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**The price of safety: food deprivation in early life influences the efficacy of chemical defence in an aposematic moth**

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**Abstract**

Aposematism is the combination of a primary signal with a secondary defence that predators must learn to associate with one another. However, variation in the level of defence, both within and between species, is very common. As secondary defences influence individual fitness, this variation in quality and quantity requires an evolutionary explanation, particularly as it may or may not correlate with variation in primary signals. The costs of defence production are expected to play a considerable role in generating this variation, yet studies of the cost of chemical defence have focused on species that sequester their defences, while studies in species that produce them *de novo* are scarce. Here we examine effects of resource availability on the production of chemical defences in female wood tiger moths *Arctia plantaginis*. This species produces defensive fluids when attacked, and advertises this protection with bright colours on its hindwings. Furthermore, the chemicals in these fluids are produced *de novo*. In order to establish if the production of this chemical protection is costly, and thus resource-limited, we manipulated resource availability (i.e. food) during larval development and measured its subsequent effects on adult chemical defence. We presented defensive fluids from female moths to wild blue tits, in the absence of any visual cues, to determine their effectiveness against avian predators. Our results demonstrate that the defensive fluids produced by female moths repel birds, and that these defences require resource investment to produce. We found that moths that were resource-limited during development had less effective chemical defences, despite producing the same volume of defensive fluids as the control moths. As a reduction in available resources negatively influences the effectiveness of the chemical defence, resource availability may explain the variation seen among individuals.

**Keywords:** aposematism, chemical defence, life-history, predator defence, predator–prey interactions, resource allocation

## Introduction

Predation is a significant threat to the survival of almost all organisms in existence. As a result, organisms have evolved a variety of strategies to avoid being predated upon. One such strategy is aposematism, which is the coupling of a warning signal with an anti-predator defence (e.g. mechanical defences, such as spines, or chemical ones, like toxins). The combination of these components, referred to as the primary signal and the secondary defence, allows would-be predators to learn to associate the two (Poulton 1890, Rojas et al. 2015, Ruxton et al. 2004). Thus, on detecting the warning signal, predators will recognise the individuals bearing it as unprofitable prey, and avoid attacking them. However, there remain several key challenges that must be resolved before we can truly understand aposematism as an evolutionary strategy. Chief among them is the maintenance of this relationship between signal and defence. Selection on warning signals, and the negative effect of signal variability on predator learning, should produce purifying selection (Lindström et al. 2001), yet unexpected levels of within-species signal variation exist. Understanding how genetic and phenotypic diversity is maintained is central to evolutionary biology, and this variation in aposematic signals provides an ideal system to study these interactions. However, while considerable research, both theoretical and quantitative, has been carried out trying to address this question (Kikuchi and Pfennig 2013, Lindström et al. 2001, Rojas et al. 2014), much of it has focused only on the variation in warning signals themselves. Meanwhile, our understanding of the second vital component of aposematism, the secondary defence lags behind that of the signals that advertise them. The possession of chemical defences is widespread across multiple taxa, from bacteria (Jousset 2011) to plants (Zagrobelyny et al. 2008) and animals (Blum 1981). Furthermore, a variety of different chemicals are used for defence, including disulfides (Andersen et al. 1982), terpenoids (Kim et al. 1975, Mosher et al. 1964), alkaloids (Santos et al. 2016, Saporito et al. 2012), cardenolides (Petschenka et al. 2011), pyrrolizidine alkaloids (Hartmann and Conner 2009), and iridoid glycosides (Bowers 1992, Lindstedt et al. 2010, Reudler et al. 2015, Rothschild et al. 1979, Rothschild et al. 1984), to name but a few. These compounds may be produced by the organism itself *de novo* (Bowers 1992), sequestered from another organism (Opitz and Müller 2009), or in some cases produced by symbiotic bacteria (Schmidt 2008). As with primary warning signals, variation in the level of secondary defence also exists within species (Speed et al. 2012). This variation may be non-adaptive and due to stochastic environmental factors (Speed et al. 2012), or it may be genetic (Eggenberger and Rowell-Rahier

