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THERMOREGULATION CONSTRAINS EFFECTIVE WARNING SIGNAL EXPRESSION

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Evolution of conspicuous signals may be constrained if animal coloration has nonsignaling as well as signaling functions. In aposematic wood tiger moth (*Parasemia plantaginis*) larvae, the size of a warning signal (orange patch on black body) varies phenotypically and genetically. Although a large warning signal is favored as an antipredator defense, we hypothesized that thermoregulation may constrain the signal size in colder habitats. To test this hypothesis, we conducted a factorial rearing experiment with two selection lines for larval coloration (small and large signal) and with two temperature manipulations (high and low temperature environment). Temperature constrained the size and brightness of the warning signal. Larvae with a small signal had an advantage in the colder environment, which was demonstrated by a faster development time and growth rate in the low temperature treatment, compared to larvae with a large signal. Interestingly, the larvae with a small signal were found more often on the plant than the ones with a large signal, suggesting higher basking activity of the melanic (small signal) individuals in the low temperature. We conclude that the expression of aposematic display is not only defined by its efficacy against predators; variation in temperature may constrain evolution of a conspicuous warning signal and maintain variation in it.

KEY WORDS: **Aposematic coloration, Arctiidae, opposing selection, thermal melanism.**

Aposematic animals have conspicuous signals as an antipredator defense to inform predators that they possess some unprofitable characters (Poulton 1890; Cott 1940; Ruxton et al. 2004). Predators learn to avoid conspicuous signals more effectively compared to less conspicuous ones (Gittleman and Harvey 1980; Forsman and Merilaita 1999; Gamberale-Stille and Tullberg 1999; Lindström et al. 1999; Riipi et al. 2001; Lindstedt et al. 2008). It is also suggested (but see e.g., Rowe et al. 2004; Ihalainen et al. 2007) that effective predator learning should select monomorphism in signal design (see e.g., Müller 1879; Beatty et al 2004; Rowland et al. 2007). Despite the observed directional selection on signal conspicuousness, warning signals can often be relatively weak (see examples in Endler and Mappes 2004) and puzzling variation exists in warning signals within (Brakefield and Liebert 1985; Holloway et al. 1995; Exnerová et al. 2006; Sandre et al. 2007) and among (Brown and Benson 1974; Brakefield 1985; Joron et al. 1999) species. This suggests that the magnitude and direction of selection for warning signal expression may vary and therefore constrain maximal signal expression.

Conspicuousness of the warning signal incurs the cost of increased attention from predators, thereby increasing the attack probability of naïve or bold predators (Riipi et al. 2001; Lindstedt et al. 2008). Therefore, differences in the predator community (e.g., differences in attack and learning rates of predators), combined with frequency-dependent selection could cause only a weak selection for conspicuousness (Endler and Mappes 2004). Moreover, the optimization of defenses can cause variation in signal intensity if there is variation in predation risks, marginal costs of signals, and efficacy of secondary defenses (Mappes et al. 2005; Speed and Ruxton 2007). In addition, based on empirical findings, warning signals can be costly to produce and maintain due to environmental factors such as diet, which could generate the

observed variation (Grill and Moore 1998; Ojala et al. 2007). Because warning signals are aimed at predators, most of the interest has been on the detection costs of the aposematic display and less effort has been placed on the fitness costs of generating aposematic displays in variable environmental conditions (Ojala et al. 2007; Speed and Ruxton 2007). However, in addition to protection from predators, animal coloration may serve other functions such as sexual signaling (Endler 1987) or thermoregulation (Majerus 1998; Williams 2007). Therefore, depending on an individual's selective environment, the importance of these different selection pressures on the signaling and other functions of coloration may vary spatially and temporally, thereby causing variation in the costs of producing a certain color pattern (Endler 1980, 1988; Endler and Mappes 2004; Mappes et al. 2005).

Thermal melanism has been shown to maintain adaptive color polymorphism in many species (Fields and McNeil 1988; Goulson 1994; Holloway et al. 1997; Windig 1999; Bittner et al. 2002; Hazel 2002; Davis et al. 2005). Darker, more melanic individuals should have an advantage in colder climates compared to paler ones as a darker color absorbs heat more effectively (Majerus 1998). In their study with *Colias* butterflies, Ellers and Boggs (2003) showed that if animal coloration has multiple functions such as sexual signaling and thermoregulation, opposing selection pressures exist and can maintain variation in coloration (see also Chunco et al. 2007). Because aposematic signals often consist of black patterns combined with bright colors such as yellow, red, or orange, developing a conspicuous and bright warning signal may decrease the size of melanized areas below the level that coloration can increase thermoregulatory efficacy. Although dark coloration increases an individual's activity (Kingsolver 1995; Van Dyck et al. 1997), it can also decrease the efficacy of the warning signal in the more melanic individuals, leading to opposing selection pressures on animal coloration.

