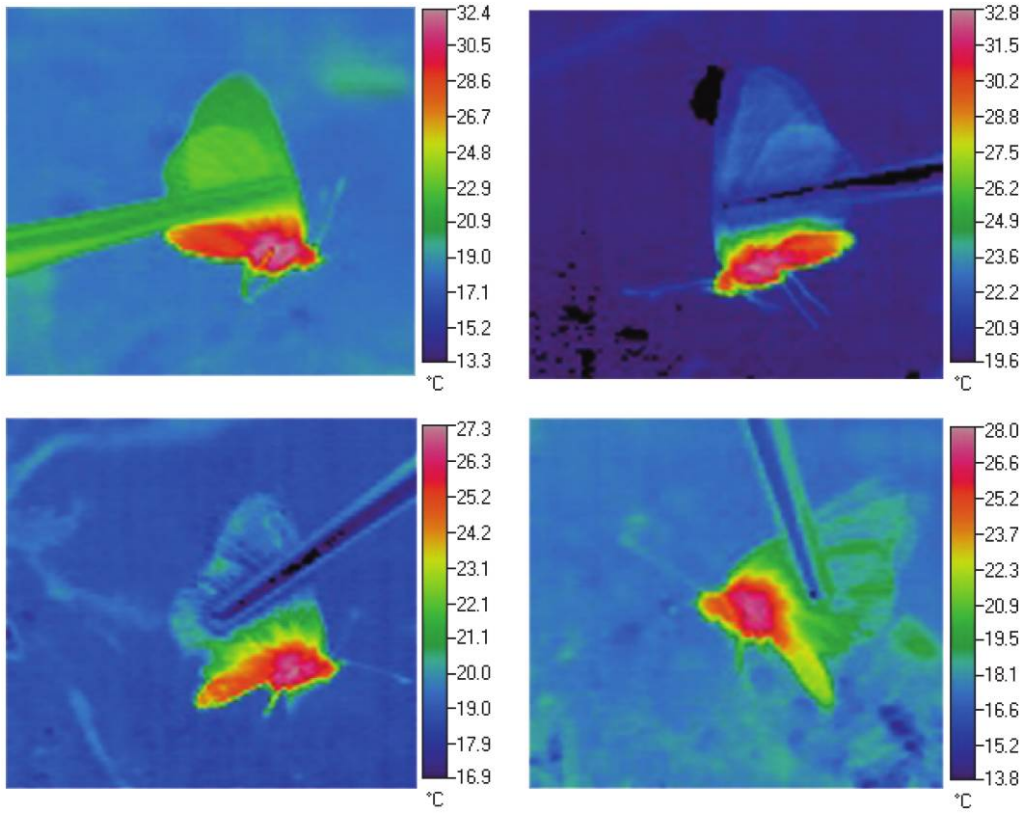

Figure 1: Thermal images of four butterflies caught at flight.

tained 20–30 ng genomic DNA, 1 μM each primer, 200 μM each dNTP, 2.5 mM $MgCl_2$, 20 ng BSA, and 0.2 U Taq DNA polymerase. An initial denaturing step (5 min at 95°C) was followed by 35 cycles of amplification with 1 min at 94°C, 1 min at the annealing temperature, and 1.5 min at 72°C. A final extension step included incubation for 15 min at 72°C. The PCR products were purified with Exo-SAP-IT (GE Healthcare, Munich) at the concentration of 1 μL/10 μL PCR. Primer extension reactions employed the SNuPe kit (GE Healthcare; Batley and Hayes 2003), following the manufacturer's instructions. Primer extension reactions were run on a Megabace 1000 (GE Healthcare) and genotypes called by SNP profiler (GE Healthcare). The calls were checked visually.

The PGI allozyme allele *f* is defined by a combination of two SNPs, AA111F and AA361R (L. Orsini, C. W. Wheat, J. Kvist, M. Frilander, and I. Hanski, unpublished manuscript). Individuals with bases AC or CC at AA111F and with bases CT or TT at AA361R correspond to PGI-*f* and are here called *Pgi-f*. Any other individuals are called *Pgi-non-f*. As the two SNPs are strongly linked, the results would have been qualitatively similar if AA111F alone had been used, in which case AC and CC individuals would have been contrasted with AA individuals. We observed no individuals with CC at AA111F in the 2005 cage material; hence the *Pgi-f* individuals were all AC at AA111F.

Statistical Analyses

Statistical analyses were performed with SAS, version 8.02 (SAS Institute 1999). All explanatory variables were normally distributed. Linear models (GLM procedure in SAS) were used to test the effect of low morning temperature on the timing of oviposition and clutch size. Only females that initiated oviposition on the day of the treatment were compared, as these females are considered to have had comparable oviposition motivation. In this analysis, the experimental group (0 = control, 1 = treatment), *Pgi* genotype (*Pgi-f* vs. *Pgi-non-f*), and their interaction were used as explanatory variables. To test whether experimental females that delayed oviposition for more than 1 day attained average clutch size and average oviposition time, a second model was constructed in which butterflies were divided into three groups (0 = control, 1 = experimental females that laid on the day of the treatment, 2 = experimental females that delayed oviposition by at

Table 1: Times of day and ambient temperatures at initiation of oviposition, and factors affecting clutch size

	2003	2004	2005	2006
Time at initiation of oviposition:				
Mean (±SD; h, min)	2:34 p.m. (1.18)	2:41 p.m. (1.44)	2:39 p.m. (1.23)	2:22 p.m. (1.19)
Minimum	11:42 a.m.	11:38 a.m.	11:30 a.m.	11:48 a.m.
Maximum	6:35 p.m.	5:43 p.m.	6:30 p.m.	5:00 p.m.
Temperature at initiation of oviposition (°C):				
Mean (±SD; °C)	22.5 (2.9)	21.3 (3.0)	22.8 (2.6)	25.7 (4.2)
Minimum	15.1	14.5	17.7	19.0
Maximum	27.5	27.3	28.5	33.0
Factors affecting clutch size:				
Female[a]	$P < .001$	$P < .04$	$P < .0001$	$P < .02$
Time at initiation of oviposition[b]	$P < .002$	$P < .001$	$P = .737$	$P < .03$

[a] Random factor.