1992). In species that sequester their chemical defences from their diet, variation in diet quality, as well as the availability of preferred host plants, can result in some individuals possessing far stronger defences than others (e.g. (Bowers M and Williams Ernest 1995)). However, this is not the only source of variation. Detoxification of defended host plants also carries costs (Després et al. 2007, Reudler et al. 2015), which individuals may be able to avoid by altering their diet at the expense of chemical protection. The existence of variation in defences in species that produce some or all of their chemical defences *de novo* is less intuitive, but could nevertheless be explained if such production is costly. In addition, variation in species with *de novo* production also hints at a possible genetic cause. Genetic variation underlying chemical defence may be maintained in a population if there are trade-offs between defence and other traits important for survival and fitness. However, evidence for physiological costs of chemical defence is not clear cut, and studies of the cost of *de novo* production are particularly lacking (Zvereva and Kozlov 2016). Furthermore, the existing literature shows a strong bias towards specialist species, i.e. those that feed on a narrow range of host plants, (Zvereva and Kozlov 2016, but see also Ojala et al. 2007, Reudler et al. 2015). Specialists, in particular those that sequester their defences, may provide an apparently simpler system, as variation in the level of defensive chemicals present in the host plant can correlate with those in the sequestering species (e.g. Brower et al. 1984). However, generalists that are capable of synthesising some or all of their defences *de novo* actually allow for more subtle manipulation, as they can survive on a greater variety of food plants, allowing them to be exposed to greater variation in nutritional content. Meanwhile, in species that sequester their chemical defence from plants, the environment (plant/diet) has a vast influence on both chemical defence and other life history traits, which is much harder to control. Thus, species that produce their defences *de novo* provide a useful system for examining the costs of chemical defence, and may give results that are widely applicable to other systems, as they can be used to test the effects of different energetic and nutritional constraints without having to consider the close co-evolutionary history often seen between specialists and their hosts.

The wood tiger moth, *Arctia plantaginis* (formerly *Parasemia plantaginis*; (Rönkä et al. 2016) is an aposematic species that produces defensive fluids when threatened. The thoracic defensive fluids (produced by glands at the “neck”) of males have been shown to be effective against avian predators (Rojas et al. 2017) due to the presence of methoxypyrazines, which the moths are able to produce *de novo* (Burdfield-Steel et al. 2018). The species is polyphagous and do

not feed as adults, instead collecting all the nutrients they need for reproduction during their larval stage, a strategy known as capital breeding (Tammaru and Haukioja 1996). Male European *A. plantaginis* have discrete colour morphs, showing variation both within and between populations (Hegna et al. 2015, Waring and Townsend 2003), while females show continuous colour variation in their hind wings, from yellow, through to orange and red (Fig. 1; (Hegna et al. 2015, Lindstedt et al. 2011)). The coloration of males and females has been shown to affect their protection against avian predators (Nokelainen et al. 2012). In particular, redder females were attacked less by great tits (Lindstedt et al. 2011) . While male colour appears to be solely under genetic control, female colour shows both heritable and environmental effects (Lindstedt et al. 2010). Thus female wood tiger moths provide an opportunity to examine patterns of investment in both primary signals and chemical defence, and how these may respond to changing resource levels.

In this study we investigate the effectiveness of female wood tiger moths' defensive fluids against bird predators and, in particular, the effect of early life resource availability on these chemical defences, and on female warning colouration. We predict that female fluids will be deterrent to birds, and that, if chemical defences are costly to produce, females routinely deprived of food during development will have less effective defensive fluids, and primary warning signals, than those who were able to feed uninterrupted.

## Materials and Methods

### *Study species*

Wood tiger moths are found throughout most of the Holarctic region (Hegna et al. 2015) in many habitats such as bogs, meadows, and alpine areas (Waring and Townsend 2003). The larvae often occur in patchy habitats, where suitable food plants may be clustered together or widely separated (pers. obs.). Thus, the resources available to them are expected to vary considerably in nature, including periods of food deprivation if larvae are forced to search for a new food plant, as well as when they emerge from overwintering in the spring. In addition, as often is the case in moths, *A. plantaginis* females do not always lay their eggs on the host plant and it is typical that larvae actively search for their host plants (O. Nokelainen pers. comm.). Moths used in this experiment were taken from a laboratory stock, founded from individuals caught in Estonia in 2010, and housed in a greenhouse at the University of Jyväskylä in Central Finland. In Estonia males are monomorphic white while females show continuous colour variation from

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yellow to red (Fig. 1). While wild individuals produce only a single generation per year and overwinter as larvae (Nokelainen 2013), in the laboratory they can produce up to three.