Phenotypic and genotypic variation in melanine-based warning coloration occurs in aposematic and hairy *Parasemia plantaginis*' (Arctiidae) larvae. The orange patch on the dorsal side of the black body forms the moderately conspicuous warning pattern (Ojala et al. 2007; Lindstedt et al. 2008) (Fig. 1). Hairiness only has a minor effect on predator learning, therefore predation selects for larger signal sizes because predators learn to avoid larvae with large signals faster than larvae with small signals (Lindstedt et al. 2008). In addition, larvae that produce a larger signal size have a shorter development time and better survival, suggesting better fitness for conspicuous individuals in terms of life history correlates in favorable light and temperature conditions (Ojala et al. 2007). However, geographical and seasonal variation in temperature could constrain the expression of a large orange warning signal and explain variation seen in signal sizes. Hence, in warm years, large signals (brighter) may have an advantage over the small signals (darker) because of a better antipredator defense,

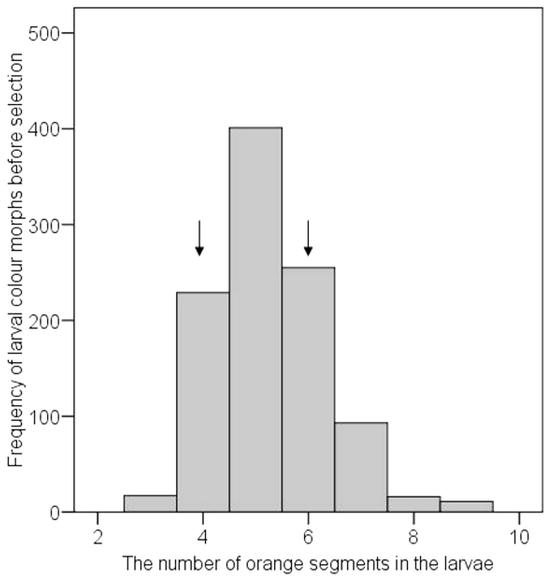

Figure 1. The variation in size of the orange color patches in last instar *P. plantaginis* larvae in P-generation. Arrows show the thresholds for the signal size in upward and downward selection lines.

whereas in cold years darker individuals may have higher fitness because of enhanced thermoregulation. Alternatively, if predation intensity is correlated with temperature (see e.g., Jeanne 1979; Reznick et al. 2001; Wüster et al. 2004; Niskanen and Mappes 2005), it is possible that in colder habitats this would further enhance the selection for smaller signals.

We conducted a two (high and low thermal environment) by two (high and low selection lines for warning signal size) factorial rearing experiment with a full sibling design. By rearing different genotypes in different radiation regimes with the full sibling design, we tested if growing in a low temperature constrains the warning signal expression in *P. plantaginis*. We tested (1) if there is phenotypic plasticity in the signal size of *P. plantaginis* larvae in response to temperature, (2) if there are costs and benefits of expressing a large warning signal compared to a small warning signal in different thermal environments, and (3) if larvae with different signal sizes differ in their basking behavior in low and high radiation environments. We predicted that if the temperature constrains the warning signal expression then larvae should be (1) darker in the low radiation treatment than in the high radiation treatment (plasticity), (2) larvae with a small signal size should have an advantage in the low temperature treatment with respect to life-history traits such as growth rate and development time compared to larvae with large signal size, and (3) larvae with a large signal size should spend longer periods basking in the low temperature treatment compared to ones with a small signal to balance the thermoregulatory costs of a large warning signal

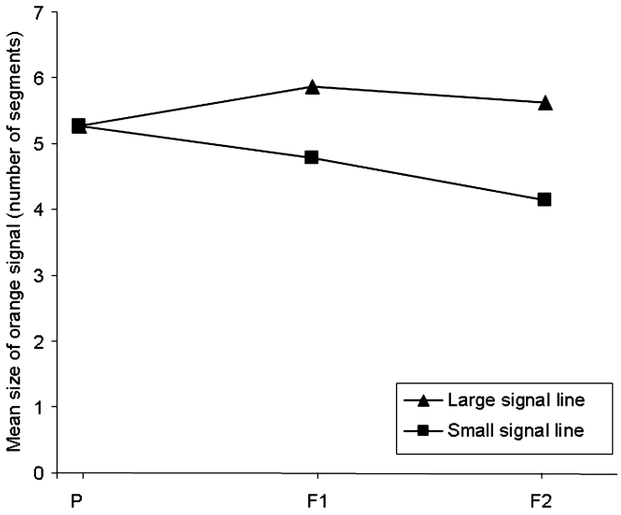

Figure 2. Response of warning signal size of *P. plantaginis* to artificial selection for two generations of selection: upward line is marked with triangles and downward line is marked with squares.