[b] F statistics for the fixed factor of time at the initiation of oviposition in 2003–2006: $F = 10.31$, df $= 1, 144$; $F = 13.43$, df $= 1, 58$; $F = 0.11$, df $= 1, 135$; and $F = 5.34$, df $= 1, 180$.

least 1 day). Egg laying history (number of clutches laid before the experiment) was included in the original models but was eliminated from the final models since it did not have a significant effect.

A linear mixed-effects model of repeated measures with female as a random factor (MIXED procedure in SAS) was used to examine the effect of *Pgi* genotype on clutch size in 2005 to account for multiple clutches laid by each female. A linear model (GLM procedure in SAS) was used to analyze factors affecting body temperature during flight, with ambient air temperature, sex, body mass (standardized for sex), and *Pgi* genotype as explanatory variables. Starting with the full model, including all interactions, model selection was done by backward elimination of nonsignificant factors. *F*-tests with Type III sum of squares were used to determine the level of significance. To analyze the effect of the time of day at the initiation of oviposition on clutch size under the natural conditions in the population cage in 2003–2006, we used a linear model (MIXED procedure in SAS) with individual female as a random factor.

To examine whether low temperatures at 11:00 a.m. affected whether females laid eggs in the same day, we used a linear mixed model with binomial error distribution (GLIMMIX procedure in SAS) and individual female as a random factor. Other explanatory factors included the *Pgi* genotype (in 2005 only) and whether the female had laid eggs on the previous day. Starting with the full model, including all interactions, model selection was done by backward elimination of nonsignificant factors. The data set was constructed by including for each female the days during which she could have oviposited. Based on our extensive observations on oviposition, we assume that females may begin to oviposit in the second day after mating and will continue to lay eggs until the day when the female was last observed. The vast majority of matings (>80%) were directly observed in the cage. If mating was not observed (but must have occurred because the female laid fertile eggs), we assumed that mating took place 2 days after the female had been released into the cage.

We used regression analysis with Type II sum of squares to analyze the effect of temperature at 11:00 a.m. on the time of oviposition in females that oviposited in that day. The latter was calculated in two different ways, as the average time when females initiated oviposition in that day and as the time by which 20% of females had initiated oviposition.

Results

Oviposition Time and Clutch Size

The time of day when females initiate oviposition and the respective average temperature were consistent over the 4 years of the study (table 1). On average, females initiated oviposition between 2:00 p.m. and 3:00 p.m., when the ambient temperature was 21°–26°C. In 3 out of 4 years, females that initiated oviposition earlier in the day laid significantly larger clutches than females that oviposited later in the afternoon (fig. 2). In all years, individual females differed significantly in their average clutch size (table 1), but there were no significant differences among the females in the time of oviposition in any of the years (ANOVA, $P = .21$–$.96$).

Pgi *Genotype and Clutch Size*

The *Pgi-f* females laid, on average, 32% larger clutches ($\overline{X} \pm \text{SE} = 144 \pm 72$ eggs) than *Pgi-non-f* females ($\overline{X} \pm \text{SE} = 109 \pm 75$ eggs). The difference was statistically significant ($F = 3.91$, df $= 1, 145$, $P < .05$). This comparison includes all females in the cage in 2005, including those that participated in the low morning temperature exper-