#### *Husbandry*

During larval development the temperature was approximately 25°C, dropping to 15-20°C at night. In every second generation larvae overwintered at the third instar, during which they were kept at 5°C. Larvae were fed with dandelion (*Taraxacum spp.*) leaves and misted with water daily. They were housed in family groups in clear plastic tubs with approximately 30 larvae per tub. Tubs were cleaned as needed and any uneaten food replaced. Upon pupation, individuals were weighed to the nearest milligram and kept individually. Pupa weight serves as an approximation of adult size, as heavier pupa tend to produce larger adults. Upon eclosion to adulthood, the sex and hindwing colour of each moth was recorded, and they were stored in a climate cabinet at 7°C. Hindwing colour was classified according to the protocol of Lindstedt et al. (2011; see below for more details).

#### *Larval diet manipulation*

We used a 2-way factorial design with family and diet treatment as the two levels. Larvae were taken from 10 families from the stock population and equally divided between the two treatments 14-15 days after hatching. A total of 500 larvae were used, 50 from each family. Larvae were initially housed in family groups of 25 per tub. Control larvae were fed daily with dandelion leaves. Larvae in the food-deprived treatment had all food removed for one day a week, and were provided with dandelion leaves *ad libitum* at all other times. Larval development time (from hatching to pupation) is typically 30-45 days when reared on dandelion. Thus the minimum time a larva in this experiment could have fed under treatment conditions is two weeks. All tubs were housed in the greenhouse. Tub position was randomised and tubs were swapped between the inner and outer shelves each day so each one spent a similar time in each position. At the 4<sup>th</sup> instar larvae were divided into individual petri dishes. All other conditions were kept constant.

#### *Adult stage*

To test the response of the larvae to food deprivation we collected measures on pupal weight, time to eclosion, and volume of thoracic fluid (hereafter referred to as defensive fluid) produced from newly emerged adult females (approximately 1 day old). To collect the fluid, each individual was held with tweezers just below the thoracic glands, and pressure was applied. This

stimulated the release of the defensive fluids, which we then collected with a glass capillary. The volume was recorded for each sample, and the fluid was transferred into a glass vial with 100µl of hexane, dehydrated using a nitrogen line, and stored at -80°C until rehydrated for testing (dehydration removed all detectable traces of hexane; see supplementary materials). Fluid samples were given individual codes to ensure the person carrying out the predator feeding trials was blind to which larval treatment they originated from. As part of laboratory protocol we also recorded hindwing colour by eye on a scale of 1-6 following the procedure of Lindstedt et al. (2011), where 1 was the most yellow, progressing through orange in 3, to the most red in 5 and 6. Classifying the colours by eye has previously been shown to correlate strongly with spectrophotometer measurements (Lindstedt et al. 2011).

#### *Predator assays*

Feeding trials were carried out at Konnevesi Research Station, Central Finland (62°N, 26°E) in the winter of 2014-2015. Blue tits, *Cyanistes caeruleus*, were used as a model predator, as they are generalist predators and have a similar distribution as *A. plantaginis* (Nokelainen et al. 2012). Birds (n= 50) were caught at feeding sites between November and December 2014, and January and March 2015 using a trap with peanuts as the food source (13 x 17 x 40 cm box) (Ham et al. 2006, Lindstedt et al. 2011, Nokelainen et al. 2012). Once trapped, all birds were housed individually in plywood cages with a daily light period of 11h:13h (light:dark), fed on sunflower seeds, peanuts and a vitamin enriched mix, and provided with fresh water *ad libitum*. Wild birds were used with permission from the Central Finland Centre for Economic Development, Transport and Environment and license from the National Animal Experiment Board (ESAVI/9114/04.10.07/2014) and the Central Finland Regional Environment Centre (VARELY/294/2015). All birds in our experiment were used according to the ASAB guidelines for the treatment of animals in behavioural research and teaching. After the experiment, all birds were aged, sexed (when possible) and ringed for identification purposes before being released at the capture site. The experimental box was made from plywood (50 x 60 x 45 cm) and lit with a light bulb showing the entire visible daylight spectrum (Nokelainen et al. 2012). Inside the box there was a perch, a water container and a small barrier that was in front of a moving hatch on which the food was presented (Nokelainen et al. 2012, Rojas et al. 2017). This barrier meant observers could clearly tell when the birds first noticed the food item presented. All birds were