(compensatory behavior). By studying the cost:benefit ratio of the warning signal expression in different environmental conditions, we can attain a better understanding of how natural selection affects the maintenance of warning signals and genetic variation in wild populations in general.

Methods

STUDY ORGANISM

The Arctiid moth *P. plantaginis* (Arctiidae) larvae are polyphagous (Marttila et al. 1996). In Finland, this species usually has only one generation per year and typically *P. plantaginis* overwinters as a larva. Larvae have five to seven instars (Ojala et al. 2007). In laboratory conditions *P. plantaginis* moth can produce two generations per year and the second generation overwinters. The first two instars are cryptically colored and orange–black coloration is reached at the third instar onward.

SELECTION LINES

We have reared *P. plantaginis* as a permanent stock established in 2003. During the laboratory rearing, the effective population size was kept as large as possible to maintain genetic variation. The selection was started in 2004 from the 51 families from which upward and downward selection lines for divergent phenotypes (i.e., the large and small orange signals) were produced by applying a truncated family selection protocol to the stock (Lynch and Walls 1998) (Fig. 1). Thus, we first selected both the individuals with large (number of segments including orange hairs = 6 or more) and small signals (number of segments including orange hairs = 4 or less) within the family and after that we have crossed the individuals exceeding the threshold value of selected signal sizes within the selection lines in the following generations. The selection lines have been reared in laboratory conditions in a greenhouse at the University of Jyväskylä in Central Finland. The temperature in the greenhouse was kept on average at 25°C during the day and during the night it decreased to 15–20°C. Larvae overwintered at the third instar in every second generation and experienced the normal winter outdoor temperatures of Central Finland (approx. +5°C to −30°C).

Signal sizes have responded to selection rather strongly already after two generations (Fig. 2). We estimated the genotypic and phenotypic variances for signal size and heritability by using the REML-animal model implemented in ASReml 2.0-software (VSN international Ltd, Hemel Hempstead, UK). We used the model $y = \mu + a + e$, in which μ is the overall mean, a is the additive genetic effect, and e is the residual. In complex pedigrees this method is powerful to estimate additive genetic variance (e.g., Lynch and Walsh 1998). Total number of individuals measured per generation (n), population mean for the signal size in different generations (±SD), phenotypic variance, environmental (residual) variance, additive variance, and the heritability estimate for the original population (±SE) are reported in Table 1.

EXPERIMENTAL PROCEDURES

Families for the experiment were taken from the third generation of lines selected for small ($N = 20$) and large ($N = 24$) signal.

Table 1. Population means for the warning signal size (i.e., number of segments). Total number of individuals measured per generation (n), population mean for the signal size (±SD). Phenotypic variance, environmental (residual) variance, additive variance, and heritability estimate for the original population (±SE).

	Generation	n	Population mean (±SD)	Vp (±SE)	Ve (±SE)	Va (±SE)	h^2 (±SE)
	P	1022	5.26 (±1.07)	0.9655 (±0.0414)	0.4568 (±0.0363)	0.5087 (±0.0615)	0.53 (±0.05)
Small signal line	F1	166	4.80 (±0.76)				
	F2	74	4.15 (±0.86)				
Large signal line	F1	310	5.87 (±1.01)				
	F2	100	5.62 (±0.75)				

The larvae from each line were randomly chosen and equally divided into two temperature treatments 10 days after hatching (8 larvae/treatment/family) resulting in 160 individuals from the small signal line and 192 individuals from the large signal line for each temperature treatment. As the larvae were still cryptic at the age of 10 days, larvae were randomly assigned to treatments rather than based on the individual signal size. During the experiment larvae were grown in family groups in containers with living plants (see below) until they reached a weight of 100 mg (VI–VII instar) after which they were moved onto the petri dishes and grown individually in temperature treatments.

Radiation intensity affects thermoregulation of insects (Digby 1955). We therefore created two temperature treatments using artificial habitats differing in both radiation intensity and temperature. We used incandescent lights placed at different heights above the plants to create temperature differences. Bulbs release heat and do not produce wavelengths usable for photosynthesis. To maintain the photosynthesis of plants, the laboratory was lighted with fluorescent daylight tubes. In the low temperature treatment the mean day temperature was 16.8 °C (± 0.05) and the 40-W incandescent light bulbs were positioned 39 cm above the plants. In the high temperature treatment, 70-W incandescent light bulbs were positioned 15 cm above the plants, increasing the temperature to 20.3 (±0.05)°C. During the night, the temperature decreased to 15°C in both treatments. These temperature manipulations mimicked the conditions in sunny and shady patches in the northern distribution ranges of the *P. plantaginis* moths.