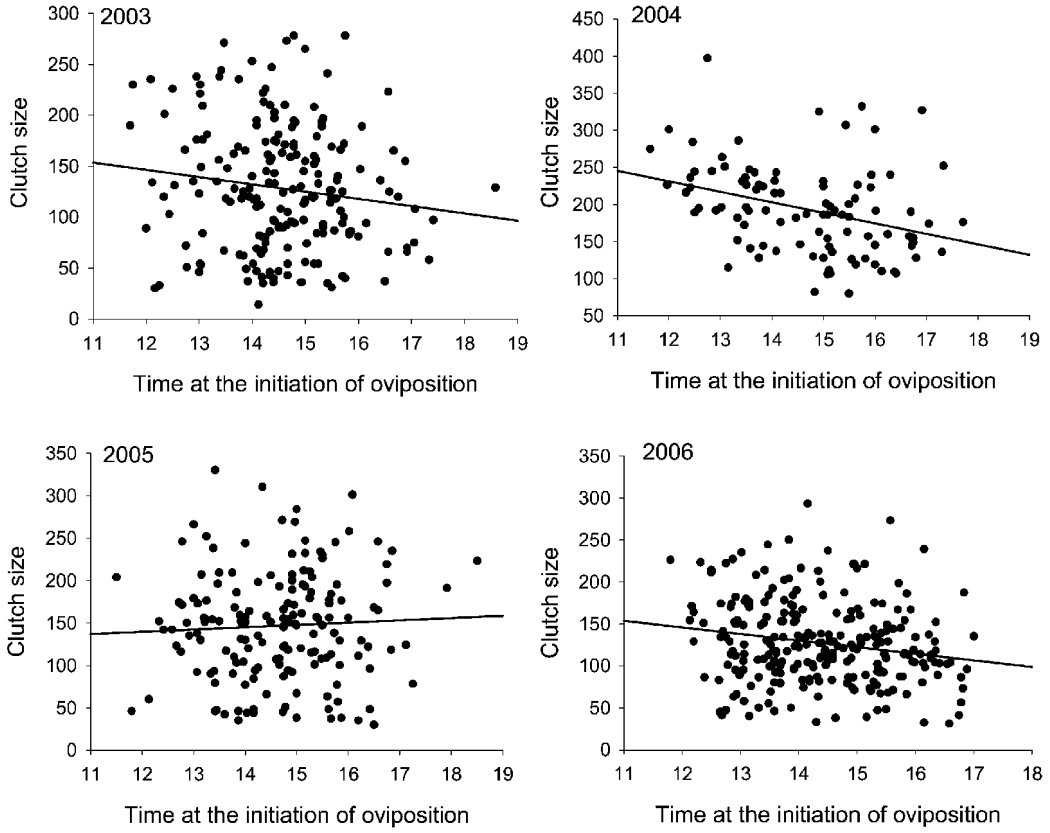

Figure 2: Relationship between time of day at initiation of oviposition and clutch size in the years 2003–2006.

iment but excluding data from the morning when a particular female was exposed to the low morning temperature. The difference in clutch size between the *Pgi* genotypes was the same when the time of oviposition was included in the model (no significant effect of the time of oviposition in 2005; fig. 2). There was no significant difference in the time of oviposition between the *Pgi* genotypes in 2005 ($P = .94$).

Effect of Low Morning Temperature on Clutch Size

Twenty-seven of the 39 females in the treatment group and 19 of the 23 females in the control group laid eggs after taking part in the experiment on low morning temperatures. Thus, the treatment did not affect the future reproduction of the butterflies (χ^2 test, $P = .66$). A greater fraction of females in the control (13 of 19) than in the treatment group (13 of 27) initiated oviposition in the day of the experiment, but the difference was not statistically significant ($P = .17$).

Females in the low morning temperature treatment that laid eggs on the same day initiated oviposition 2 h later, on average, than females that experienced the natural morning conditions (fig. 3*A*; $F = 15.92$, df $= 1, 25$, $P < .001$). On the other hand, females that experienced low morning temperatures but delayed oviposition until the following days (average delay 2 days, minimum and maximum 1 and 5 days, respectively) initiated oviposition at the same time as females in the control group ($P = .18$). The results on clutch size were as expected, based on the pattern in figure 2: females that experienced low morning temperature and laid eggs on the same day oviposited smaller clutches than control females (fig. 3*B*; $F = 4$, df $= 1, 25$, $P = .057$), but females in the treatment group that delayed oviposition until subsequent days laid similar clutches as females in the control group (fig. 3*B*). There was no difference in the initiation of oviposition between *Pgi-f* and *Pgi-non-f* females ($F = 0.07$, df $= 1, 25$, $P = .79$), nor was the interaction between the treatment and *Pgi* genotype significant ($F = 0.28$, df $= 1, 25$, $P = .60$). The *Pgi* genotype did not affect clutch size ($F = 0.16$, df $= 1, 25$, $P = .69$; interaction between *Pgi* genotype and treatment, $F = 0.82$, df $= 1, 25$, $P = .38$).

Turning to the 4-year observational data from the pop-

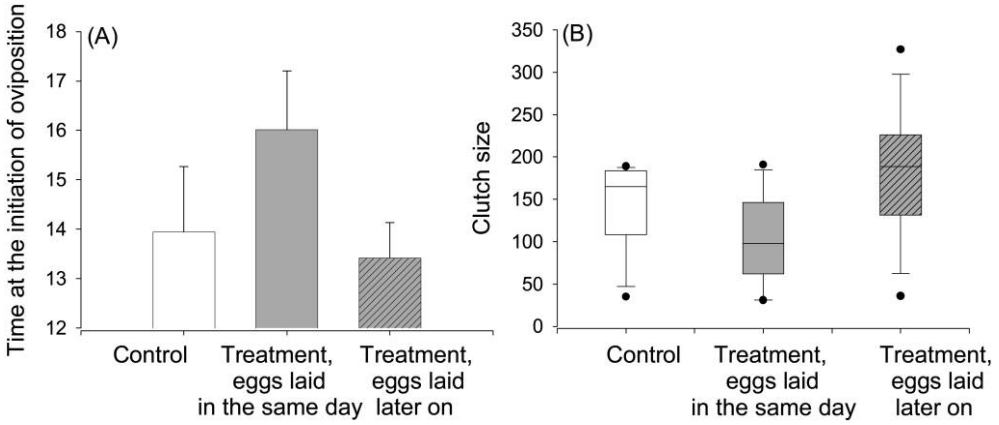

Figure 3: Consequences of experiencing low morning temperature in the experiment on the time of oviposition (*A*) and clutch size (*B*). $F = 2.63$, df $= 1, 26$, $P = .12$; $F = 10.29$, df $= 1, 26$, $P < .004$; $F = 4.0$, df $= 1, 25$, $P = .57$.

ulation cage, we first examined whether ambient temperature at 11:00 a.m., as a proxy of the morning environmental condition, influenced the likelihood of oviposition in the same day. In all 4 years, the likelihood of oviposition increased significantly with temperature at 11:00 a.m. and decreased with the incidence of oviposition in the previous day. Table 2 and figure 4 give the results for 2005, for which we also have information on the *Pgi* genotype. Significant interaction between *Pgi* genotype and morning temperature indicates that *Pgi-f* females are less affected than *Pgi-non-f* females by low morning temperatures (fig. 4). Other interactions were nonsignificant.

