Journal of Animal Ecology

British Ecological Society

Journal of Animal Ecology 2013, **82,** 653–662 doi: 10.1111/1365-2656.12037

Environment-mediated morph-linked immune and life-history responses in the aposematic wood tiger moth

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Summary

1. Warning signals are expected to evolve towards conspicuousness and monomorphism, and thereby hamper the evolution of multiple colour morphs. Here, we test fitness responses to different rearing densities to explain colour polymorphism in aposematic wood tiger moth (*Parasemia plantaginis*) males.

2. We used larval lines sired by white or yellow adult males selected for small or large melanization patterns of coloration. We reared these selected lines either solitarily (favourable conditions) or in aggregations (challenged conditions), and followed their performance to adult stage. We tested whether differences in larval density affected life-history traits, adult melanin expression, adult morph (white or yellow) survival and immunological responses.

3. We found that the aggregated environment increased mortality of larvae, but decreased larval developmental time and pupa weight. Adult wing melanin pigmentation was dependent on larval melanin expression but not rearing density. We also confirmed that adult wing coloration had a genetic basis (h^2 = 0·42) and was not influenced by larval growth density. Adult yellow males survived better from aggregations in comparison with white males, which may be related to differences in immune defence. White males had better encapsulation ability, whereas yellow males had increased lytic activity of haemolymph in the aggregations.

4. Our main results highlight, that morph-linked immune responses mediated by differential growth density may facilitate the maintenance of colour polymorphism in aposematic species. In nature, risk of diseases and parasites vary spatially and temporally. Therefore, both yellow and white adult morphs can be maintained due to their differential investment in immune defence in heterogeneous environments.

Key-words: aggregation, aposematism, colour polymorphism, immune defence, *Parasemia plantaginis*

Introduction

Elaborate colour signals represent some of the most well-documented examples of discrete phenotypic variation both in vertebrates (Seehausen & Alphen 1998; Roulin 2004) and invertebrates (Sandoval & Nosil 2005; Svensson & Abbott 2005). The maintenance of different genetic morphs within the population implies similar net benefits for all morphs (Ford 1965; Maynard Smith 1982), and therefore, different colour morphs should have equally rewarding strategies in the long term (Gross 1996; Sinervo & Lively 1996). Otherwise, morphs with lower fitness would decrease leading to fixation of the fittest morph (Fisher 1930; Stearns 1992). Although some polymorphisms are maintained by selective heterogeneity in predation pressure (Olendorf *et al.* 2006), polymorphisms can also be maintained by intraspecific competition (Calsbeek & Cox 2010), female preference (Maan & Cummings 2008) via frequency-dependent selection or differential thermoregulation benefits of morphs (Forsman 1995; Williams 2007). Alternative colour morphs can also differ in features other than colour (i.e. correlated characters) (Sinervo & Svensson 1998; Roulin 2004; Gray & McKinnon 2007; McKinnon & Pierotti 2010). For example, in side-blotched lizards (*Uta stansburiana*) hormone levels of different female morphs have been shown to respond to their social environment (Comendant *et al.* 2003).

The maintenance of colour polymorphism becomes puzzling to explain under the context of aposematism (Poulton

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1890; Cott 1940; Edmunds 1974). This is because warning colour patterns are expected to be under positive frequency-dependent selection (Endler 1988; Mallet & Joron 1999; Sherratt 2002), which should prevent the evolution of multiple colour signals (Lindström *et al*. 2001; Rowland *et al*. 2007; Marples & Mappes 2011). In spite of this there are several examples of aposematic polymorphism (Brakefield 1985; Siddiqi *et al*. 2004; Williams 2007), which may stem from a variety of reasons. For example, in a resource-limited environment genetic morphs are bound to allocate nutrient components among necessary life-history traits (Stearns 1992). Thus, if there is a cost to producing an effective warning coloration (Darst, Cummings & Cannatella 2006; Blount *et al*. 2009, 2012), limited resources may constrain signal expression (Grill & Moore 1998; Ojala, Lindström & Mappes 2007; Lindstedt *et al*. 2010b). In addition, if genetic morphs are allocating available resources in a different way (Roff 2002), it could result in a physiological trade-off between competing colour morphs in terms of immune defence (Svensson, Sinervo & Comendant 2001; Pryke *et al*. 2007).

Correlational selection may favour colour polymorphism especially if individuals are bound to produce some traits which expression is excluding some others (Roff 2002). For example, in side-blotched lizards the female throat colour polymorphism is linked to immunological defence (Svensson, Sinervo & Comendant 2001). This appears as positive relationship between survival and antibody responsiveness in the yellow morph, whereas this relationship is negative in the orange morph, suggesting that producing an orange throat colour is limiting an effective antibody response in this species. Indeed, defence against pathogens is one fundamental trait shaping the fitness of organisms (Wilson & Cotter 2008), and it has been shown to result in physiological trade-off between colour morphs in terms of immune responses (Svensson, Sinervo & Comendant 2001; Pryke *et al*. 2007).