Larvae were fed with similar-sized living dandelion (*Taraxacum* sp.) planted in a homogeneous mixture of commercial soil enriched with fertilizer (Kestomulta produced by Biolan) in ceramic pots. A plastic cylinder covered with mesh cloth (26 × 12 cm, height × diameter) surrounded each plant and prevented larvae from escaping. To minimize variation in plant quality, the dandelions were grown in a greenhouse from roots collected from the same area near the laboratory. After the plants reached a certain size (approximately same amount of 20–26 cm long leaves) we chose similar-sized plants of the same clone for each full sibling pair to ensure similar light and food conditions for the larvae. During the experiment, the position of rearing pots was randomized. The plants were also watered every second day and sprayed once a day with water. No pesticides or fertilizers were used during the experiment. Because one plant was not sufficient to feed the later instars larvae, we also added fresh leaves daily to the plants in the containers in both treatments. This ensured that the food quality was similar despite the temperature difference between the treatments. During the individual rearing in last instars, larvae were fed with collected dandelion leaves, which originated from the same location as the planted individuals. Additional food in the containers and on the petri dishes was changed daily to prevent differences in the freshness of food between the radiation manipulations.

During group rearing all the larvae in the container ($N = 8$) were weighed and their signal size was measured once a week. After larvae reached 100 mg they were reared individually, which allowed us to measure development time, maximal signal size and brightness, and the weight of the pupa of each individual. The proportional size of the warning signal was determined from the last instar larvae by counting the number of body segments that were covered by orange hairs. As larvae have the same number of segments in all instars, the possible differences between the treatments in signal size due to allometry do not contort statistical analyses and results. The hue of the signal varies from dark brownish orange to brighter yellowish orange. To obtain a quantitative measure of the brightness, the last instar larvae were photographed with a Canon D 60 digital camera (Canon, Tokyo, Japan) in standardized light conditions. During the photographing larvae were anesthetized with CO_2 and stretched to full length. The brightness of the signal was measured using ImagePro Plus 4.0 (Media Cybernetics, Bethesda, MD) from the gray scale photos. Due to high mortality during the experiment, the final sample size of last instar individuals on the high radiation treatment was 57 individuals (14 families) from the large signal selection line and 43 individuals from the small signal selection line (10 families). On the low radiation treatment we had 42 individuals from the large signal selection line (10 families) and 32 from the small signal selection line (10 families).

Behavioral observations were recorded once a week to determine how the radiation treatment and signal selection line affected the basking behavior of larvae. Basking activity was measured as a proportion of larvae spending time basking and/or feeding on the plant's leaf during daytime (i.e., number of larvae on the ground/number of larvae on the plant). We also measured the temperature during the recording from inside and on the top of the rearing container. The behavioral observations were taken once a week. The timing of observations was changed weekly, to examine the effect of diurnal variation in the temperature on the larval behavior. Behavioral observations were stopped when the larvae reached the 100 mg weigh and were moved onto the petri dishes. In the data analysis we used only the last four observation times (21, 27 July and 3, 11 August) because during those periods, most of the larvae had reached the orange–black colored instar. Thus, the earlier observations from cryptically colored instars did not measure how the size of black element affected the larvae's behavior.

DATA ANALYSIS

To test the effect of temperature treatment and selection line on the weight and signal size of the small larvae during the group rearing, we analyzed the change in weight and signal size on the

6th, 10th, and 15th week from the beginning of experiment with repeated measures analysis of variance (ANOVA). We used the mean weight and the mean signal size of the family as a dependent variable and the selection line and temperature treatment were used as between-subject factors. Due to the heteroscedastic structure of the weight and signal size data, we log-transformed the variables prior to the analyses.

We fitted a general linear model ANOVA with temperature treatment and selection line as fixed factors and family as a random factor (family nested within selection line) to analyze the effect of temperature treatment and selection line on individual (1) warning signal size, (2) brightness, (3) development time from the beginning of the experiment (10-day old) to pupa, (4) growth rate (log pupal mass/larval development time in days) from larvae to pupa and (5) pupal weight of last instar larvae. Because the data were heteroscedastic, we used log-transformed values for the pupal weight. Due to the high mortality of larvae in both temperature environments, only the families with at least two individuals in both treatments were included in the analysis, which decreased the final sample size of families to eight families from the small signal selection line and nine families from large signal selection line. To test possible family-by-temperature interactions in signal expression, we also ran the second ANOVA separately within the selection lines (because a family belonged to only the large or small signal line) and used temperature treatment as a between-subject factor and the family as a random factor in the model.

