Early development and the honesty of aposematic signals in a poison frog

Submitted by

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to the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

12th November 2012

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Juvenile of *Dendrobates auratus* at the study site in Santa Fe de Veraguas, Panama. Photo: Eric Flores, 2009.

Abstract.

The causes and consequences of variation in aposematic signals during immature stages are not clearly understood. This thesis explores the effects of early environment on the expression of aposematic signals in the green and black poison frog (*Dendrobates auratus*), and the consequences of variation in such components in the wild. It also explores how aposematic expression relates to levels of chemical defences in immature froglets. Embryos and larvae of poison frogs in the genus *Dendrobates* are known to be darkly pigmented. This thesis reports for the first time polymorphism in egg pigmentation in *D. auratus* and ontogenetic colour change through development reverting to a normally pigmented phenotype; however whether this pigmentation results from constraints or has adaptive consequences remains unclear. Evidence on how immature individuals allocate resources to growth and warning signalling is scarce. Experimental results in this thesis show that food supply during early environment affected body size and signal luminance in post-metamorphic froglets. Therefore the relative importance of these traits in relation to predation risk was further tested, using artificial prey in a field experiment. The results indicated that rates of attack by birds correlated negatively with body size, and on the contrary survival of artificial prey was independent of signal luminance. I therefore tested the hypothesis that in the wild larger, relatively well-nourished juvenile frogs are chemically better defended. I found that in fact larger juveniles are at a selective advantage conferred by their greater foraging efficiency and their superior levels of chemical defences. Overall, these results shows plasticity in aposematic traits in relation to early environmental nutrition in *D. auratus*; and suggests that acquiring large body size and similar integument colour as to adults are key determinants for survival during the early stages of their terrestrial life.

Acknowledgements

First thank to my family for their virtual company and support trough these years, in special to my mother, I love you. Many thanks to my supervisors Jonathan Blount and Allen Moore for allocating time and constant advice regarding experimental design, field work and manuscripts preparation and revisions, and also to John Hunt for his crystal clear advice as mentor. I am in debt with Martin Stevens for his big support with the modelling of vision systems, a core part of this thesis. I am also grateful to John Endler and Hannah Rowland for their suggestions and discussions on experimental designs and manuscripts. Thank you to Christopher Mitchell who did a brilliant and professional work with biochemistry analyses. Thanks to James Roper for encouraging me to do a PhD and for his solid and opportune advice, obrigado! Thanks to Luitgard Shwendenmann for her recommendations and support. In Panama Nelva Alvarado and Benjamín Name deposited their confidence on me to start this journey for what I am indebted. I would like to give special thanks to people and friends that contribute in different ways to achieve this task: Rachel Page, Janalee Caldwell, Tom Spande, Rodolfo Contreras, Luis Ureña, Alfonso Ramallo, Carlos Bonilla, Jean Baptiste Saulnier, Iker Vaquero-Alba, Alfredo Attisano, Alberto Pinto, Joseph Macedonia, Iva Fukova and Pilar Velásquez.

Many thanks to the following people that made the impossible tasks possible for me in the field: Edgar Toribio, Eliécer Pineda, Caroline Filmore, Paul Budgent, Carolyn Loeb, Georgia Croxford, Leesther Vásquez, Joelbin de la Cruz, Melva Olmos, Katherine Rodríguez, Víctor Bravo, Hortencio Palma, Jonathan

González, Luis Abrego, Carlos Abrego, and of course the Toribio´s Family and the people in Alto del Pito in Santa Fe.

I have to recognise the support from the SENACYT-IFARHU program in Panama who awarded me a PhD scholarship and in special to SENACYT for the research grant that funded my second field season. The School of Biology at the University of Panama in Veraguas, ANAM-Veraguas, ANAM-UNARGEN, and ICGES helped me to solve key technical problems while in Panama, many thanks.

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Chapter 1. General Introduction

1.1. Aposematic signals in relation to early developmental conditions

The complex and dynamic interactions between predators and prey have favoured the evolution of several defence mechanisms in animals, some of which are advertised via different signal modalities in order to avoid harmful encounters with predators (Dawkins 1993). Many chemically defended prey exhibit distinctive integument colouration that may draw the attention of predators and enable them to avoid confusing distasteful prey from edible prey (Wallace 1867). The possession of conspicuous colouration jointly with secondary defences such as toxins or physical weapons is known as aposematism (Poulton 1890). Aposematic colours are distinctive, in contrast to those whose main function is concealment or crypsis (Cott 1940). The existence of aposematic signals is well documented among invertebrates, fishes, amphibians, snakes, and birds, and has been a topic of extensive debate and research in relation to its evolution (Ruxton, Sherratt, & Speed 2004). Nevertheless much less is known about how environmental conditions may affect the expression of aposematic signals, especially during early development, and the consequences for fitness.

The conditions in which an individual develops early in its life are of critical importance in shaping the final form of its mature phenotype (Lindström 1999a; Metcalfe & Monaghan 2001). In particular, phenotype expression and fitness at adulthood can be affected by variability of the developmental environment (e.g.

temperature, nutrition, photoperiod, predation pressure) (Monaghan 2008) and the capacity of the genotype to cope with such variance (Nijhout, 2003). It is well known that the environment experienced in early life can have important effects on physiological, morphological and behavioural traits which manifest at adulthood. However, the extent to which aposematic traits are sensitive to developmental conditions remains relatively poorly understood. Aposematic signals are expected to be uniform in expression, to facilitate easy recognition and to reduce sampling error by predators. Consequently, any variation in signal expression would be expected to be selected against (Endler & Greenwood 1988; Mappes & Alatalo 1997a; Beatty, Beirinckx, & Sherratt 2004). Conversely, non-directional selection on signal expression would be expected to result if predators for example are inexperienced / unfamiliar with the signal (Rowe, Lindström, & Lyytinen 2004; Ihalainen *et al.* 2008). In fact, intraspecific variation in aposematic signal expression is widespread in nature (e.g. Summers, Cronin, & Kennedy 2003; Bezzerides *et al.* 2007; Tullberg *et al.* 2008; Mochida 2011; Wang 2011). Such variation may be expected to influence predation risk, perhaps especially during early life stages when secondary defences are incompletely developed, and animals must spent time exposed while they prioritise foraging in order to meet the demands of growth and development.

This thesis explores aspects of early life conditions affecting components of aposematism such as body size, signal expression and conspicuousness in the green and black poison frog (*Dendrobates auratus*), and the consequences of variation in such traits for predation risk in the wild. It also explores how

aposematic colouration relates to levels of chemical defences in immature froglets in the wild.