The insect immune system provides defence against different pathogens, such as parasitoids, viruses, bacteria and fungi, mainly via humoral and cellular components (Ashida & Brey 1998; Khush & Lemaitre 2000; Rolff & Reynolds 2009). The humoral immune response (i.e. the lytic activity of haemolymph) is mainly targeted against microbial pathogens (Morishima *et al*. 1995; da Silva, Dunphy & Rau 2000; Shelley 2004), and is characterized by fast production of multiple small antimicrobial peptides (Khush & Lemaitre 2000; McKean & Nunney 2001). Cell-mediated immune response (i.e. encapsulation ability), however, is primarily directed against foreign intrusions such as eggs and larvae of parasitoids, spores of fungi and microbes (Carton & Nappi 1997; Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005). Importantly, encapsulation ability is linked to phenoloxidase (PO) cascade through series of micropeptide formation starting from tyrosine being further catalysed by phenoloxidase and eventually leading to melanin (Gotz & Boman 1985; Ashida & Brey 1998; Siva-Jothy, Moret & Rolff 2005). Thereby, expression of melanin-based pigments in phenotype can link to an active immune system (Aso *et al*. 1985; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000), or alternatively, deposition of melanin in the cuticle can serve some other function like warning coloration or thermoregulation (Lindstedt, Lindström & Mappes 2009).

Males of the aposematic wood tiger moth (*Parasemia plantaginis*) show discrete phenotypic variation locally and on a broader geographic scale (Leraut 2006), and therefore provide a suitable system to study the maintenance of colour polymorphism. The most typical colour morphs in Europe are yellow and white with various degrees of melanization (Lindstedt *et al*. 2010a; Nokelainen *et al*. 2012). Larvae possess variable orange-black coloration, and the size of the orange patch functions as a warning signal (Lindstedt, Lindström & Mappes 2009). As a generalist herbivorous capital breeder (Tammaru & Haukioja 1996), this species occurs in a wide variety of habitats without strict dietary preferences (O. Nokelainen, R.H. Hegna, J. Valkonen, C. Lindstedt & J. Mappes unpublished). Both male morphs are aposematic, but the yellow males have a fitness benefit by possessing the more efficient warning signal against visually hunting bird predators, whereas white males seem to benefit from female preference (Nokelainen *et al*. 2012). It also appears that more melanized adult individuals have a fitness benefit of increased thermoregulatory properties at the cost of less efficient warning signalling (R.H. Hegna, O. Nokelainen, J.R. Hegna, & J. Mappes unpublished). A similar trade-off is also confirmed in the larvae of this species (Lindstedt, Lindström & Mappes 2009). Females show continuous variation (yellow-orange-red) in coloration (Lindstedt *et al*. 2010a), for simplicity, however, here we only focus on the maintenance of male-limited colour polymorphism.

Here, we ask whether morph-linked responses to differential larval density can facilitate the co-occurrence of white and yellow male morphs. To do so, we tracked fitness-related traits in both favourable (solitary) and unfavourable (aggregated) environments. We used lines selected for small or large melanization patterns of larval coloration, reared larvae sired by white and yellow males solitarily (favourable conditions) and in aggregations (challenged conditions) and followed their performance to adult stage. First, we estimate the heritability of adult colour pigmentation (i.e. phenotype) with parent–offspring regression to test whether the phenotypic expression of coloration in adults is genetically controlled. Secondly, we examine performance of different colour morphs once the larvae reared under different larval densities reached adulthood. From a life-history point of view a highly competitive environment is expected to be costly (Roff 2002; Zuk & Stoehr 2002; Rantala & Roff 2005). Therefore, compared with favourable conditions, larvae grown in aggregated groups are expected to have shorter developmental time (e.g. escaping the costly environment; Goulson & Cory 1995; Sheldon & Verhulst 1996) and to experience high mortality. Thirdly, as larval aggregations are prone to pathogen infections (Goulson & Cory 1995),

achieving immunity under life-history bound resource costs could allow for differential resource allocation between adult morphs in the challenging environment (Svensson, Sinervo & Comendant 2001; Zuk & Stoehr 2002). If melanin pigmentation is linked to activation of immune system in the wood tiger moth (see also Friman *et al*. 2009), increased disease risk may also constrain warning signal expression by increasing cuticular melanization (Aso *et al*. 1985; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000). Thus, we predict an increase in the area of wing melanization for individuals in the aggregated environment as an indication of investment in immune defence together with up-regulated immune responses. If adult colour morphs also have differential immune responses it could reveal alternative strategies in pathogen resistance.

Materials and methods

REARING OF *PARASEMIA PLANTAGINIS*

Parasemia plantaginis used for the experiment originated from a laboratory stock (12th generation), and experimental individuals derived from 11 generations of two divergent selection lines for larval coloration (small or large melanization pattern, see Lindstedt, Lindström & Mappes 2009); hereafter they are referred to as 'selection lines'. The experiment was conducted at the University of Jyväskylä, Central Finland (62°N, 26°E) from July until October 2008. Food (dandelion, *Taraxacum* sp.) was offered *ad libitum* during the larval period (for more details see Lindstedt *et al*. 2010b; Lindstedt, Lindström & Mappes 2009). Larvae were checked and fed, and rearing containers were cleaned daily until they reached adulthood.