We analyzed the effect of temperature treatment and selection line on the number of larvae surviving from the beginning of experiment to the (1) last instar, (2) pupal stage, and (3) adult stage with a general linear model ANOVA. To test that mortality was equal across the signal sizes, we analyzed the relationship between likelihood of survival to pupal stage (dependent factor) and signal size and temperature treatment by using binary logistics.

To test how larval behavior differed between temperature treatments and selection lines during group rearing, we analyzed the differences in larval behavior between the temperature treatments and selection lines using the mean proportion of the larvae on the plant per family (larvae on the plant/total number of larvae per container) as a dependent variable because of the differential mortality between the full sibling pairs. Because the behavioral observations were repeated several times for the same family during the experiment, we used a repeated measure ANOVA, which takes into account the dependent structure of the data.

Results

SIGNAL CONSPICUOUSNESS

Early instar larvae

The mean signal size of families increased as the larvae grew larger ($F_{2,25} = 12.028$, $P < 0.001$). Moreover, signal sizes increased similarly during the larval growth in both temperature treatments as well as both selection lines, and there were no three-way interactions (time × selection line × radiation treatment) (all P-values > 0.265). Temperature did not affect the signal size of small larvae but the signal size difference between the selection lines was already visible as the young larvae had a larger signal in the large signal selection line compared to the small signal selection line. Thus, the response of signal size to selection had been sufficient. There was no interaction between selection line and temperature treatment (Table 2).

Table 2. Analysis of variance for signal expression and performance of small instars larvae. As larvae were reared in groups, we analyzed the family means.

Source	df	MS	F	P
Mean signal size of family				
Temperature	1, 26	0.073	0.732	0.400
Selection line	1, 26	1.791	17.970	<0.001
Temperature × selection line	1, 26	0.000097	0.001	0.975
Mean weight of family				
Temperature	1, 26	1.828	8.476	0.007
Selection line	1, 26	0.526	2.441	0.130
Temperature × selection line	1, 26	0.034	0.157	0.695

Late instar larvae

As larvae reached final instars, when the warning signal is at the most effective stage, both the selection line and temperature manipulation affected the signal size (Fig. 3). Signal sizes

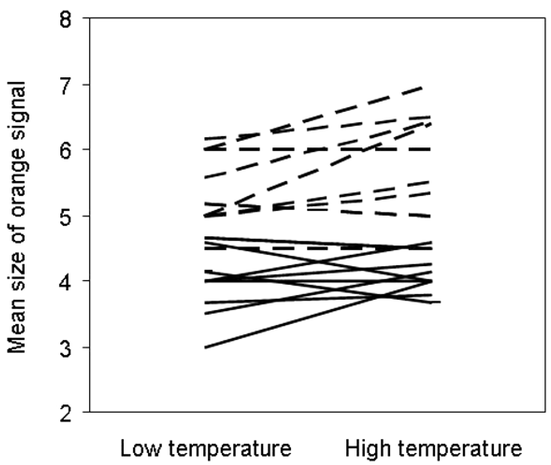

Figure 3. The reaction norms for the signal expression between different temperature treatments: dashed lines show the mean signal sizes for the families from the large signal selection line and solid lines show the mean signal sizes for the families from the small signal selection line.

Table 3. Nested analysis of variance for warning signal expression and performance of last instars larvae.

Source	df	MS	*F*	*P*
Signal size				
Temperature	1, 24.661	2.093	4.545	0.042
Selection line	23, 20.401	5.346	11.895	<0.001
Temperature × selection line	19, 130	0.447	0.854	0.639
Brightness				
Temperature	1, 27.809	0.969	4.290	0.048
Selection line	23, 20.130	0.474	2.102	0.049
Temperature × selection line	19, 129	0.226	0.992	0.475
Development time				
Temperature	1, 29.973	4201.832	34.194	<0.001
Selection line	22, 8.400	609.678	7,182	0.003
Temperature × selection line	13, 77	96.919	0.410	0.962
Pupal weight				
Temperature	1, 17.492	81255.318	23.173	<0.001
Selection line	22, 11.814	6463.147	1.710	0.171
Temperature × selection line	13, 76	3705.721	1.406	0.176
Growth rate				
Temperature	1, 35.088	0.002	75.636	<0.001
Selection line	22, 8.181	0.00002	7.109	0.003
Temperature × selection line	13, 77	0.00003	0.321	0.987

were smaller in low temperature than in high temperature in both selection lines, suggesting that temperature constrained the warning signal expression in late instar larvae (Table 3). However, the plasticity of the signal size was low because the signal sizes were always larger in the large signal line than in the small signal line in both temperature treatments, and there was no interaction between the signal selection line and temperature treatment (Table 3).