introduced to eating oat flakes while still in their home boxes. They were then familiarised with the experimental box and trained to eat oats from the feeding hatch prior to the experiment. Each bird experienced four trials, the first and last were control trials in which the oat was soaked in water. Birds were exposed to the chemical defences of a single moth across trials 2 and 3. We rehydrated samples with 15 $\mu$ l of water. In all trials 7 $\mu$ l of fluid (either water or rehydrated defensive fluid) was pipetted onto the oat and allowed to soak in for 5 minutes before being presented to the bird. If the bird did not eat the entire oat within five minutes during trial 1, the test did not continue. The maximum duration of each trial was five minutes from when the bird first saw the oat. The trial ended either when the bird had eaten the whole oat, or after five minutes had passed. For each trial, predator attack latency (i.e., hesitation time between the bird seeing the oat and it tasting it) was measured, as was the time taken to finish the oat. In trials 2 and 3, if the bird did not finish the oat within five minutes, the proportion eaten was recorded. As a proxy for distaste, the number of times the birds cleaned their beak (recorded as the number of bouts of beak cleaning, i.e. occasions where the birds wiped their beaks repeatedly against any surface) was recorded. The final control trial ensured that the birds were still motivated and hungry, and thus any reaction to the middle trials was due to the fluid sample rather than satiation. All trials were recorded with a camera positioned on the roof of the box, allowing any missed behaviours to be checked after the trial was finished. Birds were only used for a single experiment and had not been previously used in any other experiment involving exposure to the moths' defensive fluids.

#### *Statistical analysis*

#### *Food deprivation and life history traits*

In order to assess the severity of the costs of food deprivation, particularly in terms of growth, we examined the effect of treatment on female life-history in a semi-random sample containing a total of 97 moths (49 food deprived, 48 control) from all ten families. We tested the effect of larval treatment on time to eclosion with a mixed effects Cox model, with a Gaussian distribution for the random effects with larval treatment as the explanatory variable and family as a random factor. We modelled pupae weight and defensive fluid with generalised linear models with larval treatment as the explanatory variable. The GLM for defensive fluid volume also included pupae weight and the interaction between pupal weight and larval treatment in the explanatory variables, as prior work suggests that larger moths produce greater volumes of fluid (K. Suisto pers. obs.).

The effect of larval treatment on adult colour morph was examined with a linear mixed-effects model (LME) with larval treatment (food deprived/control) as the fixed effects predictor variable, and family as the random effects predictor variable.

#### *Predator response to female fluids*

The remaining analyses based on the predator response assay were performed on data collected from a subset of 50 moths (24 food deprived, 26 control). As moths were not evenly distributed across the colour classifications, and only a small number of moths in categories 1, 2 and 5 were available for the experiment, moth colour was not included in the analysis of predator response (but see Supplementary figure 2 for the patterns of predator response according to the colour categories). Bird ID was included as a random variable in all subsequent models.

The effect of female defensive fluid on bird behaviour was tested by comparing the trials in which fluids were presented to birds (i.e. trials 2 and 3) with the control trials. For the purposes of this analysis fluids from food deprived and control moths were grouped together. Given that birds had to consume all of the oat in trial 1 in order to progress with the experiment, trial 4 was set as the intercept for all models. Differences in predator attack latency (i.e. time between seeing and attacking the oat) between trials were tested with a mixed effects Cox model with a Gaussian distribution for the random effects. Trial was set as an explanatory variable. The same was then done for trial duration.

We used generalized linear mixed-effects models (GLMMs) with a negative binomial distribution to test the effect that trial had on the number of times birds cleaned their beak and drank water. Trial was included as an explanatory variable with duration as an offset. Given the low frequency of both behaviours, duration was scaled to minutes. Only birds that ate at least part of the oat flake were included in these analyses. The proportion of oat consumed by birds during the trials was also analysed using a GLMM with binomial distribution, where trial was included as the explanatory variable. As the proportion eaten in trial 1 was always 100% this trial was excluded from this analysis.

#### *Food deprivation and predator response*

Analysis of the effect of treatment, and other variables such as fluid volume was then done using the results from trials 2 and 3 as these were the trials in which birds were exposed to the fluids. Differences in predator attack latency between larval treatments were tested with a mixed effects Cox model, again with a Gaussian distribution for the random effects. Larval treatment,

trial number, defensive fluid volume and the possible 2-way interaction between trial number and larval treatment were used as the explanatory variables. Trial number was included to account for any learning, i.e. an increase or decrease in aversion across trials. The interaction between trial and treatment was included to look for any differences in predator learning between the two treatments. The same was then done for trial duration.