1.2. Phenotypic plasticity and aposematic signals

Phenotypic plasticity is a mechanism that enables individuals to adapt to environmental changes, and also operates during early development (West-Eberhard 2005). Indeed, considerable phenotypic plasticity of aposematic coloration has been found in response to prey population density (Sword 1999, 2000). Also panic moth caterpillars (Grant 2007) and poison frogs (Hoffman & Blouin 2000) show temporal variation in aposematic colouration as result of ontogenetic colour change. Similarly, in the striated shieldbug (*Graphosoma lineatum*) there are seasonal changes in aposematic colouration (Tullberg *et al.* 2008). Furthermore, the expression of aposematic colouration can be influenced by variability in ambient temperature during development (Lindstedt, Lindström, & Mappes 2009), and in response to the quantity or quality of early nutrition (Blount et al., 2012; Grill, 1999; Grill & Moore, 1998; Lindstedt et al., 2010; Ojala, Lindström, & Mappes, 2007). Chemical defences are also subject to phenotypic plasticity. For example, variation in the diet experienced during early development can influence the accumulation of diet-derived toxins at adulthood (e.g. Daly *et al.* 1994; Sime, Feeny, & Haribal 2000). The fact that not all individuals of aposematic species are maximally conspicuous or toxic suggests that there are costs associated with aposematism. One such cost is conspicuousness to predators

(Sherratt 2002). Nevertheless there could also be physiological costs related to aposematic signals and chemical defences.

1.3. Physiological constraints and expression of aposematic signals

One possible explanation for why aposematic traits vary within species is that the expression of such traits is subject to physiological resource allocation trade-offs. Investment in aposematic traits as an anti-predator strategy can trade-off with life-history traits, reducing the effectiveness of the signal (Endler & Mappes 2004). Allocation of resources to enable the storage and secretion of chemical defences, and detoxification mechanisms can divert resources necessary for metabolic maintenance and growth (Ahmad, 1992; Dobler, 2001; Grill & Moore, 1998; Nishida, 2002). Also, allocation of resources to aposematic signals can compete with other uses like thermoregulation (Lindstedt *et al.* 2009) or immunity against parasites (del Campo, Smedley, & Eisner 2005; Lindsey & Altizer 2009). Integument colouration in aposematic animals is largely the result of deposition of diet-derived pigments (Fox 1976; Nijhout 1991; Sandre *et al.* 2007a), which may function in colouration and also as antioxidants (McGraw 2005). Demanding metabolic activities like rapid growth during early development can increase the production of reactive oxygen species (ROS) (Alonso-Alvarez *et al.* 2007; Menon & Rozman 2007; Nussey *et al.* 2009). ROS can be neutralized in part by endogenous enzymatic and non-enzymatic antioxidants and/or antioxidants derived from the diet (reviewed in Selman *et al.* 2012). Despite the fact that availability of

antioxidants and levels of oxidative stress might be expected to impair early expression of aposematic signals, this mechanism has not been fully explored. Ojala *et al.* (2005) demonstrated potential trade-offs in the allocation of antioxidant pigments to aposematic signal expression versus other life history traits. However, the role of oxidative stress as a physiological mechanism mediating such trade-offs remains unclear.

1.4. Design of aposematic signals and predators response

The expression of warning signals may reflect production and maintenance costs (Dawkins 1993). In turn the receiver of such information (i.e. the predator) may itself incur opportunity costs in terms of the time needed to assess the reliability of signals, which could otherwise be spent on other activities (Dawkins & Guilford 1991). Consequently, it is in the interests of both the signaller (prey) and the receiver (predator) that a signal has efficacy, resulting in avoidance by the predator and no harm to the prey. The strength and durability of the association between a noxious quality such as existence of chemical defence and warning advertising can be affected by different components of the signal, for example conspicuousness (Roper & Redston, 1987; Roper, 1994), colouration (Ham *et al.* 2006), size (Gamberale & Tullberg 1998), and pattern (Aronsson & Gamberale-Stille 2012). An aposematic signal looks conspicuous when it contrasts against the visual background where it is seen, or when contrast results from comparison among the various markings of the prey itself (Endler, 1990; Stevens & Ruxton, 2012). In this

regard prey might vary the signal itself or the type of background in order to appear more or less conspicuous (Gamberale-Stille, 2001; Roper & Redston, 1987; Uy & Endler, 2004). Evidence from empirical studies shows that non-cryptic colours (e.g. yellow, red, orange) facilitate predator learning and enhance unlearnt avoidance in visual-oriented predators (Ruxton *et al.* 2004). On the other hand, achromatic contrast (i.e. brightness variation) seems to facilitate speed and duration of aversion learning of aposematic signals (Prudic, Skemp, & Papaj 2007). In addition, predator bias toward aposematic signals can be enhanced by large patterns (Forsman & Merilaita 1999), increases in the body sizes of prey (Hagman & Forsman 2003; Nilsson & Forsman 2003) or prey aggregations (Gamberale-Stille, 2000; Lindström, Alatalo, & Mappes, 1999; Riipi, Alatalo, Lindström, & Mappes, 2001). Although some aspects of the pattern elements of aposematic signals can be considered disruptive (Stevens 2007), the specific layout of such markings may cause aversion in predators (Wüster *et al.* 2004). In some instances it has been demonstrated that symmetric pattern elements enhance the anti-predator value of aposematic signals (Forsman & Merilaita 1999), whereas in other circumstances no difference has been found (Stevens, Castor-Perry, & Price, 2008).

Predators can show innate wariness toward aposematic signals as result of phobia to novel items (neophobia; Schlenoff 1984; Exnerová *et al.* 2007), or an aversion to include new items in the diet (dietary conservatism; Marples, Roper, & Harper 1998; Thomas *et al.* 2003, 2010). Nevertheless, naïve predators can learn to associate an aposematic signal with unprofitability faster if the signal is easy to recognize and is memorable (Ruxton *et al.* 2004). Birds, for example, have shown innate rejection toward conspicuous phenotypes (e.g. Marples, Van Veelen, &

Brakefield 1994; Darst, Cummings, & Cannatella 2006; Skelhorn & Rowe 2006). In some instances, however, initial sampling occurs before the bird learns to avoid the unpalatable prey (e.g. Schuler & Roper 1992, Chai 1996); here, the possession of adequate levels of secondary defences capable of causing deterrence is crucial to reduce predation risk. This is also important since certain potential predators of aposematic species, such as spiders, may not assess prey profitability using visual cues (Brodie & Tumbarello, 1978; Gray, Kaiser, & Green, 2010; Summers, 1999). Predators can vary in their sensory systems and therefore in how they perceive the aposematic signal, but also in their tolerance to chemical defences (Brodie & Ridenhour, 2002; Endler & Mappes, 2004). Indeed, differences in predator communities is likely to be one of the most important factors that drives variation in components of aposematic signalling within species, because it can relax selection towards uniformity and instead generate heterogeneous selection on signal form (Allen & Greenwood, 1988; Endler & Mappes, 2004; Losey, Harmon, Ballantyne, & Brown, 1997; Mappes, Marples, & Endler, 2005).