We ran a second analysis for both selection lines separately to test possible family-by-temperature interactions. There was a significant variation in signal sizes among the families within the large signal line (ANOVA: $F_{13,76} = 5.155$, $P = 0.005$), but within the small signal selection line, the among-family variation was nonsignificant ($F_{9,54} = 2.080$, $P = 0.145$). There were no family-by-environment interactions in either of the selection lines (large signal selection line: family × radiation treatment: $F_{9,76} = 0.945$, $P = 0.492$; small signal selection line: $F_{9,54} = 0.707$, $P = 0.700$). However, we found that larvae from the large signal selection line had a smaller signal size in the low temperature treatment than in the high temperature treatment ($F_{1,76} = 5.619$, $P = 0.032$), but the temperature treatment did not affect the signal sizes within the small signal selection line ($F_{1,54} = 0.503$, $P = 0.491$). Taken together, these results suggest that there was more genetical variation and environmental plasticity in the signal size among the families in the large signal selection line than in the small signal selection line, but no family-by-environment interaction between signal size and growing temperature.

Temperature and selection line also affected the brightness of the signal (Table 3). Orange signals were darker in the low temperature than in the high temperature treatment. In addition, individuals from the small signal selection line had a darker signal compared to individuals from the large signal selection line. No interaction effects were observed.

LARVAL PERFORMANCE

Survival

Selection line and temperature did not affect an individual's survival to the last instar, nor were there interactions. In addition, the selection line did not affect the survival to pupal or adult stage, but the temperature decreased the survival significantly in both of these developmental stages. Survival was lower in the low temperature than in the high temperature and did not show an interaction between the selection line and temperature treatment in later developmental stages (Table 4). Also, in the binary logistic model, only the temperature explained the survival of individuals to the pupal stage (*Wald* = 1.788, $P = 0.019$). Signal size (*Wald* = 1.776. $P = 0.183$) or interaction between signal size and temperature treatment (*Wald* = 1.214, $P = 0.271$) did not explain the survival of individuals to the pupal stage in temperature treatments. Thus, the mortality did not depend on the signal size.

Development time and growth rate

Both the temperature treatment and selection line affected the larvae's development time. Larva developed slower in the low

Table 4. Survival (number of survived/family) of the larvae to different developmental stages.

Source	df	MS	*F*	*P*
Survival to last instar				
Selection line	1, 40	2.045	0.473	0.495
Temperature	1, 40	2.045	0.473	0.495
Selection line × temperature	1, 40	4.752	1.101	0.300
Survival to pupa				
Selection line	1, 40	1.723	0.487	0.489
Temperature	1, 40	52.123	14.745	<0.001
Selection line × temperature	1, 40	3.877	1.097	0.301
Survival to adult				
Selection line	1, 40	0.462	0.154	0.697
Temperature	1, 40	56.616	18.847	<0.001
Selection line × temperature	1, 40	1.016	0.338	0.564

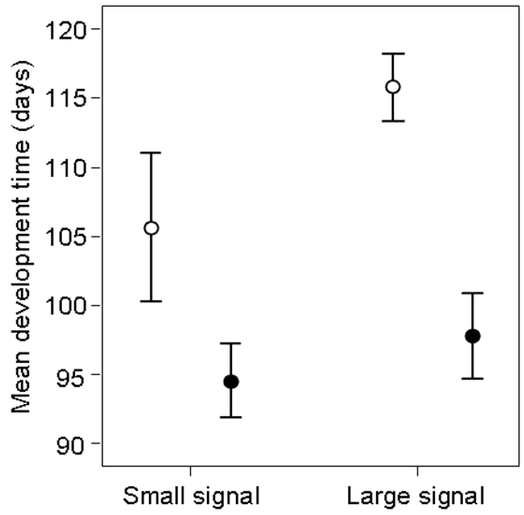

Figure 4. The mean development time from hatching to pupa (open circles: low temperature treatment; filled circles: high temperature treatment). Bars show ± SE.