We used generalised linear mixed-effect models (GLMMs) to test the effect that larval treatment had on beak cleaning, drinking, and the proportion of oat consumed by birds during the trials. As before, a negative binomial distribution was assumed for beak cleaning and drinking rate due to the occurrence of many zero scores in the data, and duration, scaled to minutes, was included as an offset. The proportion of oat consumed was modelled with a binomial distribution as described above. For each model, the comparison was initially made with larval treatment 'control' as the baseline. Explanatory variables were as above. All statistical analyses were done in R version 3.3.3 using the packages *coxme* (Therneau 2015), *MASS* (Venables and Ripley 1994) and *lme4* (Bates et al. 2015).

## Results

### *Food deprivation and life history traits*

Pupae weight was significantly affected by larval treatment ( $t = -3.021$ ,  $n=97$ ,  $P = 0.003$ ) as pupae from the food deprived larval treatment weighed less, on average, than those from the control (means  $\pm$  standard deviation were  $232.6 \pm 31.2$  and  $250 \pm 39.6$  mg respectively).

However this was the only life history trait to show an effect of the treatment.

Neither hindwing colour in adult females ( $t = 0.026$ ,  $n=97$ ,  $P=0.980$ ) nor time taken to eclosure ( $z = -0.820$ ,  $n=97$ ,  $P=0.410$ ) were significantly affected by larval diet treatment (the number of moths in each category are shown in Supplementary table 11.). However, when included as a random effect, family accounted for approximately 22% of the variance in hindwing colouration.

Similarly, the volume of defensive fluid produced was not affected by larval treatment ( $t$ -value= $-1.557$ ,  $n=97$ ,  $P=0.122$ ), pupae weight ( $t = 0.038$ ,  $n=97$ ,  $P=0.970$ ), or the interaction between them ( $t = 1.429$ ,  $n=97$ ,  $P=0.157$ ).

### *Predator response to female fluids*

Latency to attack decreased over the course of the experiment, with birds showing higher latencies in trial 1 than all subsequent trials (trial 4 compared to trial 1,  $z$ -value= $-2.31$ ,  $n=50$ ,  $P=0.021$ , Fig 2). No other explanatory variables in this model influenced hesitation time ( $p > 0.05$

for all, see supplementary Table 1). While the total proportion of the oat eaten did not show a significant effect of trial ( $P > 0.05$  for all comparisons, see supplementary Table 2), trial duration differed significantly between trials 2 and 4 ( $z$ -value=-3.57,  $n=50$ ,  $P < 0.001$ , see supplementary Table 3) and trials 3 and 4 ( $z$ -value=-2.57,  $n=50$ ,  $P=0.012$ ), but not between trials 1 and 4 ( $z$ -value=-0.12,  $n=50$ ,  $P=0.910$ ). Trials where the birds were presented with fluids were on average longer than those where the birds were exposed to only water, indicating that the birds ate water soaked oats faster.

Bouts of beak cleaning and drinking did not show any significant effects of trial ( $P > 0.05$  for all values, see supplementary Tables 4 and 5).

#### *Food deprivation and predator response*

Food deprivation during development had an effect on the defence fluid quality as the bird latency to attack the oat varied in response to larval treatment ( $z$ -value=2.460,  $n=50$ ,  $P=0.014$ ). Birds attacked the oats soaked with fluids from moths deprived of food more quickly than the those with fluids from moths fed *ad libitum* (Fig. 3). None of the other behavioural measures (i.e. beak cleaning, drinking, trial duration or the proportion of the oat eaten) were affected by larval treatment ( $P > 0.05$  for all values, see supplementary Tables 6-10 for full details). In addition the volume of the defensive fluid did not significantly influence any of the bird behaviours measured.