1.5. Honest signalling in aposematic species

Conventionally, aposematic signals have been considered to have relatively low information content, on the basis that predators show wariness simply because they associate the signal with a previous distasteful experience, without a necessary assessment of its quality (Grafen 1990; Guilford & Dawkins 1993). It is now clear that, in fact, predators do make assessments as to the level of defence before deciding whether to ingest prey (Skelhorn & Rowe 2006; Barnett *et al.* 2011;

Halpin, Skelhorn, & Rowe 2012). Nevertheless, whether aposematic signals can be considered to provide detailed and honest information about the defensive capacities of prey remains controversial (Stevens & Ruxton, 2012). Theoretical models have offered alternative ways to explain variation in aposematic signals and its association with level of defence. Aposematic signals seem to be 'qualitatively honest' in the sense that only well-defended prey can bear the conspicuousness cost incurred by signalling (Sherratt 2002). However, there has also been speculation that aposematic signals may be 'quantitatively honest', in the sense that they provide detailed information about the strength of prey defence (Guilford & Dawkins 1993; Blount *et al.* 2009; Lee, Speed, & Stephens 2011). A positive correlation between aposematic signal expression and levels of chemical defence has been reported both within (Bezzerides et al., 2007; Blount et al., 2012; Maan & Cummings, 2012) and across aposematic species (Summers & Clough, 2001; Cortesi & Cheney, 2010). Yet most theoretical models of aposematism predict the opposite pattern, i.e. well-defended prey should reduce investment in aposematic signals because they have a good chance of surviving attacks and can therefore avoid the conspicuousness costs of signals (Leimar, Enquist, & Sillen-Tullberg 1986; Speed & Ruxton 2005a, 2007). Empirical evidence in support of this prediction shows an inverse correlation between aposematic signalling and the level of secondary defence (across dendrobatid species: Darst et al., 2006; within the seven spot ladybird, *Coccinella septempunctata*: Blount et al., 2012; within the granular poison frog, *Oophaga* [*Dendrobates*] *granulifera*: Wang, 2011). To address this lack of consensus, recently new theoretical models have been developed which generate predictions of both signal 'honesty' and negative signal-

defence correlations (sometimes referred to as ‘dishonesty’) (Blount *et al.* 2009; Lee *et al.* 2011). These ‘resource competition’ models are based on the assumption that production and maintenance of aposematic signals and secondary defences both use-up a shared resource. Where the resource is in limited supply neither signalling nor defence can alone provide sufficient protection; here, the optimal strategy for prey is to invest equally in signals and defences resulting in a positive correlation between these traits (i.e. ‘honest’ signalling). However, when resources are abundant the model predicts a major investment in defences and a reduction in signalling, consistent with earlier theoretical models (Leimar *et al.* 1986; Speed & Ruxton 2005a, 2007). The shared resource could be energy, but an alternative possibility is antioxidant pigments, which must be partitioned between signals and protection against autotoxicity due to storage of toxins (Blount *et al.* 2009). In a recent paper (Blount *et al.* 2012), found that in a food-limited environment the seven-spot ladybird showed a positive correlation between body levels of precoccinelline, a defensive alkaloid, and elytra carotenoid content, as predicted by the resource competition model. They also found that correlations between components of the aposematic signal and chemical defences were not only affected by direct variation in resource (i.e. food) supply, but also by sex differences in this sexually size-dimorphic species. Females are larger and thus more likely to become resource-constrained than males, suggesting that females are more likely to signal honestly (Blount *et al.* 2012). While this study provides some support for the resource competition models (Blount *et al.* 2009; Lee *et al.* 2011), the role of oxidative stress as a mechanism underlying variation in aposematic signal expression remains unclear. Moreover, there is clearly a need

for more studies to assess the potential generality of these findings in other species and at different life stages. In particular, the resource competition models of aposematic signal honesty do not take into account the fact that during early development many prey species lack secondary defences and/or go through a process of ontogenetic colour change as discussed above (see **§ 1.2**). 'Honest' signalling cannot, by definition, apply in the absence of secondary defences.

1.6. Study species and field site

One interesting group of aposematic animals are the poison frogs of the family Dendrobatidae, which possess a striking variety of intra- and interspecific integument colouration and patterning coupled with toxic substances sequestered from the diet (Lötters *et al.* 2007). As in many other amphibians, dendrobatids undergo a complex early life, derived from an evolutionary history involving aquatic ancestors and more recent terrestrial-adapted phenotypes (Summers & McKeon 2004). The evolution of the aposematic syndrome in poison frogs has been the subject of extensive research (e.g. Darst, Menéndez-Guerrero, Coloma, & Cannatella, 2005; Hagman & Forsman, 2003; Santos, Coloma, & Cannatella, 2003; Summers & Clough, 2001). However, the relationships between early developmental conditions and the diversity of aposematic signal designs between and within species requires further study.

The green and black poison frog *Dendrobates auratus* is a diurnal terrestrial frog found on both slopes in Central America, from Nicaragua to northwestern

South America (Savage 2002). Typically males tend clutches of 2 - 8 eggs (Summers, 1989; Wells, 1978) before carrying larvae to phytotelmata (holes in small trees, shells of fallen fruits or the leaf axis of bromeliads), where they develop without parental care over a period of 39-89 days (Summers, 1990). The tadpoles are cryptic and palatable during development, but after metamorphosis they are conspicuously coloured (Pope 1941), yet lack toxic defences because they need to be sequestered from dietary arthropods on the ground (Daly *et al.* 1994b). However, the proximate mechanisms during early development that may give rise to variation in aposematic traits and its consequences for predation risk are poorly understood.

The research presented in this thesis was conducted at a shade coffee plantation in the area of Cañablancal (8° 31' N, 81° 03' W), Santa Fe district, Veraguas province, Republic of Panama, where a population of the green and black poison frog can be found (**Figure 1.1**).

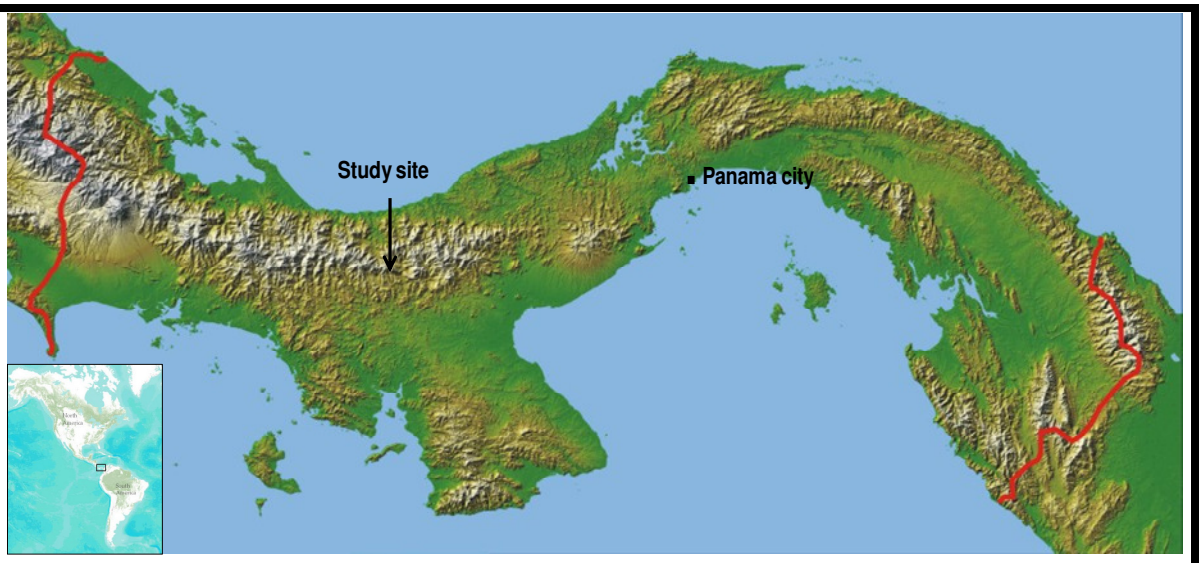

Figure 1.1 Location of Cañablancal study site in the Republic of Panama.