Considering females that oviposited on a particular day in the population cage, the observational data demonstrate that naturally occurring low morning temperatures are associated with a delay in the time of oviposition, as ovipositions started later in days when the ambient temperature was lower at 11:00 a.m. The dependent variable in this analysis is the time by which 20% of the females that laid eggs on that day had initiated oviposition (results were qualitatively similar when the average time at the initiation of oviposition was used as the response variable instead of the 20% tail). The R^2 values ranged from 0.26 to 0.59 in the 4 years (all P values significant at the 5% level). Figure 5 gives detailed results for 2004 and 2005, the two summers with the highest and lowest frequencies of low morning temperatures, respectively.

Table 2: Factors that affect the likelihood of oviposition in 2005 (see fig. 4)

	df	F	P
Ambient air temperature at 11:00 a.m. (°C)	1, 474	68.35	<.001
Pgi genotype	1, 474	3.27	.071
Oviposition (yes/no) in the previous day	1, 474	19.24	<.001
Ambient temperature at 11:00 a.m. × *Pgi* genotype (°C)	1, 474	4.45	.035

Body Temperature during Flight

Body surface temperature during flight increased with ambient temperature (fig. 6), and there was a significant difference between the sexes (table 3): the average thoracic temperature was 28.4° ± 0.34°C ($\overline{X} \pm$ SE; $n = 94$) in males and 30.1° ± 0.36°C in females ($\overline{X} \pm$ SE; $n = 36$). A significant interaction between ambient air temperature and *Pgi* genotype indicates that *Pgi-f* butterflies had higher body temperature at low ambient temperatures (table 3; fig. 6). Body mass, standardized for sex, did not affect body temperature ($F = 0.23$, df $= 1, 29$, $P = .63$), nor were any second-order interactions involving body mass significant. Interactions between sex and *Pgi* genotype and between sex and ambient air temperature were not significant. However, males were apparently flying more frequently at low ambient temperatures than females, as 18% of males ($n = 17$) but only 3% of females ($n = 1$) were caught at flight when the ambient temperature was <15°C (χ^2, $P = .05$).

Discussion

We observed that the clutches laid by *Pgi-f* females were, on average, 32% larger than clutches laid by *Pgi-non-f* females. Saastamoinen (2007*a*) found a comparable difference: *Pgi-f* females laying 21% larger clutches than *Pgi-non-f* females. The question is why. We set out to test the

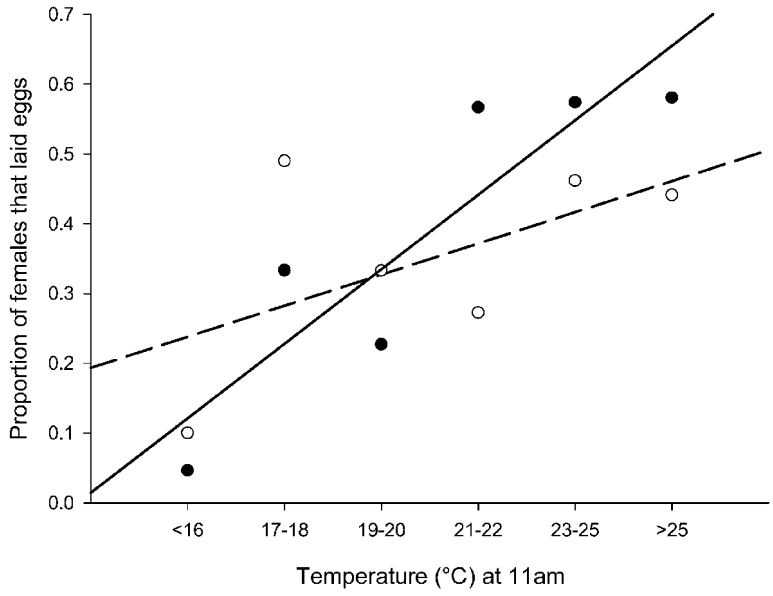

Figure 4: Relationship between proportion of females laying eggs and ambient air temperature at 11:00 a.m. during the 17 days in 2005 when females oviposited in the population cage. Open and filled circles represent individuals with and without the *Pgi-f* genotype, respectively.

hypotheses that, first, the larger clutches laid by *Pgi-f* females are due to their being able to initiate oviposition earlier in the day than *Pgi-non-f* females, thereby being able to take advantage of the favorable conditions for oviposition in the early afternoon or, second, the *Pgi-f* females lay larger clutches for some other inherent difference between the genotypes that is unrelated to the prevailing environmental conditions.

We summarize the results in five points. First, both experimental and observational results (in 3 of 4 years) show a general decrease in clutch size with the time of day at initiation of oviposition. Second, both experimental and observational results show that, in females in general, oviposition occurs later in the day when morning temperatures are low, and from this it follows that generally clutch size is reduced in the days when morning temperatures are low. Third, individuals with the *Pgi-f* genotype had higher thoracic surface temperature than *Pgi-non-f* individuals when caught at flight in mornings with low or relatively low ambient temperature, from which we infer that *Pgi-f* females are less constrained by low morning temperatures than *Pgi-non-f* females. Fourth, observational data from the population cage show that the likelihood of oviposition is less affected by morning temperatures in *Pgi-f* than in *Pgi-non-f* females. Finally, in 2005, which was the exceptional year when there was no relationship between time of oviposition and clutch size, *Pgi-f* females nonetheless laid larger clutches than *Pgi-non-f* females. From these results we conclude that, due to their ability to fly in lower morning temperatures, *Pgi-f* females are able to initiate oviposition earlier in the day than *Pgi-non-f* females, which generally leads to larger clutches; however, *Pgi-f* females also tend to lay larger clutches otherwise. Thus, there is support for both hypotheses about the causes of larger clutches in *Pgi-f* females.