#### **Discussion**

Understanding the costs associated with the production of chemical defences in a species is crucial to understanding how it may be selected and maintained. Our results show that early life resource availability influences the efficacy of chemical defence in the wood tiger moth although this species synthesizes their chemical defence *de novo* (Burdfield-Steel et al. 2018). The defensive fluids of female wood tiger moths are deterrent to bird predators, but this deterrent effect was reduced when moths were moderately food-deprived during development. Thus, environmental effects could, indirectly, play a significant role in creating variation in chemical defence in this species. Given that these effects are not the direct result of changes in diet chemistry, as is the case when host plant chemistry influences the defence of sequestering species, there is even the potential for sibling competition over resources to lead to variation in the defence levels of individuals from the same clutch, feeding on the same host plants. While adults that were food deprived as larvae were smaller than those from the control (but still within the normal size range commonly observed in the laboratory), no other life history traits were

affected by the treatment. In addition, while we did not measure overall survival in this experiment, a subsequent study using the same population and food deprivation method found no differences in survival between the two treatments (Furlanetto 2017). Nevertheless, larval food deprivation did affect the response of wild-caught blue tits to the defensive fluids of female moths. While the fluids from both treatments deterred blue tits, the birds hesitated longer before attacking artificial prey soaked in the fluids from control moths (suggesting that the chemical defences of food restricted moths were a less potent predator deterrent).

A recent meta-analysis found that, in the three studies of physiological costs of chemical defence in non-sequestering species, there was evidence for low but significant costs associated with the defence (Zvereva and Kozlov 2016). Our findings in the wood tiger moth support this by showing that a reduction in resources available during development leads to a reduction in the efficacy of chemical defence. However, the costs of *de novo* defence production may carry other, less direct costs, if it trades-off with sequestration. A 2007 study in *Heliconius* found that sequestration ability and *de novo* production were negatively correlated, with species that were able to produce part of their defences *de novo* showing reduced sequestration from host plants (Engler-Chaouat and Gilbert 2007). Thus, species may be constrained in which defence acquisition mechanisms they can use. These constraints link more broadly to the challenges facing generalist species, as feeding on a wide variety of hosts not-only requires the ability to defend against, or otherwise mitigate, multiple plant defences (Ali and Agrawal 2012), but also makes it more challenging for species to maintain their own anti-predator defences. While food deprivation did influence latency to attack, a crucial predator behaviour thought to have a significant effect on prey survival in natural conditions (Gamberale-Stille 2000, Moore and Hassall 2016), it did not affect behaviours indicative of distastefulness, such as the amount of oat eaten. Interestingly, there were no significant differences in hesitation between trials 2 and 3, suggesting that hesitation was not a response to their previous experience with the fluids, but rather was induced before the birds even tasted the fluids. This strongly implies an odour cue. Indeed, the defensive fluids of the wood tiger moth have a strong, distinctive smell (pers. obs.), and have been shown to contain 2-sec-butyl-3-methoxypyrazine (Rojas et al. 2017) and 2-isobutyl-3-methoxypyrazine (Burdfield-Steel et al. 2018). Pyrazines are known for their distinctive odour (Guilford et al. 1987, Kaye et al. 1989, Moore et al. 1990, Rothschild et al. 1984), which has been shown to trigger colour aversions in birds (Rowe and Guilford 1996). It seems therefore that the defensive fluids themselves may be multimodal, stimulating both

olfaction and taste. Multimodal signalling has been suggested to increase predator learning and, subsequently, prey defence (Siddall and Marples 2008). While the two are expected to be closely intertwined, they nevertheless differ in their detectability at different stages of predation. Olfactory cues can be detected prior to an attack, and may therefore allow prey to avoid damage, and even death, if they can deter predators. In contrast, taste cue can only be detected after the initial attack. The wood tiger moth typically releases its thoracic defensive fluids in response to pressure, but it has occasionally been observed to do so in the absence of physical cues when stressed or startled (XXX pers. obs.), although it remains unknown if this is commonplace in the wild. Thus it is possible that the odour cues may be used as a “second line of defence” (Roper and Marples 1997), or simply to increase the efficacy of the colourful warning signal (Jetz et al. 2001, Rowe and Guilford 1996). If this is indeed the case then our results suggest that food deprivation has the greatest effect on defence before the attack takes place. This would suggest that the odour and taste of the fluids are the result of two or more different compounds, showing different patterns of investment. However, preliminary studies with wild-caught blue tits suggest that 2-sec-butyl-3-methoxypyrazine may also elicit distaste behaviours in birds (Rojas et al. 2017). Thus further study is needed to disentangle the modes by which the wood tiger moth’s defensive fluids work, and the chemical compounds underlying them.