Larvae were divided into treatment groups as follows: Larvae were first reared together in family groups consisting on average of 143 (SD ± 101) individuals (total number of families = 50) until their third instar under regular greenhouse conditions. On the third instar, larvae were divided into two different densities (solitary or aggregations). Solitary larvae were reared individually on petri dishes (diameter 90 mm) representing favourable (i.e. less stressful) growing conditions with typical density for later larvae instars. Aggregated larvae were reared in groups of 40 individuals in plastic rearing containers (100 × 130 × 120 mm). In total we had 1517 individuals divided among each treatment group (Fig. 1). After division, all experimental larvae were then reared in controlled environmental chambers (MLR-351; Sanyo, Etten-Leur, Netherlands, Table S1).

Wood tiger moth eggs are usually laid in egg clusters located in close vicinity, and all together contribution of one female can be up to 400 larvae. Given this, it is possible that in their early development larvae are in even more dense conditions than in our experiment's aggregations, but as larvae grow, they soon start to disperse and larval clusters will be scattered. In this experiment, however, our goal was to seek for correlated fitness characters by manipulating growing conditions of larvae.

ADULT PHENOTYPE DETERMINATION

The visual difference between adult yellow and white male colour morph was confirmed by spectrophotometer measurements

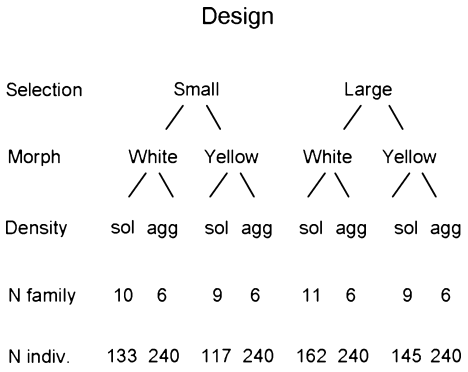

Fig. 1. Schematic illustration of the study design used to examine density-dependent effects on mortality and immune defence in two colour morphs of wood tiger moth males (white, yellow). *Selection* represents the origin of the larvae based on selection lines for more or less melanization (or conversely small or large warning signal patch) deposited on larval coloration. *Morph* represents the colour morph of fathers. *Density* refers to the larval rearing density treatment (solitary treatment (*sol*) larvae were grown individually and aggregated treated (*agg*) larvae were grown in groups of 40 individuals). Next, *n family* indicates number of families in treatments, and *n indiv*. stands for total number of individuals at the start of experiment. Each family consists of on average 14 members. In aggregations we used 24 rearing containers resulting in a total six high-density containers in each treatment. In each of high-density containers we divided members into four different families to control for family-based close relatedness.

(Nokelainen *et al*. 2012), allowing easy classification of colour morphs of eclosed adult moths by eye. We used digital photographing to quantitatively measure the variation in wing melanization pattern in adult males. Photographs were taken of dead specimens with a FujiFilmFinepix S3 Pro UVIR digital camera with standard illumination (Arcadia Reptile D3; Salfords, Redhill, UK). A subsample of adult individuals was used to measure variation in melanization pattern between treatment groups (Table S2). The coverage of wing melanization pattern was measured with Paint.Net – software (dotPDN LLC) – from right fore- and hind wing. The proportion of melanization pattern on the wings was then calculated by dividing the area of melanization pattern by the total area of the wings.

HERITABILITY OF COLOUR PIGMENT

Heritability of wing coloration was estimated using the regression between mean trait values of sire and their singly reared offspring. First, the average colour expression of a family from known sire was calculated. Then, the colour of male progeny (white, yellow) was regressed against the colour morph of the sire in linear regression. The obtained heritability estimate then equals twice the slope of the regression line (Lynch & Walsh 1998). This approach was chosen for categorical trait value, as the colour polymorphism in wood tiger moth is likely the result of two or more linked genes that reside on the sex chromosomes (Honkola *et al*. unpublished).

Relationships between larval density, colour morph of the sire (white, yellow) and selection line (small, large melanin patch) on adult wing colour (white or yellow) of the eclosed moths was analysed in binary logistic regression starting with the full model,

Table 1. Binary logistic model fitting of the eclosion proportions of two male colour morphs of wood tiger moth: relationships between colour morph of the sire, larval density, larval selection line and their interactions. Chi-square statistics describe the difference between log-likelihood function for the current model and initial model; the lower the chi-square statistics the more accurate the model. The degrees of freedom for the model chi-square statistics are equal to the difference between the numbers of parameters estimated in the current model. Difference χ^2 *df* 1 describes the χ^2 improvement to the next step if one term is removed from the model. Underline describes the best model fit

Model	Term removed	χ^2	DF	Difference χ^2 df 1
1. D + S + C + D × S + D × C + C × S + D × C × S		32·178	7	
2. D + S + C + D × S + D × C + C × S	D × C × S	31·691	6	−0·487
3. D + S + C + D × S + D × C	C × S	31·575	5	−0·116
4. D + S + C + D × S	D × C	31·388	4	−0·187
5. D + S + C	D × S	30·928	3	−0·460
6. S + C	D	29·864	2	−1·064
7. C	S	28·413	1	−1·451
8. intercept	C	<0·001	0	−28·413*

D = density, S = larval selection line & C = Colour morph of the sire. + describes the main effects, whereas × stands for interactions. All models incorporate constant, but in the last step it is highlighted. * Sig < 0·001 departure from Chi-square distribution

and omitting non-significant ($P > 0{\cdot}05$) covariates stepwise (Table 1). This test was done using IBM SPSS statistics 19·0 software (Armonk, NY, USA).