Daily Time of Oviposition and Clutch Size

These results suggest that environmental conditions experienced in the morning can greatly influence the reproductive performance of females in the same day. In the experiment on low morning temperatures, females from the treatment group were released back to the population cage at noon, 3 h after the release of the control females. Females in the treatment group had about 2 h to experience the natural environmental conditions before the control females started to lay eggs, at around 2:00 p.m., but clearly this was not enough to offset the experience of low morning temperatures. A roughly similar 2-h delay due to low morning temperatures was observed in the observational data (fig. 5*A*).

Low temperatures generally occur in the morning, but naturally low temperatures during other times of the day also may have consequences for oviposition and clutch size. Low temperatures constrain activity and reduce opportunities for nectar feeding, which females typically do before oviposition (M. Saastamoinen, personal observation). Low temperature may also reduce the rate of egg

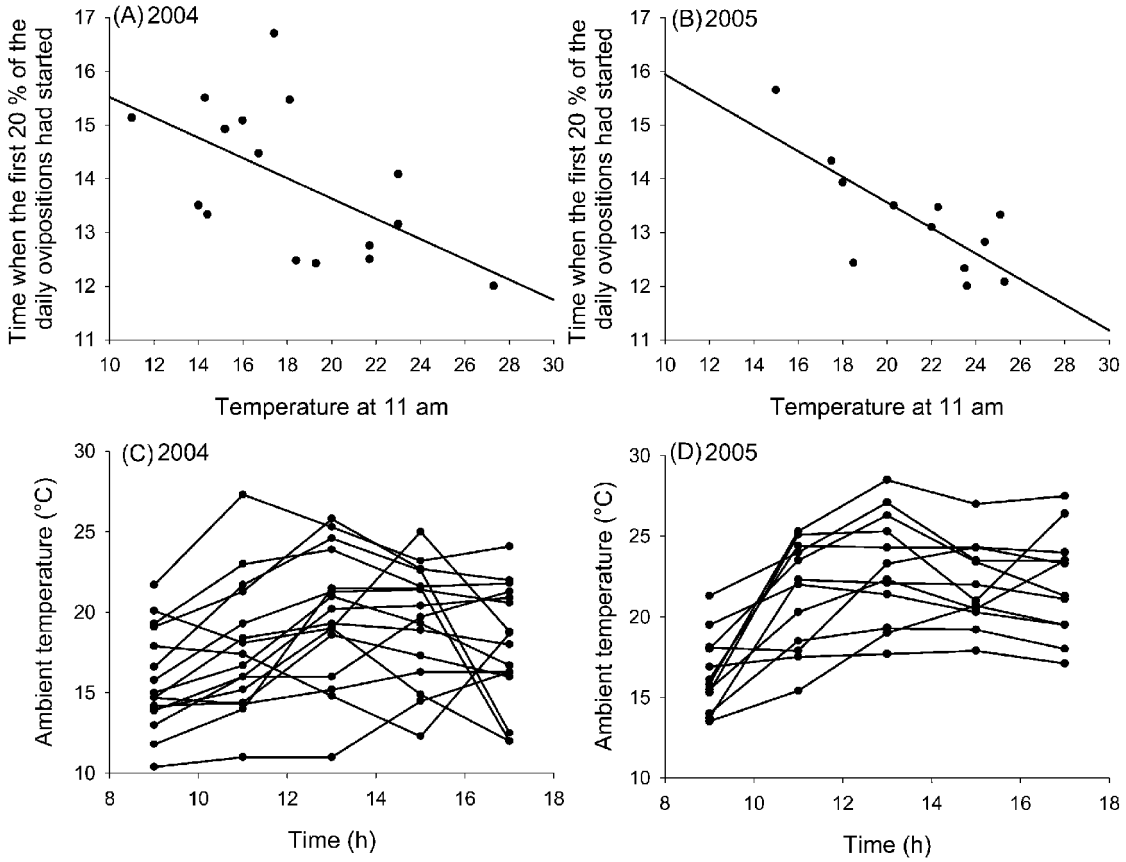

Figure 5: Relationship between ambient air temperature at 11:00 a.m. and time of day when the first 20% of daily ovipositions started in 2004 (*A*) and 2005 (*B*). *C* and *D* show ambient air temperatures for days during which more than five females laid eggs in 2004 and 2005, respectively.

maturation. Night temperatures during the flight season are so low (11°C on average in June 2005; M. Saastamoinen, personal observation) that egg maturation is unlikely to occur during the night. In any case, the environmental conditions experienced by butterflies in the morning are of greater importance than the conditions during the night because ovipositions have not been initiated before noon even after warm nights, and ovipositions start at the usual time following cold nights if the morning is sufficiently warm (M. Saastamoinen, personal observation).

Delayed oviposition in the experiment with low morning temperature led to reduced clutch size, in agreement with generally declining clutch size in naturally occurring ovipositions with the time of the day. The latter was observed in 3 out of 4 years. Exceptionally, in 2005, the year of our experiment, there was no such decline. The likely reason is the generally favorable weather conditions in 2005, when temperature at 11:00 a.m. exceeded 17°C in all but one day when many (more than five) females oviposited (fig. 5*D*; cf. fig. 5*C* for 2004).