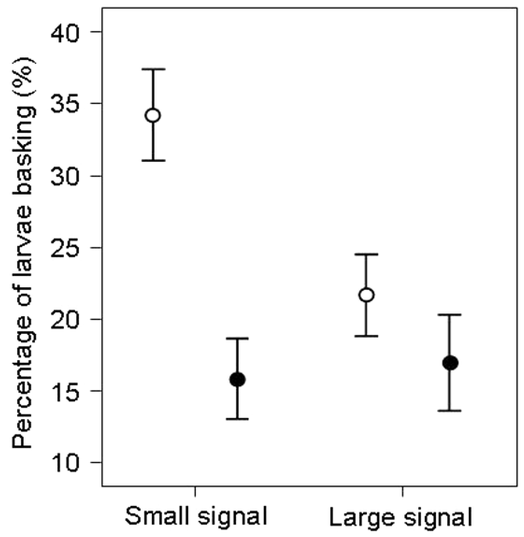

Figure 5. Percentage of larvae basking on the plant (open circles: low temperature treatment; filled circles: high temperature treatment). Bars show ± SE.

temperature than in high temperature (Table 3) (Fig. 4). Individuals in the small signal line developed faster in both temperatures compared to less melanic individuals with a large signal line. There was no significant interaction between temperature treatment and selection line (Table 3). Similarly, the growth rate (see data description) of late instar larvae was higher in the high temperature than in the low temperature and the individuals from the small signal line grew faster (i.e., they were gaining more weight per time unit) than individuals from the large signal line. There was no interaction between the temperature and selection line (Table 3).

Weight

Early instar larvae gained weight more slowly in the low temperature than in the high temperature treatment (Table 2). The mean weights of families increased similarly in both selection lines and there were no interaction between the time, selection lines, and temperature treatment (all P-values > 0.246). Consequently, the late instar larvae were heavier in the high temperature treatment than in the low temperature treatment, but the mean weight did not differ between the selection lines nor was there an interaction between the selection line and temperature (Table 3). Similarly, the pupal weights were greater in the high temperature than in the low temperature. However, there were no significant differences between the selection lines and no interactions. Thus, the signal size did not depend on the pupal weight.

BASKING BEHAVIOR OF SMALL INSTARS LARVAE

There was a significant interaction between selection line and rearing temperature for larval basking behavior (repeated measure ANOVA $F_{1,55} = 5.386$, $P = 0.024$) and therefore we performed separate analyses within the temperature treatments. In the high temperature treatment, the basking behavior of larvae did not change during the larval development (repeated measure ANOVA $F_{2,27} = 1.031$, $P = 0.370$) and selection line did not affect larval behavior (time × selection line $F_{2,27} = 1.469$, $P = 0.248$). In addition, the basking behavior was at the same level for both selection lines in the high temperature treatment (selection line, main effect $F_{1,28} = 0.145$, $P = 0.706$). In contrast, within the low temperature treatment, there was a nonsignificant trend with basking activity of larvae decreasing during the experiment as larvae grew larger (time $F_{2,26} = 2.773$, $P = 0.081$). Basking behavior did not differ between the selection lines during the larval development (time × selection line $F_{2,26} = 0.376$, $P = 0.690$). However, overall, the larvae from the small signal selection line were basking more than larvae from the large signal selection line (selection line, main effect: $F_{1,27} = 8.792$, $P = 0.006$) within the low temperature treatment (Fig. 5).

Discussion

The high heritability of the warning signal size in *P. plantaginis* larvae (Table 1) and its rather strong response to directional selection (Fig. 2) suggests that selection by predators for larger signal sizes (Lindstedt et al. 2008) should rapidly lead to more and more conspicuous warning signal expression. However, larvae of this species have considerable variation in the size of the warning signals, which suggests that there are other selective forces besides predation that may constrain the conspicuousness of the warning signal (Fig. 1; see also Ojala et al. 2007). The results from the

present study suggest that one explanation for this observed variation could be thermal constraints. The orange warning signals of *P. plantaginis* larvae were smaller and darker (i.e., larvae were more melanic and less conspicuous) when reared at a low temperature. This increased melanism can potentially decrease an individual's fitness in terms of increased predation risk (Lindstedt et al. 2008) because a smaller signal impairs avoidance learning efficiency of predators. However, the benefit of expressing the small and dark warning signal is, as our results show, an enhanced growth rate and shortened development time in colder environments (Fig. 4).

Because no defense is perfect (Calvert 1979; Fink and Brower 1981; Yosef and Whitman 1992), the overall benefit of shorter development time can be significant because it decreases the period when larvae are vulnerable to predation and parasitism and increases the probability of the individual surviving until the reproductive life stage (Damman 1987; Teder and Tammaru 2001). For species hibernating in its larval stage like *P. plantaginis*, an efficient growth rate during the spring and autumn may be especially important in terms of successful reproduction. On the other hand, because larvae were smaller (lower pupa weights) in the low radiation treatment than in the high radiation treatment, temperature may constrain the effectiveness of aposematic display by simply reducing the size of the larvae. It has been shown that a larger size of the aposematic prey increases the defense efficiency against visual predators (Gamberale and Tullberg 1996) and therefore the environmental constraints reducing the body size of aposematic prey can also decrease its warning signal efficiency.