REARING DENSITY AND FITNESS

To measure the costs and benefits affecting the fitness of colour morph of the eclosed adult moth, we measured life-history responses from larvae and adult males. The overall larval mortality was calculated from all individuals who died before the adult stage. Developmental time from larva to pupation was measured in number of days. As soon as individuals reached the pupation stage they were weighted to the closest milligram (mg). In addition, we counted amounts of eclosed adult moths and their colour morphs. As colour morphs cannot be distinguished before adult stage, the measure of eclosed adult moths serves as an indirect measure of morph-dependent mortality.

Total larval mortality was analysed using a Chi-square test, as it is frequency-based data. In addition, frequencies of eclosed adult moths were analysed using a Chi-square test. First, frequencies of eclosed adult moths were tested over all treatment groups, and then eclosions were tested on both density groups. Linear mixed-effects (LME) analysis was used to examine the life-history responses of interest using R version 2·12·2 (R Development Core Team 2009). Models were simplified starting from the full model and sequentially removing non-significant interactions ($P > 0{\cdot}05$). All main effects were kept in the final model. To account for the fact that individuals from the same rearing containers may express similar kind of responses, we incorporated rearing container (i.e. the jar effect) as a random effect in the intercept. As there is no within-jar variance for individuals reared solitarily, variance for the low-density individuals was fixed to zero, but allowed to be estimated for the aggregated treatment. We constructed linear mixed-effects models for two response variables: developmental time and pupa weight. The explanatory variables were colour morph of the eclosed adult (*morph*), growth density (*density*) and selection line for larval coloration (*selection*). We incorporated the jar as a random effect in the intercept as indicated above.

IMMUNE DEFENCE AND MELANIN EXPRESSION

For immune defence assays, a subsample of individuals from each treatment was taken to measure the variation in immunological parameters (Table S2). Encapsulation assessment (i.e. induced response) is a commonly used method to stimulate an animal's response against foreign intrusions (e.g. parasitoids) (Schmid-Hempel 2005). Encapsulation assessment was conducted using the fifth instar larvae, which were reared on average 29 days and reaching a mean weight of 208·62 mg (SD ± 39·38) before the immune assays. Larvae were anaesthetized with carbon dioxide (CO^2), after which a sterilized nylon implant (diameter = 0·11 mm, length = c. 6 mm) was inserted inside the larvae between the second and the third segments from the dorsal side of the larva (Ojala *et al.* 2005; Friman *et al.* 2009). The larva was kept still by hand under the microscope while approximately two thirds of the implant was inserted with tweezers into the larva. The immune system of the larvae was allowed to react for 5 h before the implant was removed by pulling on the remaining one third of the implant outside the larva. The resulting encapsulating reaction was seen as a darkening of the implant. Five hours reaction time was selected because it yields optimal darkening of samples for later analysis with image software. Shorter time would produce too pale and longer times completely blackened samples (Ojala *et al.* 2005; Friman *et al.* 2009). The implant was dried and photographed under a microscope with 10× magnification using a Panasonic wv-CL702 (Panasonic, Osaka, Japan) video recorder. The mean grey value of the implant was measured with ImagePro Plus 4·0 (Media Cybernetics, Rockville, MD, USA) on 1 mm of the implant measured from the end implanted inside the larva to avoid measuring melanized tissue formed at the wounding sites. The grey value of the background was subtracted from the grey value of the implant to correct for any variation in lighting during photography. Three measurements were taken from each implant and their average was used. Higher grey values (darker implant) indicated stronger response of encapsulation.

Lytic activity against bacterial pathogens was determined using the area of inhibition (i.e. lytic zone) assay. Unexpectedly, the traditional method testing lytic activity (e.g. Kurtz & Sauer 1999) did not succeed to give a response, and thus we modified the method as follows. Prior to the experiment agar plates were prepared containing a solution of 10 g of Nutrient Broth, 2·5 g of yeast extract, 15 g of agar and 1 L of distilled water after which the solution was autoclaved for 25 min. The bacteria solution (*Micrococcus luteus*, ATCC strain 4698) was grown in the LB medium in 34 degrees Celsius until the lag phase. After this it was kept in the stationary phase to keep the bacterial concentra-

tion stable. Next, we injected 500 μL of the live bacterial solution onto each petri dish (diameter = 90 mm) yielding a dense film of bacteria on top of the plate. To attain a homogenous coverage of the culture across the agar solution we smeared manually with a heat sterilized spreader. Plates were then left to dry for 15 min to attain gelatinous finishing, after which five sterile filter discs (diameter = 5 mm) were placed on the plate in cross formation to designate spots for haemolymph samples.