Why do females that are able to initiate oviposition earlier in the day lay larger clutches? The reason may be ambient temperature, which typically is highest during early afternoon and declines toward the evening. As Glanville fritillary females oviposit on the undermost leaves of the host plant, they are often in shade (M. Saastamoinen, personal observation), and their body temperature is likely to decrease over the course of oviposition, which typically lasts 45 min (M. Saastamoinen, personal observation). In the afternoon, females may prematurely terminate oviposition when their body temperature falls below a threshold level, and thus higher ambient temperature would be beneficial by maintaining higher body temperature during oviposition. On the other hand, increased clutch sizes at higher ambient temperatures might be due to a higher rate of oviposition. Other factors such as humidity, solar radiation, and/or wind may also be causally related to variation in clutch size, as these conditions too vary in the course of the day. In any case, the reasons why temperature affects oviposition rate in a clutch-laying species such as the Glanville fritillary are different from those in, for ex-

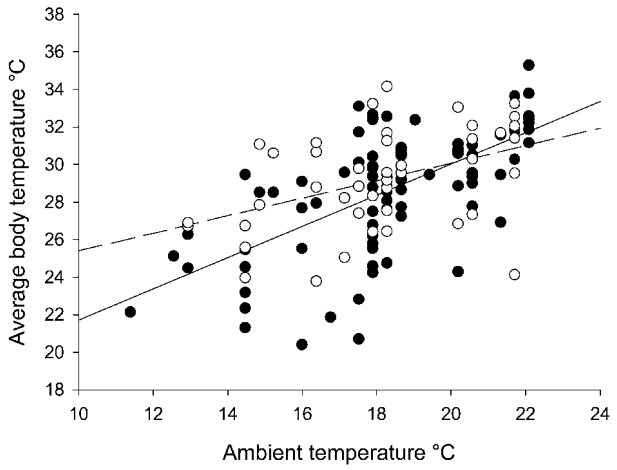

Figure 6: Relationship between body surface temperature immediately after flight and ambient air temperature. Open and filled circles represent individuals with and without the *Pgi-f* genotype, respectively.

ample, *Colias* butterflies (Watt 1992) because the latter lay eggs singly and need to fly between each oviposition.

Saastamoinen (2007*a*) showed with observational data collected in 2004 that there was a relationship between the time of oviposition and clutch size, that *Pgi-f* females initiated oviposition earlier in the day than *Pgi-non-f* females, and that *Pgi-f* females laid larger clutches on average than *Pgi-non-f* females. In the present study, the first two results were not observed, but the third one was, demonstrating that *Pgi-f* females lay larger clutches also for reasons other than the daily timing of oviposition. On the other hand, in the experiment *Pgi-f* females laid smaller clutches when their oviposition was delayed due to low morning temperatures, strongly suggesting that environmental conditions experienced by females in the day of oviposition have an influence also on *Pgi-f* females. This is further supported by the probability of ovipositing in a particular day increasing with the 11:00 a.m. temperature in *Pgi-f* females, though this effect was significantly less strong in *Pgi-f* than in *Pgi-non-f* females (fig. 4).

Pgi *Genotype and Body Temperature during Flight*

Glanville fritillary females typically feed before oviposition (M. Saastamoinen, personal observation). Individuals that are able to fly at lower ambient temperatures have an advantage because they can feed earlier in the day and therefore begin to oviposit sooner. We used the body temperature of butterflies caught at flight in the morning as an indication of their flight capacity in low ambient temperatures.

Note that as the body temperature of butterflies caught at flight was measured with the thermal image camera, the measurement reflects the body surface temperature and gives an underestimate of the temperature of the flight muscles. Measurements were made in shade to prevent heat transfer from direct sunlight. The photographs (fig. 1) reveal that the warmest part of the body surface was the center of the thorax, which shows that the thoracic measurement reflects the heat transferred from flight muscles to the body surface. Other things being equal, higher surface temperature indicates higher temperature of flight muscles.

The body surface temperature was greatly affected by ambient air temperature, consistent with numerous other studies on insects. This result is explained by ectothermic organisms needing an external heat source to warm up (e.g., Heinrich 1986*a*, 1986*b*; Shreeve 1992; Merckx et al. 2007) and by increased heat loss during flight at low ambient temperatures (Gilchrist 1990).

Our main result is that individuals with the *Pgi-f* genotype had higher body temperature during flight than *Pgi-non-f* individuals under low ambient temperatures. This could be because *Pgi-f* individuals basked longer before flight and hence took off warmer. Several species of butterflies have been shown to "buy" more time and distance per individual flight by storing more heat in the thorax before takeoff (Heinrich 1986*a*, 1986*b*). Morphological differences in solar absorptivity (melanization), fur thickness, and/or body size influence thermoregulation in *Colias* butterflies (Kingsolver and Watt 1984) and in the speckled wood butterfly (*Parage aegeria*; Van Dyck and Matthysen 1998; Merckx et al. 2006). In the latter species, dark males heated up faster than pale ones, but there was no difference in the thoracic temperature at which they started to fly (Van Dyck and Matthysen 1998). In the Glanville fritillary, we have not observed differences in melanization or fur thickness between the different *Pgi* genotypes.

An alternative hypothesis is that higher flight metabolic rate maintains higher thoracic temperature during flight (Heinrich 1993). In the Glanville fritillary, *Pgi-f* individuals have a peak metabolic rate about 17% higher than *Pgi-non-f* individuals (Haag et al. 2005). According to this

Table 3: Factors that affect body temperature immediately after flight in the morning (see fig. 6)

	df	*F*	*P*
Ambient air temperature (°C)	1, 129	37.35	<.0001
Pgi genotype	1, 129	6.09	.015
Sex	1, 129	5.44	.021
Ambient air temperature × *Pgi* genotype	2, 129	5.37	.022

hypothesis, individuals with different *Pgi* genotypes take off with similar body temperatures, but *Pgi-f* individuals do not cool down as fast as *Pgi-non-f* individuals. Heinrich (1986*a*) showed that *Colias eurytheme*, a slightly larger butterfly than the Glanville fritillary, can fly nearly continuously at the ambient temperatures of 17°–18°C at noon, because in those conditions their mass is sufficiently large to retard heat loss in flight. An even larger species, *Nymphalis antiopa*, can fly at low ambient temperatures because it can stabilize thoracic temperature near 35°C by flight metabolism alone (Heinrich 1986*a*).