1.7. Aims and structure of the thesis

The present thesis explores the effects of the early developmental environment on phenotypic plasticity in growth, body size and aposematic traits in *D. auratus*. It also examines the consequences of variation in body size and aposematic signal expression for survival in terms of predation risk in the wild. Finally, it investigates how the expression of aposematic signals and levels of chemical defences relate to foraging efficiency, and how signals and toxins correlate in free-living froglets.

Chapter 2 starts with the description of an observation of an ontogenetic change in egg and larvae pigmentation in wild-caught *D. auratus* raised in captivity. This novel observation adds to our understanding of the natural history of the species and the potential adaptive basis of colour polymorphism is discussed.

In **Chapter 3**, plasticity in aposematic traits induced by early developmental conditions (i.e. variation in food supply) is addressed. Using a controlled experiment the amount of food was supplied at relatively low or higher levels, and the resultant investment in growth versus aposematic signals was measured in post-metamorphic froglets. In addition, levels of antioxidants and oxidative damage were measured to examine the possible role of oxidative stress as a mediator of developmental trade-offs. Specifically, I test the prediction that high-food froglets would grow relatively large, and would consequently reduce investment in warning signalling compared to low-food froglets. This is because it is anticipated that both large body size and high investment in aposematic signals may both attract the attention of predators during the vulnerable period post-metamorphosis when chemical defences are absent or incompletely developed.

The potential adaptive significance of variation in body size and aposematic signal expression is evaluated in **Chapter 4** in a field study of predation on model post-metamorphic froglets (i.e. artificial prey). This chapter tests the hypothesis that larger prey and those with more conspicuous signal expression should have lower survival. In both **Chapters 3 & 4** we used a putative bird predator to model the visual perception of aposematic signals, as birds seem to be an important selective force in the evolution of discrete aposematic phenotypes (Exnerová et al., 2008; Stevens & Ruxton, 2012) and extensive work has been done to evaluate their perceptual sensitivity (Vorobyev & Osorio 1998; Osorio, Miklósi, & Gonda 1999; Hart *et al.* 2000; Cuthill 2006). Furthermore, there is a large body of evidence relating to the psychology of birds toward aposematic signals (Marples *et al.* 1998; Lindström *et al.* 1999a; Skelhorn & Rowe 2006).

One important benefit of aposematism is that it allows animals the ultimate freedom to go about their lives while exposed; unlike cryptic species, the aposematic animal is able to pursue its behaviour actively in daylight, enjoying some immunity from attack (Speed, Brockhurst, & Ruxton, 2010). **Chapter 5** focuses on behavioural observations of immature *D. auratus* in the wild, coupled with measurements of body size, aposematic signal expression and biochemical analyses of chemical defences. Based on results of previous work (**Chapters 3 & 4**), here I test (1) whether larger juveniles have lower signal conspicuousness than smaller individuals; (2) whether larger juveniles have higher levels of chemical defenses and a greater feeding rate compared to smaller juveniles; and (3) whether larger, more toxic juveniles spend more time exposed on the forest floor. I also examined whether there was any evidence of 'honest' (or 'dishonest') aposematic signalling in this sample of wild juveniles.

Finally, **Chapter 6** summarizes the main results of the thesis and discusses the implications of early developmental conditions for the honesty of the aposematic syndrome and fitness in terms of predation risk in *D. auratus*.

Chapter 2. Unusual whitish eggs in the poison frog Dendrobates auratus Girard, 1855[1]

2.1. Abstract

Poison frogs in the genus *Dendrobates* (sensu Grant et al. 2006) are known to lay black pigmented eggs. During a field study in May 2010 in central Panama, a captive pair of wild-caught adult *Dendrobates auratus* laid a clutch of whitish eggs. The eggs developed and metamorphic froglets were similar in size and colour to that of age-matched normal-coloured tadpoles produced by different parents and reared in exactly the same conditions. The observation of a pale pigmented tadpole in the wild suggests that polymorphism in the degree of melanism is not simply an artifact of laboratory rearing. Our study is the first to report the production of viable whitish eggs by any species in the genus *Dendrobates*. Whether this coloration arises due to constraint or is a polymorphism that has adaptive significance awaits further study.

[1] This chapter has been published as: Flores, E. E., Moore, A. J. and Blount, J. (2012). Unusual whitish eggs in the poison frog Dendrobates auratus Girard, 1855. *Tropical Zoology* 25(2): 67-73.

2.2. Introduction

Species of the genus *Dendrobates* Wagler, 1830 (sensu Grant et al. 2006) share some characters during early development, such as egg size, tadpole size and parental care, and are known to lay darkly pigmented eggs (Grant et al. 2006; Lötters et al. 2007). Mature oocyte pigmentation is considered one character state used to construct phylogenies in the Dendrobatidae (Grant et al. 2006), although some controversy exists concerning the adaptive significance of egg pigments in anurans. For example the hypothesis that dark pigmentation protects against ultraviolet (UV) damage (e.g. Duellman and Trueb 1986), has been questioned because the extent of damage to anuran embryos by UV radiation has been considered minimal under natural conditions (Licht 2003). However, the synergistic interaction of UV radiation with other factors (e.g. chemicals, parasites) may have negative consequences at early stages of development (Blaustein *et al.* 2003; Blaustein & Johnson 2003). The UV damage hypothesis may not equally apply to all dendrobatids since many species lay eggs on the ground or may be hidden by leaf litter, and other adaptive explanations may be important. The appearance of a novel phenotypic trait at a low frequency can be considered an anomaly or an artefact, unless it provides advantages under unusual environmental conditions (West-Eberhard 2003). During a study of early developmental influences on aposematic traits in the green and black poison frog, *Dendrobates auratus* Girard, 1855, we found a clutch of whitish eggs, produced by a breeding pair kept in captivity, which developed into normal froglets showing characteristic dark pigmentation.