As a measure of the lytic activity of haemolymph we measured the area of inhibition on the agar plates. 10 μL of sterile water was first added to the centremost filter paper as a negative control, which was used to control that any produced zones of inhibition were due to antimicrobial activity and not a failure in bacterial growth. The haemolymph sample was taken from the larva from the insertion site of the nylon implant by injecting a sterile needle between the second and third segment of the larva, and allowing the haemolymph to form a droplet. This was done before the nylon implant (see above) was inserted, and thereby, lytic activity of haemolymph serves as a measure of constitutive immunity (Schmid-Hempel 2005). We then withdrew 10 μL of haemolymph with a pipette and ejected it on a filter paper disc on the agar plate. Some of the samples expressed a rapid melanization reaction of haemolymph, which is often due to a strong phenoloxidase reaction (PO; Cerenius & Söderhäll 2004), and this was categorized as absent or present based on whether it occurred less than five minutes after the sample was ejected onto the petri dish. All petri dishes were kept at room temperature (+25 °C) for 3 days, after that, the plates were photographed and the area of inhibition was measured using Image J software. We subtracted the maximum area of the inhibition from the area of the filter disc as a data point. Area of inhibition was always visible around the filter disc when it occurred, but some samples never expressed the area of inhibition. Larval weight at immune assays did not differ between colour morphs of the eventually eclosed adult moths ($F_{1,68}$ = 0·047, P = 0·829) or between larval selection lines ($F_{1,68}$ = 0·680, P = 0·413).

Linear mixed-effects (LME) models with R version 2·12·2 (R Development Core Team 2009) were used to examine encapsulation and lytic activity. All models were simplified starting from the full model and removing non-significant interactions (discard P > 0·05, retain P < 0·05). All main effects were kept in the final model. All models incorporate jar as a random effect in the intercept to control for the similarity within rearing containers. As there is no within-jar variance for individuals reared solitarily, within-jar variance for the low-density individuals was fixed to zero yet allowed to be estimated for the aggregated treatment. LME analyses were carried out to explain both encapsulation response and lytic activity of haemolymph (i.e. area of inhibition) in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Generalized linear mixed model (GLMM) with a binomial response variable (absent or present) and a logit link function were used to test rapid melanization of haemolymph (PO) reaction, where colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*) were set as fixed factors. The model was fit by Laplace approximation using the lmer function in R package lme4 (Bates & Maechler 2009). Proportional melanization of adult wings was analysed using LME analysis, where wing melanin expression was set as dependent variable, and colour morph of eclosed adult individual (*morph*), larval density (*density*) and selection line for larval coloration (*selection*) were fixed factors. Finally, immunological traits and wing melanization were tested with Spearman correlation coefficients, to examine potential relationships between them. All reported P-values are two-tailed tests.

Results

HERITABILITY OF COLOUR PIGMENT

The heritability of adult colour pigmentation was h^2 = 0·422. This was obtained from the average colour of male progeny, which was regressed against the colour morph of the sire in linear regression ($F_{1,35}$ = 4·462, P = 0·042, B = 0·211, SE = 0·100). Colour pigmentation of eclosed males was best explained by colour morph of the sire (*Wald* = 27·184, DF = 1, P < 0·001, OR = 0·306), which reflects fathers getting higher proportion of sons similar in phenotype (Fig. 2). In spite of this, white males still sired 38·2% of yellows and yellow males sired 33·1% of whites of those individuals that survived to eclosion. Density, larval selection line and possible interactions were non-significant when colour morph of eclosed adult moth was included in the model (Table 1).

REARING DENSITY AND FITNESS

High larval density increased larval mortality (χ^2 = 405·258, DF = 1, P < 0·001): 17·2% of solitary reared larvae (N = 557) died during rearing, whereas 70·8% larvae grown in aggregations died (N = 960). Development time was 5 days (or 8·1%) faster in aggregations (Table 2), but was not dependent on selection line or colour morph of the eclosed adult moth. On average, developmental time for solitarily grown white and yellow males was 54 (SE ± 1·445) and 54 (SE ± 1·537) days, compared to 50 (SE ± 1·942) and 48 (SE ± 1·625) days in aggregations respectively. The average pupa weight of solitary larvae (mean = 207 mg, SE ± 3·031) was 27·4%

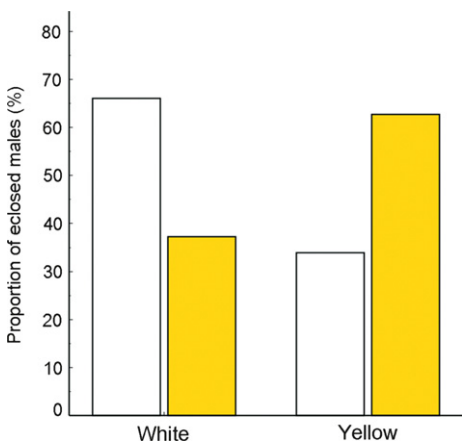

Fig. 2. The proportion of eclosed white and yellow adult morphs. Pooled data accounting both growth environments are used. On the *x*-axis, the colour morph of the sire, and on the *y*-axis the proportion of eclosed colour morphs. White bars represent the white males and yellow bars the yellow males (grey bars in print version).