In our study, females had higher thoracic temperature than males, consistent with previous results for butterflies (Pivnick and McNeil 1986; Gilchrist 1990) and probably due to the difference in body size (females 52% heavier in the Glanville fritillary). There is no difference in body mass between *Pgi-f* and *Pgi-non-f* individuals ($P = .47$), but the 17% difference in flight metabolic rate may be sufficiently large to make a difference for the rate of cooling while the butterfly is flying.

Conclusion

Although the proximate reasons remain to be clarified, these results demonstrate that *Pgi-f* individuals have higher body temperature during flight at low ambient temperatures than *Pgi-non-f* individuals. This may allow *Pgi-f* individuals to initiate flight earlier in the day and in general to be active for a longer time under unfavorable conditions than *Pgi-non-f* individuals. Similar results have been reported for *Colias* butterflies, in which individuals with a certain PGI genotype (3/4 heterozygotes) were found in higher frequencies than other common genotypes in the morning and at other times when temperature was low (Watt et al. 1983). Watt et al. (1983) suggested that these differences in the ability to fly at low ambient temperatures are due to differences in the kinetic performance of the different isoforms of the PGI enzyme at different temperatures. Our sample is not appropriate for drawing conclusions about possible changes in the frequencies of different genotypes among the actively flying butterflies in the course of the day, but the prediction for the Glanville fritillary is the same as for *Colias*.

Capacity to be active at low ambient temperatures can greatly enhance fitness of temperate butterflies that often experience unfavorable conditions. For instance, during this study, from June 7 to 25 and between 10:00 a.m. and 2:00 p.m., 29% and 63% of the time the ambient air temperature was below 14° and 18°C, respectively. Low morning temperatures reduced the probability of oviposition in that day and clutch size (the latter via a delay in the time of oviposition), and these effects were stronger in *Pgi-non-f* than in *Pgi-f* females. Thus, the capacity to fly under unfavorable conditions has consequences for reproduction, but it has consequences also for dispersal. Other things being equal, individuals that are able to fly under a broader range of weather conditions should have a greater rate and range of realized dispersal among populations. *Pgi-f* females are indeed more dispersive in low ambient temperatures than *Pgi-non-f* females (K. Niitepõld, A. D. Smith, J. L. Osborne, D. Reynolds, N. L. Carreck, A. P. Martin, J. H. Marden, O. Ovaskainen, and I. Hanski, unpublished manuscript), and *Pgi-f* individuals are more frequent in newly established populations than *Pgi-non-f* individuals (Haag et al. 2005; Hanski and Saccheri 2006). Just as with clutch size, the *Pgi* genotype may be associated with high dispersal and colonization rates for several reasons, but regardless of the actual molecular and physiological mechanisms, genetic variation segregating at the *Pgi* locus is strongly associated with fitness under temporally varying environmental conditions. At present, it is not clear what maintains the *Pgi-non-f* alleles in the metapopulation, but these mechanisms may occur at the molecular (overdominance in SNPs AA111F and AA361R; L. Orsini et al., unpublished manuscript; differences in kinetic performance of the different isoforms of the PGI enzyme as in *Colias*), individual (Hanski et al. 2006), and population levels (high emigration rate of *Pgi-f* females in highly fragmented landscapes).

Acknowledgments

We thank T. Merckx, K. Niitepõld, M. Singer, W. B. Watt, C. W. Wheat, and two anonymous referees for comments on the manuscript. C. Parmesan and M. Singer are thanked for discussions about the use of the thermal image camera. We acknowledge A. Holma, A. Jussila, and L. Maanavilja for their help in the cage experiment in 2005. The Regional Council of the Åland Islands is thanked for permission to use its land for the experiment. The research was supported by the Academy of Finland (Finnish Centre of Excellence Programme 2003–2005 and 2006–2011, grants 20286 and 213457, respectively). Partial support for equipment was provided by National Science Foundation grant EF-0412651 (to J. Marden and I.H.).

Literature Cited

Batley, J., and P. K. Hayes. 2003. Development of high throughput single nucleotide polymorphism genotyping for the analysis of *Nodularia* (cyanobacteria) population genetics. Journal of Phycology 39:248–252.

Boggs, C. L., and M. Nieminen. 2004. Checkerspot reproductive biology. Pages 92–111 *in* P. R. Ehrlich and I. Hanski, eds. On the wings of the checkerspots: a model system for population biology. Oxford University Press, Oxford.

Dennis, R. L. H. 1993. Butterflies and climate change. Manchester University Press, Manchester.

Gilchrist, G. W. 1990. The consequences of sexual dimorphism in body size for butterfly flight and thermoregulation. Functional Ecology 4:475–487.

Haag, C. R., M. Saastamoinen, J. H. Marden, and I. Hanski. 2005. A candidate locus for variation in dispersal rate in a butterfly metapopulation. Proceedings of the Royal Society B: Biological Sciences 272:2449–2456.

Hanski, I. 1999. Metapopulation ecology. Oxford University Press, New York.