Interestingly, despite a previous study showing that female colour is affected by changes in diet quality (Lindstedt et al. 2010), we detected no differences in colour between food deprived and control females using the by eye colour classification. Thus, our results do not support the idea of honest signalling, where the quality of the chemical defence reflects the primary signalling (Holen and Svennungsen 2012, Summers et al. 2015) because our study suggests a decoupled effect of larval food reduction on the secondary defense but not the primary warning signal. This does not preclude the possibility that more subtle colour changes were occurring. Lindstedt et al. (2010) found that, when fed on a diet high in iridoid glycosides (IGs), the adult colour of female wood tiger moths was lighter, suggesting that the energetic costs of digesting and excreting these toxins may cause a weaker warning signal (Lindstedt et al. 2010). Such changes in diet quality may also be a common scenario for polyphagous species. In keeping with previous studies (Lindstedt et al. 2016), however, we did find that family accounted for a significant proportion of the variance in female colour. While both food deprivation and detoxification are both expected to be costly, the form of these costs may be very different and

hence have different effects on adult phenotype. Thus the difference in findings between this study and that of Lindstedt et al. (2010) is perhaps not surprising. While we did not have sufficient power in this study to examine the relationship between hindwing colouration and chemical defence, our data did offer a hint that such a relationship may exist (see supplementary figure 2). Given the heritable component of female colouration, it may be possible to select families based on the phenotype of the mother in order to obtain a more balanced sample across the colour range. Thus, further study is needed to examine the effect of food deprivation on colour in more detail, and to determine whether colour and chemical strength are correlated. Such details will shed more light on optimal allocation between unpalatability and pigmentation for predator defence.

Our results from females align well with previous work on the male defensive fluids in this species, showing that the fluids are clearly distasteful to birds (Nokelainen et al. 2012, Rojas et al. 2017, Rönkä et al. 2018). Wood tiger moths can survive attacks by birds such as blue tits (K. Rönkä & J. Mappes, in prep.), so it is likely that taste rejection (Skelhorn and Rowe 2006, Skelhorn and Rowe 2006, Skelhorn and Rowe 2009) plays a role in the survival of this species. Female tiger moths live more sedentary lives than males, as they signal to males with pheromones while males can fly long distances in search of mates. This difference in lifestyle may contribute to the sexual dimorphism in this species. It has previously been observed that females produce smaller volumes of defensive fluid than males (XXX. obsv.), and that this may indicate that they rely more on their red colouration for defense against predators. However we found no effect of fluid volume on the response of wild caught birds in this experiment, suggesting that volume may be a poor measure of chemical defence.

Our findings address the gap in our current knowledge of the costs of *de novo* production of chemical defences by illustrating that the defences of the wood tiger moth are affected by resource limitations in early life. While the exact mechanism by which the moths are producing their defences remains unclear, future studies can build on this by manipulating individuals components of the diet, for example nitrogen availability, and examine the chemical changes underlying the changes in predator response observed in this study.

#### **Declarations**

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**Author contributions** — JM, BR and EBS designed the study. MB raised the larvae and did the bird experiments. MB and EBS collected the defensive fluids. EBS and MB did the statistical analyses. EBS and MB wrote the paper, and all authors contributed to its final version.

**Data Accessibility** - Upon acceptance, data will be available from the JYU repository

**Conflicts of Interest** – None

**Permits** - Wild birds were used with permission from the Central Finland Centre for Economic Development, Transport and Environment and licence from the National Animal Experiment Board (ESAVI/9114/ 04.10.07/2014) and the Central Finland Regional Environment Centre (VARELY/294/2015).

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**Figures Legends**

**Figure 1.** Continuous colour variation in female *Arctia plantaginis* hind wing (Lindstedt et al. 2011). (Photo: Samuel Waldron; taken with permission from Nokelainen (2013)).

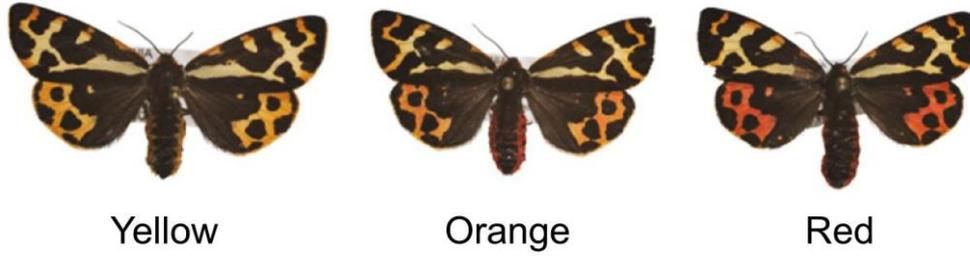


Figure 2. Measures of bird aversion in response to food items soaked in water (trials 1 and 4) or female defensive chemicals (trials 2 and 3). Top Right- Predator attack latency in. Top Left - Mean trial duration (maximum 300 seconds) for each of the four trials. Bottom Left - The number of beak cleaning events. Bottom Right - The number of drinking. The boxplots show minimum, maximum and upper and lower quartiles around the median.