The increased melanism was not sufficient to cover the overall costs of the colder environment compared to the warmer environment, as shown by the lower performance and survival rates of individuals from both signal selection lines in the colder environment. In this experiment, the conditions in the low radiation treatment could be expected to be harsher than in the high radiation treatment. In addition, melanin is a nitrogen-rich pigment (Nijhout 1991) and often butterfly growth is nitrogen-limited (e.g., Mattson 1980). Therefore, increased production of black melanin can be costly (Talloen et al. 2004), thereby decreasing the performance of melanized individuals as shown in a negative correlation between adult melanization and development time (Talloen et al. 2004) or adult size (Safranek and Riddiford 1975) or both (Windig 1999). Ojala et al. (2007) found indirect evidence for a "costly melanization" hypothesis as darker larvae of *P. plantaginis* had lower survival and growth rates when the thermoregulatory benefit was excluded during the rearing of larvae in favorable light and temperature conditions in the greenhouse. However, in the present study the more melanic individuals from the small signal line grew faster in the low radiation environment compared to the individuals from the large signal line. Results of the present experiment further strengthen the hypothesis already suggested by Ojala et al. (2007) that individuals with small signals are better adapted to colder climes.

It has been shown in several studies of ectothermic species that darker forms warm faster compared to paler ones, because darker colors absorb heat more effectively (e.g., Forsman 1997; Goulson 1994; Bittner et al. 2002; Pereboom and Biesmeijer 2003). Thus, it is intriguing to ask why the larvae with a large signal (less melanic) did not try to balance their thermoregulatory costs through behavioral responses by increasing their basking activity. For instance, in speckled wood butterflies (*Pararge aegeria*) pale males spend more time resting within the sunlit patches than darker males that are more active and spend more time in shady patches (Van Dyck and Matthysen 1998). We have several hypotheses for the larval behavior observed in our study. First, the basking activity of the individuals could be directly correlated to their ability to obtain heat, which would slow down the growth of less melanic individuals because of decreased heat absorbance. Second, darker surfaces also radiate heat more rapidly compared to paler ones (Pereboom and Biesmeijer 2003), thus to maintain a certain activity level, the individuals with more melanic coloration may spend more time near the heat source compared to less melanic ones to decrease the heat loss (see also Forsman 2000).

The third possibility is that the thermal preference is genetically linked to the coloration of the individual (see Forsman 2000; Forsman et al. 2002 for similar results with pygmy grasshoppers), suggesting that the optimum temperature would be lower for less melanic individuals than melanic ones. However, this is contradictory to the finding that individuals with large signals grow faster in optimal temperatures (Ojala et al. 2007). We suggest that this hypothesis can also work the other way around. High basking activity and melanism are both beneficial traits to maintain in colder conditions and a genetic correlation between them could explain the behavioral patterns observed. Furthermore, if the melanism genetically correlates with other traits that increase cold tolerance, we cannot know if the benefit of increased melanin via faster development is due to (1) physical properties of coloration (i.e., higher absorption efficiency), or (2) cold tolerance traits genetically linked to melanic appearance. Irrespective of the mechanism, the small signaling individuals seem to be better adapted to the colder environments in the absence of predation. In addition, even though this study did not give support for the benefits of paler coloration (i.e., a large signal size in warmer environments), it is possible that the larger signals could be favored in the case in which they decrease the risk of overheating in sunny and warm patches (see also Pereboom and Biesmeijer 2003).

The results presented here suggest that variation in temperature can maintain variation both in the genotypic and phenotypic levels of warning coloration via two mechanisms: (1) through adaptive plasticity in melanization (larger signaling

larvae developed more melanic in colder environment) and, moreover, (2) through thermoregulatory benefits of darker individuals shown by higher growth rates. Thus, in warmer periods and habitats, the frequency of large signal genotypes may increase as selection by predators favors larger pattern elements (Lindstedt et al. 2008). Consequently, if the environment changes (i.e., climate gets cooler), the individuals with a small signal can have an advantage and the adaptive peak in signal sizes shifts to a different range of values (i.e., opposing selection pressures exist). However, because we do not know how the variation in temperature interacts with the predator's behavior or with other selection pressures such as parasites or pathogens, we want to emphasize that this is a very simplified scenario. Nevertheless, our results strongly underline the suggestion that an aposematic display is not only defined by its effectiveness against predators, but that environmental constraints and opposing selection pressures on signaling and other functions of coloration are important as well.

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