Hanski, I., and I. Saccheri. 2006. Molecular-level variation affects population growth in a butterfly metapopulation. PLoS Biology, vol. 4, e129 doi:10.1371/journal.pbio.0040129.

Hanski, I., C. Erälahti, M. Kankare, O. Ovaskainen, and H. Siren. 2004. Variation in migration propensity among individuals maintained by the landscape structure. Ecology Letters 7:958–966.

Hanski, I., M. Saastamoinen, and O. Ovaskainen. 2006. Dispersal-related life-history trade-offs in a butterfly metapopulation. Journal of Animal Ecology 75:91–100.

Heinrich, B. 1986*a*. Comparative thermoregulation of four montane butterflies of different mass. Physiological Zoology 59:616–626.

———. 1986*b*. Thermoregulation and flight activity of a satyrine, *Coenonympha inornata* (Lepidoptera: Satyridae). Ecology 67:593–597.

———. 1993. The hot-blooded insects. Harvard University Press, Cambridge, MA.

Kingsolver, J. G. 1983. Ecological significance of flight activity in *Colias* butterflies: implications for reproductive strategy. Ecology 64:546–551.

Kingsolver, J. G., and W. B. Watt. 1984. Mechanistic constraints and optimality models: thermoregulatory strategies in *Colias* butterflies. Ecology 65:1835–1839.

Kuussaari, M., S. van Nouhuys, J. Hellmann, and M. Singer. 2004. Larval biology. Pages 138–160 *in* P. R. Ehrlich and I. Hanski, eds. On the wings of checkerspots: a model system for population biology. Oxford University Press, Oxford.

Merckx, T., B. Karlsson, and H. Van Dyck. 2006. Sex- and landscape-related differences in flight ability under suboptimal temperatures in a woodland butterfly. Functional Ecology 20:436–441.

Merckx, T., S. Van Dongen, E. Matthysen, and H. Van Dyck. 2007. Thermal flight budget of a woodland butterfly in woodland versus agricultural landscapes: an experimental assessment. Basic and Applied Ecology, doi:10.1016/j.baae.2007.03.009.

Nieminen, M., M. Siljander, and I. Hanski. 2004. Structure and dynamics of *Melitaea cinxia* metapopulations. Pages 63–91 *in* P. R. Ehrlich and I. Hanski, eds. On the wings of checkerspots: a model system for population biology. Oxford University Press, Oxford.

Pivnick, K. A., and J. N. McNeil. 1986. Sexual differences in the thermoregulation of *Thymelicus lineola* adults (Lepidoptera: Hesperiidae). Ecology 67:1024–1035.

Saastamoinen, M. 2007*a*. Life-history, genotypic, and environmental correlates of clutch size in the Glanville fritillary butterfly. Ecological Entomology 32:235–242.

———. 2007*b*. Mobility and lifetime fecundity in new versus old populations of the Glanville fritillary butterfly. Oecologia (Berlin) 153:569–578.

Saccheri, I., M. Kuussaari, M. Kankare, P. Vikman, W. Fortelius, and I. Hanski. 1998. Inbreeding and extinction in a butterfly metapopulation. Nature 392:491–494.

SAS Institute. 1999. SAS/STAT software user's guide, release 8.00. SAS Institute, Cary, NC.

Shine, R. 2005. Life-history evolution in reptiles. Annual Review of Ecology, Evolution, and Systematics 36:23–46.

Shreeve, T. G. 1992. Adult behaviour. Pages 22–45 *in* R. L. H. Dennis, ed. The ecology of butterflies in Britain. Oxford University Press, Oxford.

Sinclair, B. J., P. Vernon, C. J. Klok, and S. L. Chown. 2003. Insects at low temperatures: an ecological perspective. Trends in Ecology & Evolution 18:257–262.

Sokolov, B. P. 1990. Primer extension technique for the detection of single nucleotides in genomic DNA. Nucleic Acids Research 18: 3671.

Suarez, R. K. 2000. Energy metabolism during insect flight: biochemical design and physiological performance. Physiological and Biochemical Zoology 73:765–771.

Van Dyck, H. 2003. Mate location: a matter of design? adaptive morphological variation in the speckled wood butterfly. Pages 353–366 *in* C. L. Boggs, W. B. Watt, and P. Ehrlich, eds. Butterflies: ecology and evolution taking flight. University of Chicago Press, Chicago.

Van Dyck, H., and E. Matthysen. 1998. Thermoregulatory differences between phenotypes in the speckled wood butterfly: hot perchers and cold patrollers? Oecologia (Berlin) 114:326–334.

Watt, W. B. 1973. Adaptive significance of pigment polymorphism in *Colias* butterflies. III. Progress in the study of the "alba" variant. Evolution 27:537–548.

———. 1992. Eggs, enzymes, and evolution: natural genetic variants change insect fecundity. Proceedings of the National Academy of Sciences of the USA 89:10608–10612.

———. 2003. Mechanistic studies of butterfly adaptations. Pages 319–352 *in* C. L. Boggs, W. B. Watt, and P. R. Ehrlich, eds. Butterflies: ecology and evolution taking flight. University of Chicago Press, Chicago.

Watt, W. B., R. C. Cassin, and M. S. Swan. 1983. Adaptation at specific loci. III. Field behaviour and survivorship differences among *Colias* PGI genotypes are predictable from in vitro biochemistry. Genetics 103:725–739.

Watt, W. B., P. A. Carter, and S. M. Blower. 1985. Adaptation at specific loci. IV. Differential mating success among glycolytic allozyme genotypes of *Colias* butterflies. Genetics 109:157–175.

Associate Editor: George W. Gilchrist
Editor: Michael C. Whitlock