Table 2. Linear mixed-effects model analysis of examined life-history responses. The model represents the best fit model to explain life-history response (developmental time and pupa weight) in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	T	*P*
Developmental time (intercept)[a]	54·933	1·717	31·976	< 0·001*
Morph	−1·158	1·607	−0·720	0·472
Density	−7·702	3·780	−1·859	0·064
Selection	−0·914	1·988	−0·459	0·646
Pupa weight (intercept)[a]	205·981	5·084	40·513	< 0·001*
Morph	2·071	4·738	0·437	0·663
Density	−34·487	11·175	−3·085	0·002*
Selection	1·857	5·879	0·315	0·752

*$P < 0·05$.
[a]Intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

heavier than in aggregations (mean = 181 mg, SE ± 3·685, Table 2).

The eclosion of the different adult colour morphs was density-dependent ($\chi^2 = 3·941$, DF = 1, $P = 0·047$). Colour morphs eclosed at the same proportions from solitary treatment ($N_{white} = 112$, $N_{yellow} = 104$, $\chi^2 = 0·296$, DF = 1, $P = 0·586$), but more yellow males eclosed from aggregated treatment ($N_{white} = 50$, $N_{yellow} = 73$, $\chi^2 = 4·301$, DF = 1, $P = 0·038$). In solitary treatment 51·9% of eclosed males were white and 48·1% were yellow, whereas in aggregations 40·7% of eclosed males were white and 59·3% were yellow.

IMMUNE DEFENCE AND MELANIN EXPRESSION

From the eclosed adult moths white males had seemingly higher encapsulating ability levels while larvae in aggregations than yellow males (Fig. 3), and encapsulation was in general increased by density (Table 3). Nevertheless, yellow males had a larger zone of inhibition while larvae than white males in aggregations (Fig. 3, Table 3). The rapid melanization of haemolymph was affected by an interaction of melanin and colour morph of the eclosed adult moth (Table 4), as white males that were less melanized as larvae were less able to produce a more rapid PO reaction than rest of the groups.

The wings of yellow males in general were more melanized (60·1%, SE = 0·01) than white males (54·6%, SE = 0·01). There was also an interaction between colour morph of eclosed adult moth and larval selection line affecting the area of wing melanization (Table 3) because white males that were less melanized as larvae were less melanized in general compared with other groups. In general, however, the more melanin expressed in the larval stage translated to more melanin expressed in the adult stage (Fig. 4, Table 3). The rapid melanization of haemolymph (PO) was correlated with encapsulation ability but not with haemolymph's lytic activity or wing melanization (Table 5).

Discussion

These results demonstrate that wood tiger moth males have differential morph-linked immune responses. First, we confirm heritability of wing colour ($h^2 = 0·42$), which indicates that the colour polymorphism of wood tiger moth is genetically determined, and not favoured by a plastic response of colour expression to differing environments. The eclosion of adult morphs, however, was dependent on growth environment. White adult morphs have increased encapsulation ability when reared in aggregated larval environments, whereas yellow morphs express increased lytic activity of haemolymph.

Immune responses are generally stronger when individuals are grown in challenged conditions (Wilson & Cotter 2008), and thus, immune defence can have consequential trade-offs with life-history traits (Rantala & Roff 2005; but see Freitak *et al.* 2005) or competence (Kraaijeveld & Godfray 1997; Kortet, Rantala & Hedrick 2007). We observed a 53 percentage point higher larval mortality in aggregations than in solitary treatment suggesting that the aggregated environment was costly (Kazimirova 1992; Wilson *et al.* 2002; Cotter *et al.* 2008). Furthermore, there was a trend that aggregated larvae pupated faster than solitary ones, which may suggest a life-history strategy of quickly escaping the highly competitive and potentially contagious environment (Goulson & Cory 1995; Longson

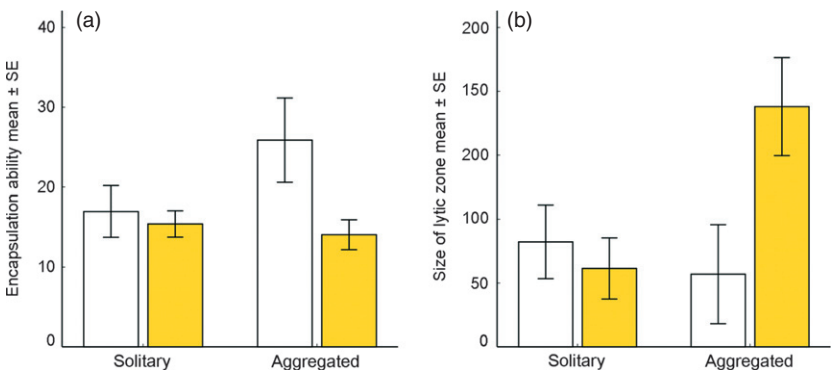

Fig. 3. The immunological responses of eclosed male wood tiger moth colour morphs to density treatment. White bars represent the white males and yellow bars yellow males (grey bars in print version). (a) On the *x*-axis, the larval growth density, and on the *y*-axis the mean encapsulation ability. (b) On the *x*-axis, the larval growth density, and on the *y*-axis the size of lytic zone ability (mm^2).