2.3. Methods

During a field season in the Republic of Panama, on 6 May 2010, a pair of adult *D. auratus* were captured at a shade coffee plantation in the Santa Fe district, Veraguas province, near the small town of Alto del Pito (8°31' N 81°03'W) (**Figure 1.1**). These frogs were returned to the laboratory and reared under standardized conditions. The female (snout-vent-length – SVL = 36.968 mm, body weight = 3.987 g), and the male (SVL = 34.571 mm, body weight = 2.574 g) were transferred to a glass terrarium (26 x 50 x 35 cm, L x W x H) with a layer of *Sphagnum sp.* Linnaeus, 1753 moss as substrate and two bromeliads (*Catopsis wangerini* (Mez & Wercklé 1904)) providing natural phytotelmata. These frogs were provided with water from a natural spring and filtered using reverse osmosis (RO) ad libitum in Petri dishes (10 cm diameter). Each terrarium was provided with two Petri dishes, one allocated in the middle of the terrarium and other half filled under a black pot upturned as shelter for egg deposition. Freshly collected soldierless termites *Nasutitermes nigriceps* Haldeman, 1853 were provided *ad libitum* as food. On 9 May 2010, three days after collection from the field, the pair laid a clutch of seven eggs, which were white in colour. Although the eggs lacked the characteristic formation of white and black poles, all reached stage 22 (sensu Gosner 1960) where the development of the neural tube and head were visible (**Figure 2.1**), and thereafter developed as normal embryos and hatched after thirteen days of embryogenesis.

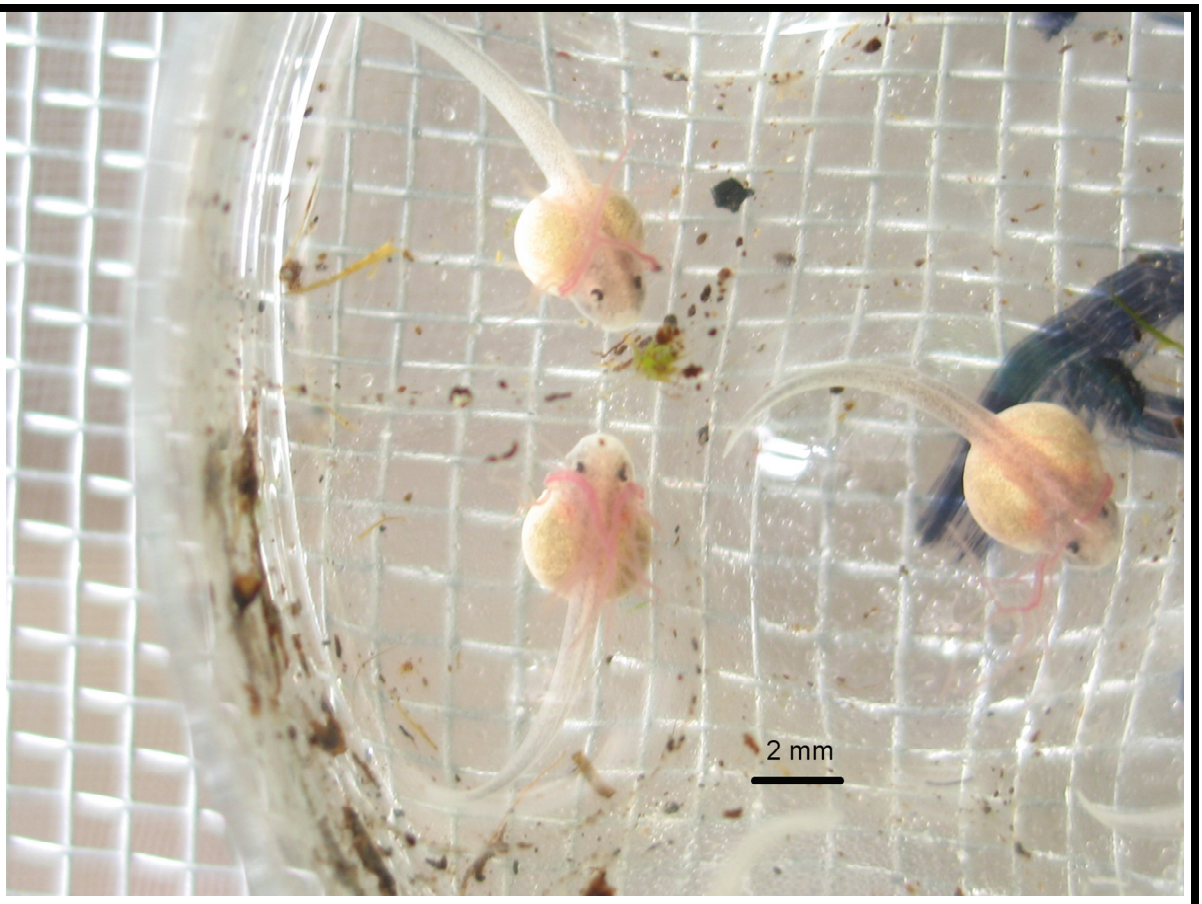

Figure 2.1 Whitish tadpoles at stage 22 (sensu Gosner 1960).

Once the tadpoles hatched (n = 7), they were individually weighed and photographed with a Cannon Power shot G6 (7.1 megapixel) digital camera (Cannon Inc., Japan) and transferred to a 700 ml plastic container containing 100 ml RO water and covered with a mosquito net. The tadpoles were fed on a diet of King British cichlid fish flakes (Fish and Fins Ltd., East Sussex, UK) *ad libitum*. After tadpoles completed metamorphosis (n = 5, 65 ± 2.3 days, mean ± s.e., range, 60 - 73), they were photographed, weighed and spectral reflectance of the dorsal

integument was measured in duplicate using a USB2000 spectrometer (Ocean Optics Inc. FL, USA) in order to calculate coloration metrics. These metrics were compared with those from a sample of n = 57 metamorphic froglets coming from pigmented eggs. These eggs came from parents that were captured in the wild at the same location, reared under the same standardized conditions and their tadpoles fed with the same diet of King British cichlid fish flakes provided *ad libitum*.

2.3.1. Data analyses

Statistical analyses were conducted using R v.2.12.1 (R Development Core Team 2010). Mean morphological characteristics for froglets from whitish eggs and those from pigmented eggs were compared for equal variances and their means analyzed using a Student´s *t*-test. Spectral reflectance data were not normally distributed, and therefore were compared using the non-parametric Spearman´s rank correlation test.

2.4. Results

At hatching the tadpoles (n = 7) had an average total length of 14.718 (14.19 – 15.35) ± 0.21 mm (mean (range) ± s.e.) and an average body weight of 28.33 (25 – 32) ± 1.23 mg (mean (range) ± s.e.), meanwhile pigmented tadpoles (n = 57) had

an average total length of 14.758 (11.84 – 16.65) ± 0.16 mm, and an average body weight of 29.16 (16 – 47) ± 0.80 mg. The tadpoles had brownish pigmentation which clearly differed from the black colour of tadpoles observed in the wild and unrelated tadpoles raised in captivity under identical conditions. At metamorphosis (n = 5) average SVL (16.082 (15.16 – 17.23) ± 0.40 mm) and average body weight (491 (433 – 596) ± 0.03 mg) were not significantly different to that of age-matched coloured tadpoles produced by different parents and reared in exactly the same conditions (n = 57), mean SVL (15.726 (13.79 – 18.68) ± 0.13 mm) and mean body weight (430 (218 – 646) ± 0.01 mg) (t-test, [Weight] $t_{5.026} = 1.817$, $P = 0.13$; [SVL] $t_{4.884} = 0.851$, $P = 0.43$) The metamorphic froglets also resembled normal conspecifics in external coloration and pattern (**Figure 2.2**). Comparison of spectral reflectance of the dorsal greenish-blue integument of the unusual and pigmented tadpoles showed that their coloration post-metamorphosis was statistically indistinguishable (Spearman's rank correlation test based on mean values of the two groups, n = 451, $r_s = 0.99$, $P < 0.001$) (**Figure 2.3**). In both cases the wavelength of maximum reflectance was 521 nm.