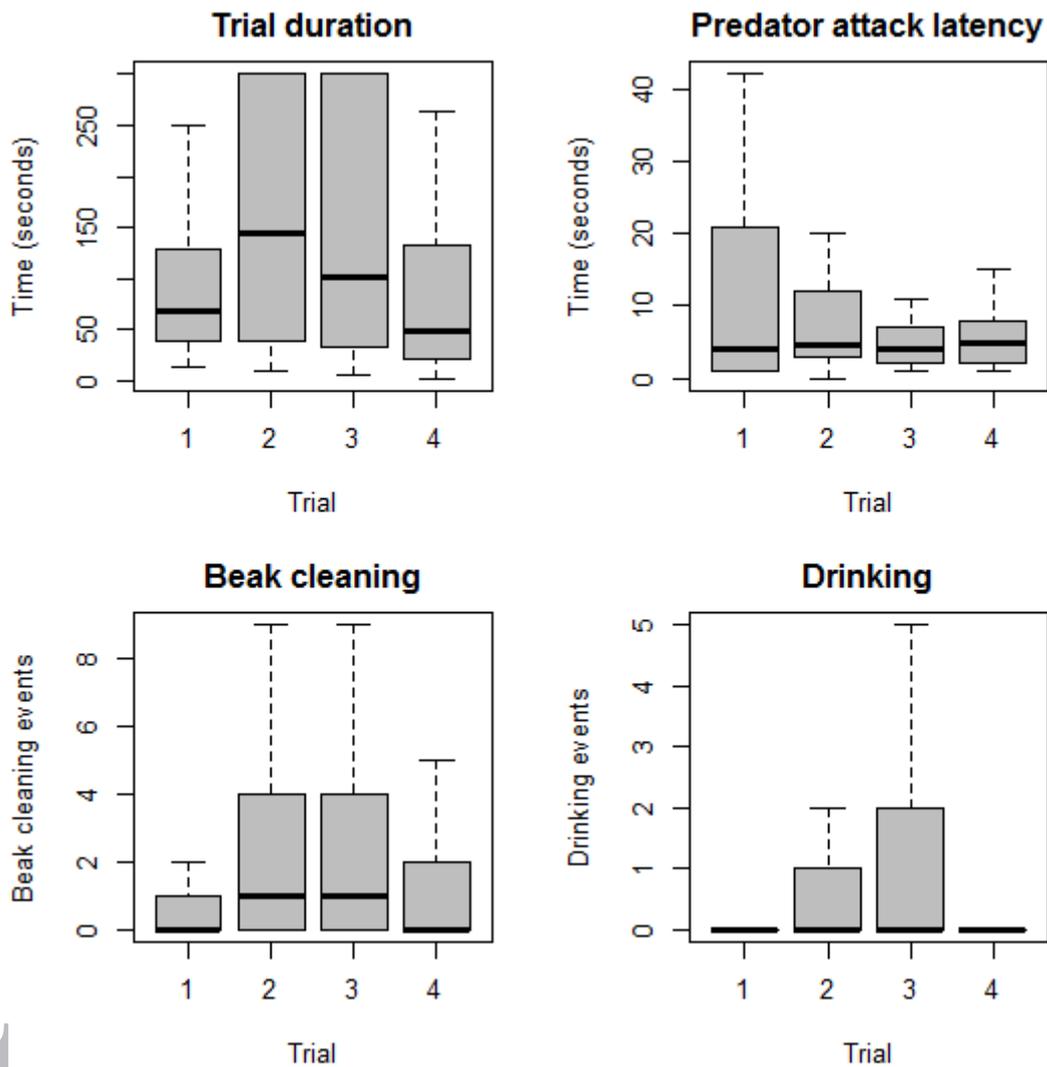


Figure 3. Predator latency (seconds) to attack a food item soaked in a moth's defensive fluids was significantly affected by the diet treatment experienced by the moth during development. The boxplot shows minimum, maximum and upper and lower quartiles around the median.