Table 3. Linear mixed-effects model analysis of examined immunological responses and phenotypic melanin expression of wood tiger moth males to density treatment. The model represents the best model fit to explain assigned responses in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	T	P
Encapsulation ability				
(intercept)[a]	13·747	3·979	3·623	< 0·001*
Morph	1·101	5·242	0·193	0·849
Density	16·726	7·096	2·357	0·022*
Selection	5·886	5·137	1·145	0·257
Morph × density	−15·118	8·049	−1·878	0·084
Morph × selection	−4·434	7·581	−0·572	0·577
Density × selection	−14·340	9·792	−1·464	0·149
Morph × density × selection	13·339	10·780	1·237	0·239
Lytic activity				
(intercept)[a]	71·124	31·729	2·241	0·002*
Morph	−17·030	38·684	−0·440	0·664
Density	−7·863	58·080	−0·135	0·892
Selection	19·916	34·798	0·572	0·569
Morph × density	156·543	66·071	2·369	0·028*
Wing melanin expression				
(intercept)[a]	0·627	0·016	39·013	< 0·001*
Morph	0·012	0·018	0·640	0·525
Density	−0·005	0·017	−0·029	0·772
Selection	−0·103	0·018	−5·568	< 0·001*
Morph × selection	0·050	0·023	2·141	0·037*

*$P < 0·05$.
[a]Intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

Table 4. Generalized linear mixed model analysis of rapid melanization of haemolymph (PO). The model represents the best model fit to explain haemolymph melanization response in relation to colour morph of the eclosed adult (*morph*), larval density (*density*) and selection line for larval coloration (*selection*). Rearing jar is incorporated as a random effect in the intercept

Response variable	Estimate	SE	Z	P
Melanization of haemolymph				
(intercept)[a]	−0·142	0·526	−0·271	0·786
Morph	−0·506	0·659	−0·767	0·442
Density	0·457	0·501	0·913	0·361
Selection	−1·952	0·803	−2·431	0·015*
Morph × selection	2·450	1·024	2·392	0·016*

*$P < 0·05$.
[a]intercept includes factor levels: morph [white], density [solitary], selection [less melanized].

& Joss 2006; Ojala, Lindström & Mappes 2007). This evidence would also explain why pupae in aggregations were 27·4% smaller than solitary ones (Cotter, Kruuk & Wilson 2004; Cotter *et al*. 2004). Although individuals were given food *ad libitum*, solitary larvae likely had more resources *per capita*, as competition was excluded. Interestingly, we observed morph-dependent mortality in aggregations where there was a 18·6 percentage point difference in eclosion frequencies, whereas in solitary treatment there was no difference suggesting divergence in underlying traits in challenging conditions.

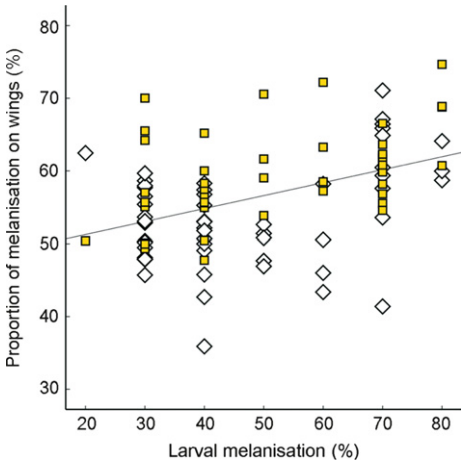

Fig. 4. Relationship between melanization of larvae (*x*-axis) and the proportion of melanization pattern on the adult wings (*y*-axis). White diamonds represent white adult males and yellow squares represent yellow males (grey squares in print version). The linear fit line $R^2 = 0·187$.

The immunological assays revealed a difference between two colour morphs of eclosed adult moths in two immune defences. Larvae that later eclosed as white males had a higher encapsulating response than individuals that eclosed as yellow males when grown in aggregated treatment (see Fig. 3). This hints of competent parasitoid resistance of white males suggesting that while larvae they are well able to encapsulate foreign intrusions (e.g. eggs of parasitoids) from their bodies (Rantala & Roff 2005; Siva-Jothy, Moret & Rolff 2005; Wilson & Cotter 2008). In comparison to encapsulation ability, the lytic zone assay indicated yellow males being better in mounting their lytic activity of haemolymph in aggregations than white males, which in turn suggests an increased defence against bacterial pathogens (Morishima *et al*. 1995; da Silva, Dunphy & Rau 2000; Shelley 2004). This pattern hints of correlational selection on larval stage. It could be that larvae have intrinsic genetic mechanism that regulate their resource-use physiologically depending on which morph individual is going to develop into. Also, morph may be bound to store their resources differently regarding the use of resources later in life history, which can result in differential investment in immune defence.