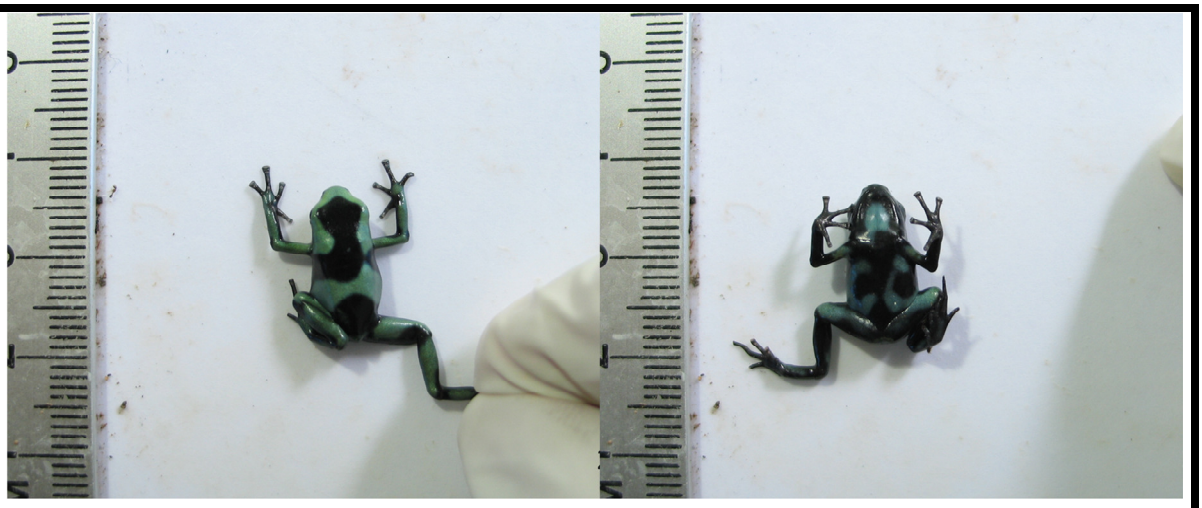

Figure 2.2 Metamorphosed froglet of *Dendrobates auratus* derived from whitish eggs in dorsal (left) and ventral views.

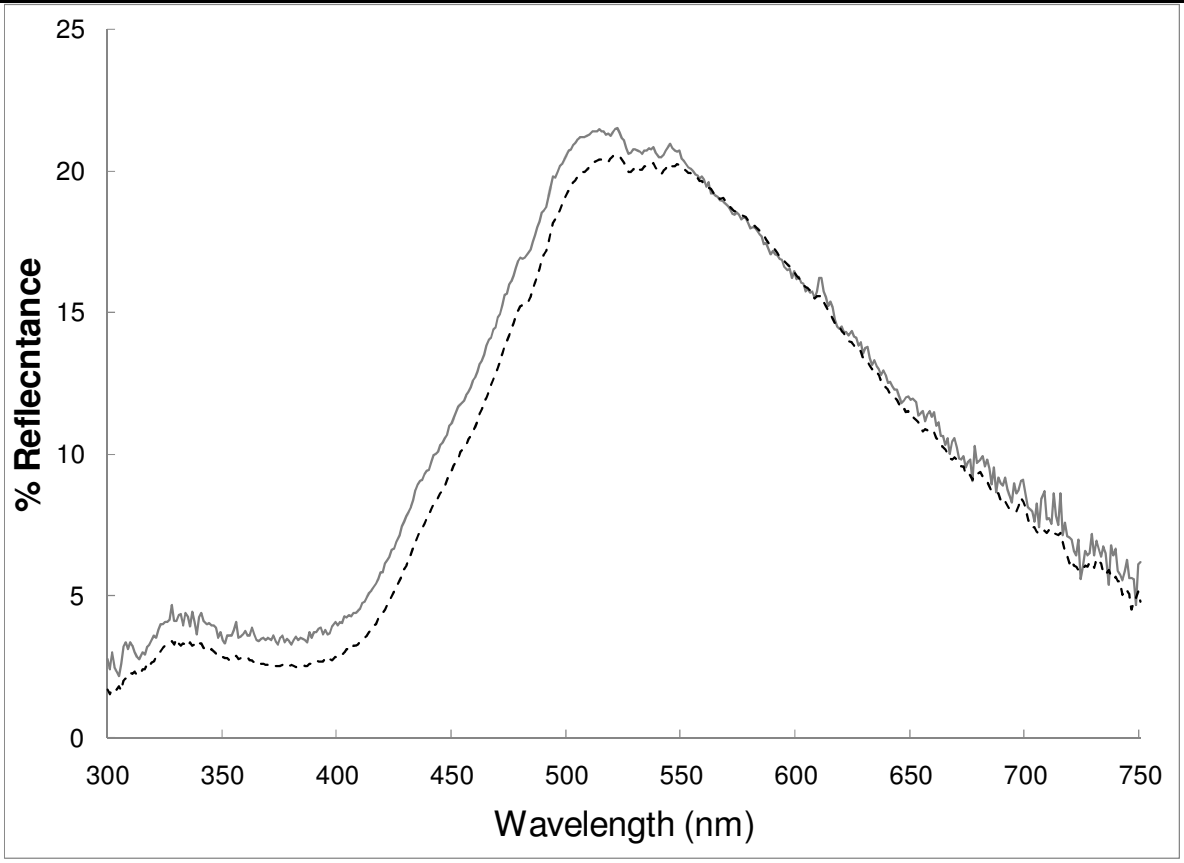

Figure 2.3 Mean spectral reflectance of dorsal (greenish-blue) coloration of tadpoles derived from whitish eggs (solid gray line) and normal-pigmented eggs (dashed black line) raised in captivity.

2.5. Discussion

Our study is the first to report the deposition of viable whitish eggs by any species in the genus *Dendrobates*. Interestingly the existence of creamy pigmented eggs and whitish tadpoles has been reported recently in other genera within the family Dendrobatidae (Brown *et al.* 2011). Whitish eggs/embryos are presumably a consequence of a lack of melanin formation. This could be due to an absence of

tyrosinase activity, which has been shown to be necessary for oocyte pigmentation in *Xenopus laevis* Daudin, 1802 (Kidson & Fabian 1989). On the other hand, the activity of the pituitary gland is known to promote melanophore expansion in anurans which has a direct effect on the dispersion of pigments (Hogben & Slome 1931). Albinism is a genetically-based disorder which is characterized by a lack of skin melanin; in anurans this becomes evident at tadpole stages and persists through development and adulthood (Browder 1972). Indeed, albinism has previously been reported in several anuran species including poison frogs (Federighi 1938; Gill, Richards, & Nace 1970; Browder 1972; Mitchell & McGranaghan 2005; Lötters *et al.* 2007; Sanabria, Quiroga, & Laspiur 2010). However, our observation of whitish eggs is striking because the resultant tadpoles were brownish, and the froglets had the same phenotype as froglets that developed from pigmented eggs and not the characteristic albino phenotype (i.e., creamy skin, orange skin spots, pink eye and golden iris) (**Figure 2.2**). Ontogenetic colour changes can be the product of variation in levels of steroid hormones (i.e. testosterone, estrogen) during early development (Hayes 1997; Hayes & Menendez 1999). Alternatively, there could have been differential expression of genes responsible for skin pigmentation at different life stages (Lee et al. 2009).

By whatever mechanism the ability to revert to a pigmented phenotype at adulthood indicates plasticity in ontogenetic colour change in *D. auratus*. This could be in response to adverse environmental effects on melanocyte differentiation at early developmental stages (Hoffman & Blouin 2000), in the absence of which the response is not elicited (see West-Eberhard 2005). However there could be other, adaptive explanations. For example, aposematic species

such as coccinellids and the freshwater apple snail (*Pomacea canaliculata* Lamarck, 1822) produce eggs that are warningly coloured and contain chemical defences (Dreon, Ituarte, & Heras 2010). However, whitish eggs in *D. auratus* seem unlikely to function as an aposematic signal. For aposematism to provide protection against predators, it needs to be widespread in the population, facilitating predator learning, memory and avoidance (Ruxton *et al.* 2004), which is not the case in our study population. Furthermore, in dendrobatids secondary defences come from dietary sources of alkaloid compounds, and thus there is a general consensus that during early developmental stages *D. auratus* lacks toxicity (Daly et al. 2000, Saporito et al. 2009). *Dendrobates auratus* deposits eggs on a variety of substrates and localities, including leaf litter, or in a small pool of water on a fallen log or the hollow of a tree (Summers 1989; Summers 1990). Melanic coloration in developing anurans is considered to confer crypsis, thermoregulation and/or to protect against UV solar radiation (Rose 1962; Smith-Gill, Richards, & Nace 1972; Duellman & Trueb 1986; Blaustein *et al.* 2003). However, melanin synthesis must incur production costs, requiring amino acid precursors (Griffith, Parker, & Olson 2006) and incurring generation of pro-oxidant species (Rozanowska *et al.* 1999). An intriguing possibility, therefore, is that the genes which code for melanin synthesis are expressed to a greater extent in environments where embryos and tadpoles are more exposed to natural light and to visually hunting predators. It has been demonstrated for example, that adult males of *D. auratus* tend to avoid places with relatively high levels of sunlight UV radiation when displaying during the day (Han *et al.* 2007). Other species in the family that exhibit polymorphisms in egg and tadpole pigmentation (i.e. *Ranitomeya*

vanzolinii Myers, 1982 and *R. flavovitatta* Schulte, 1999) are known to exploit a range of altitudinal gradients along their range (Brown *et al.* 2011), therefore facing different levels of sunlight UV radiation. It is conceivable that production of melanin for egg pigmentation is reduced when exposure to harmful radiation or predation risk are minimal.

2.5.1. Conclusions

One of us (EF) has observed a single unusual brownish colored tadpole of *D. auratus* in the field; therefore variation in the degree of melanism is not simply an artifact of laboratory rearing. There is much controversy about the phylogenetic relationships in the family Dendrobatidae, due to the highly polymorphic nature of their integument coloration amongst other factors (Santos *et al.* 2009). Our study adds to this controversy in that we have identified what seems to be polymorphism in respect of egg coloration in *D. auratus*. However, whether this variation in egg coloration has adaptive significance in the Dendrobatidae family requires further study.

Chapter 3. Diet, development and the optimisation of warning signals in post-metamorphic green and black poison frogs[2]

3.1. Abstract

Many prey species are chemically defended and have conspicuous appearance to deter predators (i.e. aposematism). Such warning signals work because predators pay attention to the colour and size of signals, which they associate with unprofitability. Paradoxically, in early life stages aposematic species are often warningly coloured, but their chemical defences are lacking because they have yet to be acquired through the diet or synthesised endogenously. This state of being conspicuous yet poorly defended must place individuals at increased risk of predation, but how they minimise this risk during development is unclear. We reared larval green and black poison frogs (Dendrobates auratus) on a relatively low or a higher food supply, and tested the hypothesis that individuals with more resources should grow larger while reducing their investment in warning signals at metamorphic completion. We also assayed markers of oxidative balance (malondialdehyde, superoxide dismutase, and total antioxidant capacity) to ascertain whether there were resource allocation trade-offs that differed with diet treatments. Low-food froglets were relatively small, and their body size and signal

[2] This chapter is in press as: Flores, E. E., Stevens, M., Moore, A. J. and Blount, J. D. (2012). Diet, development and the optimisation of warning signals in post-metamorphic green and black poison frogs. *Functional Ecology*.(in press).

luminance (perceived brightness) were positively correlated. In contrast, in high-food froglets body size and warning signal luminance were negatively correlated, suggesting either a resource allocation trade-off, or alternatively a facultative reduction in luminance exhibited by larger froglets. The reduction in luminance in relatively large, high-food froglets did not appear to arise because of oxidative stress: signal luminance and markers of oxidative stress were positively correlated in high-food froglets, but were negatively correlated in low-food froglets suggesting a trade-off. Our results highlight developmental plasticity in body size and colouration as affected by resource (i.e. food) supply. Such plasticity seems likely to minimize predation risk during the vulnerable period early in life when individuals are warningly coloured and must make the transition from an undefended phenotype to a mature aposematic state.

3.2. Introduction

Many prey species are defended, and have conspicuous colour, pattern, acoustic or olfactory signals which advertise unprofitability to predators (i.e. aposematism; (Poulton 1890; Eisner & Grant 1981; Ratcliffe & Fullard 2005). Until recently aposematic signals were assumed to not vary within species in order to facilitate predator learning and avoidance (reviewed in Ruxton, Sherratt, & Speed 2004). However, contrary to this expectation, it is becoming clear that intraspecific variation in aposematic colour and pattern is widespread (e.g. Summers, Cronin, & Kennedy 2003; Bezzerides et al. 2007; Tullberg et al. 2008; Mochida 2011; Wang

2011). While many mechanisms have been suggested for the initial evolution of aposematism (receiver psychology: Speed 2001; adaptive predator behaviour: Sherratt 2002; dietary conservatism: Thomas et al. 2003; physical defences: Speed & Ruxton 2005; defensive secretions: Gohli & Högstedt 2009), the developmental factors that give rise to phenotypic variation in aposematic signals remain little studied (Mappes *et al.* 2005; Stevens & Ruxton 2012). Predators vary in hunting strategies, perceptual sensitivities or experience, which may contribute to polymorphism in aposematic signals (reviewed by Endler & Mappes 2004) and differ according to the local predator assemblage (Wang & Shaffer 2008; Mochida 2011; Valkonen et al. 2012). In addition variation in aposematic signals may result from genetic drift (Reynolds & Fitzpatrick 2007), sexual selection (Rudh, Rogell, & Höglund 2007; Maan & Cummings 2008), or environmental factors such as developmental diet (Grill & Moore 1998; Blount et al. 2012), prey density (Sword *et al.* 2000), parasitism (Losey *et al.* 1997; Lindsey & Altizer 2009) or thermoregulation (Lindstedt *et al.* 2009).