The encapsulation ability is usually linked to PO cascade via regulated series of micropeptide formation initiating from phenoloxidase proceeding with an oxidation of tyrosine to dopaquinone and further polymerization to melanin (Ashida & Brey 1998; Cerenius & Söderhäll 2004). Hence, low encapsulation of yellow males may be due to early depletion of tyrosine that prevents the completion of the PO cascade (Riley 1997). Furthermore, if yellow pigmentation is more costly to produce (Blount

Table 5. Correlations between immunological traits and wing melanization area

	Encapsulation	Phenoloxidase	Lytic activity	Wing melanization
Encapsulation				
Phenoloxidase	0·295* (74)			
Lytic activity	−0·014 ns (74)	0·040 ns (85)		
Wing melanization	0·055 ns (13)	−0·113 ns (17)	0·421 ns (17)	

*$P < 0{\cdot}05$.
ns = non-significant effect. Sample sizes are indicated inside brackets. All correlations are two-tailed nonparametric Spearman correlation coefficients.

et al. 2009), it can suggest a trade-off between PO activity and cuticular pigmentation (Gotz & Boman 1985; Rolff & Siva-Jothy 2003; Siva-Jothy, Moret & Rolff 2005; Cotter *et al*. 2008). On the other hand, possibly all immune defence traits simply cannot be simultaneously up-regulated (Cotter, Kruuk & Wilson 2004; Rantala & Roff 2005) or there can also be a potential cost of autoimmunity associated with immune responses (e.g. Sadd & Siva-Jothy 2006). To confirm whether encapsulation reaction trade-offs with lytic activity, we would need to perform a more detailed infection experiment to exclude the possibility that measured immunological pathways are merely two ways to obtain equal pathogen resistance.

Immune responses are often found to correlate with other individual traits. Abundant evidence suggests that cuticular melanization is an indication of investment in immunity (e.g. Reeson *et al*. 1998; Wilson *et al*. 2001; Cotter *et al*. 2008; Friman *et al*. 2009; but see Jacot *et al*. 2005; Joop *et al*. 2006; Karl, Hoffmann & Fischer 2010). However, here we did not find evidence that wing melanization would have indicated an investment in immunity, but instead, more melanin expression on the larval stage yielded larger area of wing melanization in the adult stage. Furthermore, white males from the less melanized selection line comprise less melanized wing patterning compared with respective yellow males resulting in a significant interaction. Perhaps yellow males share partly the same metabolic pathway to produce yellow and melanin pigmentation, but this needs further confirmation. One reason why we did not observe the cuticular melanization correlating with immune responses could be that the cuticular pigments are synthesized independently of the immune response. The rapid melanization of haemolymph (PO) had a significant interaction effect between colour morph and selection line because white males from less melanized larval selection lines expressed rapid melanization of haemolymph less often than the rest of the groups. We do not have plausible explanation of this interaction, and future work will be needed to understand if the difference between morphs that eclose from high melanin larvae has any biological significance. Haemolymph's phenoloxidase (PO) activity and encapsulation reaction were the only correlations found between the immune traits. However, as we measured PO reaction as presence–absence trait and not gradual accumulation of phenoloxidase activity, we must be cautious interpreting its role (Ashida & Brey 1998; Cerenius & Söderhäll 2004; Shelley 2004). Also, it is possible that sample sizes of the correlation analysis set limitations to its further interpretation.

There are a wide variety of mechanisms driving the maintenance of colour polymorphism (Gray & McKinnon 2007), and likely many different selection pressures influence to the puzzle of polymorphism in aposematic species. For instance, confirmed warning signal efficacy and mating success trade-off in the wood tiger moth is a plausible explanation to the maintenance of colour polymorphism (Nokelainen *et al*. 2012). Furthermore, the efficacy of warning signal likely depends on heterogeneity in predator community (Endler & Mappes 2004). It is also possible that spatial heterogeneity of the parasitic and disease community can create variable selection on immune responses. As immune responses are morph linked, variable enemy communities can indirectly contribute to colour polymorphism maintenance. It would be interesting to study the role of immune defence directly to see how susceptible colour morphs are against different parasitoids and pathogens, and furthermore, to examine in the wild whether the abundances of parasitoids and pathogens would tie in with the colour morph frequencies of the wood tiger moth. Regardless of these mechanisms our results suggest that genes underlying the phenotypic traits can be selected differently in heterogeneous environment, which may cause selection to favour polymorphism in aposematic species. The maintenance of polymorphism in aposematic species can therefore be contributed by environment through trade-offs, or even alternative strategies that are advantageous in different environmental conditions.

Acknowledgements

We are grateful to members of the Darwin Club of the Department of Biological and Environmental Science in Jyväskylä who gave constructive feedback to improve this manuscript. We thank Kaisa Suisto for the maintenance of laboratory stock. We also thank Joachim Kurtz for his insightful comments for discussion. Tarmo Ketola gave valuable comments and Swanne Gordon kindly corrected the language. Two anonymous reviewers kindly helped to improve the clarity and quality of this manuscript. Andrés López-Sepulcre kindly offered his statistical knowledge regarding mixed model analysis in R. This study was financed by the Academy of Finland (Finnish Centres of Excellence in Evolutionary research) CoE BIOINT SA project number 21000002561.

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Received 17 April 2012; accepted 20 November 2012
Handling Editor: Stewart Plaistow

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Table S1. Standardised rearing conditions.

Table S2. Sample sizes of three different data sets: proportional melanisation of wings (*wing melanin*), encapsulation assessment (*encapsulation*), and lytic activity of haemolymph (*lytic activity*).