The environment in which early development occurs is fundamental for shaping the form of the mature phenotype (Cheverud & Moore 1994; Rossiter 1996; Monaghan 2008), and may elicit the expression of plastic phenotypes adapted to one or a set of environments (Nijhout 2003; West-Eberhard 2003). Yet, in aposematic species, only a handful of studies have considered the effects of early development on adult phenotypes. Early nutrition in particular has been shown to affect the size or colour of aposematic signals at adulthood in ladybird beetles (Grill & Moore 1998; Grill 1999; Blount *et al.* 2012), and arctiid moths (Ojala *et al.* 2007; Lindstedt *et al.* 2010). Furthermore, variability in the quantity of defensive

compounds sequestered from the diet or metabolically transformed from dietary precursors during early development may directly affect the amounts of such chemicals in the body at adulthood (e.g. Daly et al. 1994; Sime, Feeny, & Haribal 2000). The development of aposematic signals may also be constrained if integument colour has additional functions, such as thermoregulation (Lindstedt *et al.* 2009). Variation in aposematic colouration has been also found in response to prey population density (e.g. Sword et al. 2000) and across seasons (e.g. Tullberg et al. 2008). In amphibians, integument colouration is largely the result of light-absorbing (e.g. xanthophores, melanophores) or light-reflecting (e.g. leucophores, iridophores) chromatic cells, and their arrangement and dispersion in the skin (Grether, Kolluru, & Nersissian 2004). The expression of aposematic colouration is known to change with age in some amphibians (Hoffman & Blouin 2000) and potentially can be affected by the amount of particular pigments inside the chromatic cells (e.g. carotenoids, melanins and pterines) (Fox 1976; Grether *et al.* 2004)

While it is clear that there is plasticity in aposematic traits, what is less clear is how variation in resource availability during early development may drive relative investment in warning signals versus growth. Early development may be a risky stage of life in aposematic species, because chemical or other forms of secondary defences must either be produced de novo or sequestered through the diet, and therefore may be absent or incompletely developed in juveniles (Sime *et al.* 2000; Nylin, Gamberale-Stille, & Tullberg 2001; Nishida 2002). This state of being conspicuous yet poorly defended must place individuals at increased risk of predation, but how they minimise this risk during development is unclear. Hence, if

predators can discern poorly defended froglets from well-defended adults the optimal strategy during development could be to invest maximally in growth, but to reduce investment in aposematic signals and thereby minimize detection.

Body size has been considered to evolve in concert with aposematic signals in poison frogs (Hagman & Forsman 2003), particularly in highly toxic species (Santos & Cannatella 2011). It seems unlikely that restricting resource allocation to growth would be favoured by selection, because larger froglets will be better able to meet the metabolic demands of foraging for toxic dietary items during the first few, critical days following metamorphosis (Taigen & Pough 1983). Chemical defences in post-metamorphic poison frogs are in the form of neurotoxic alkaloids acquired from dietary arthropods (Daly *et al.* 1994b; Saporito *et al.* 2009). However, during early development the mechanisms of alkaloid acquisition may vary amongst species and environmental contexts. For example, maternal provision of alkaloids to embryos via the egg has been reported in the aposematic bufonid *Atelopus chiriquiensis* (Pavelka, Kim, & Mosher 1977), and apparently to tadpoles via trophic eggs in *Oophaga* [*Dendrobates*] *pumilio* (Stynoski 2012). Nevertheless, in many poison frog species, the larval diet comprises alkaloid-free foods (Caldwell 1993; Caldwell & de Araújo 1998). This is the case with the green and black poison frog (*Dendrobates auratus*) whose tadpoles are palatable to odonate and mosquito larvae (Fincke 1994, 1999) and which lacks maternal provision of trophic eggs (Summers 1990); as such sequestration of alkaloids during larval stages seems unlikely, although this remains to be conclusively demonstrated. Acquisition and storage of toxins could be costly (reviewed in Ruxton et al. 2004) especially during immature life stages when the anatomical organisation of poison glands is

incomplete (Angel, Delfino, & Parra 2003; Saporito *et al.* 2010) and resources should be mainly devoted to meet growth demands. In addition other anatomical (e.g. mouth size), behavioural (e.g. foraging capacity), and physiological (e.g. metabolic rate) factors may constrain the acquisition of toxins in juveniles (Donnelly 1991; Saporito *et al.* 2010).

Relative investment in growth and aposematic signals is likely to be influenced by environmental conditions, such as the quality of the developmental diet. High levels of investment in growth or signals may be physiologically costly. In particular, rapid growth may incur production of reactive oxygen species (ROS) (Alonso-Alvarez *et al.* 2007; Menon & Rozman 2007). ROS are atoms or molecules that are important for intracellular signalling (e.g. Hurd & Murphy 2009), but also can cause serious damage to DNA, proteins and lipids (reviewed in Selman et al. 2012). Where there is an imbalance between ROS production and the capacity of the antioxidant defence system to inactivate ROS, a state of oxidative stress results (reviewed in Selman et al. 2012). The antioxidant system is complex and includes both endogenous (e.g. glutathione, catalase, superoxide dismutase) and exogenous, diet-derived components (e.g. vitamin E, carotenoids) (reviewed in Selman et al. 2012). However, all antioxidants are potentially limiting resources for wild animals, because antioxidants or the resources (amino acids, energy) required for their biosynthesis must be obtained in the diet, and/or because antioxidants are traded amongst competing physiological demands (reviewed in Selman et al. 2012). Indeed, all types of pigments responsible for integument colouration in animals, including poison frogs (e.g. carotenoids, melanins, pterins) (Fox 1976; Hoffman & Blouin 2000; Grether *et al.* 2004), may function as antioxidants in vivo

(McGraw 2005). It has recently become clear that oxidative stress can explain variation in the expression of sexual signals (Alonso-Alvarez *et al.* 2004; Mougeot *et al.* 2010). It therefore seems possible that developmental trade-offs in aposematic animals may be modulated by oxidative stress, as affected by diet. Previous studies have shown that investment in life history traits such as growth rate, development time and size can trade against development of aposematic signals (e.g. Grill & Moore 1998; Ojala, Lindström, & Mappes 2007; Lindstedt et al. 2010). However, whether biomarkers of oxidative stress correlate with warning signal production has not been studied before.

Here we assessed the effects of variation in food supply during early development on post-metamorphic body size and aposematic colouration in the green and black poison frog. Dendrobatids undergo a complex early development, from an aquatic-cryptic phenotype to a conspicuous terrestrial one, yet only after reaching this latter stage does dietary sequestration of toxins begin (Daly *et al.* 1994b; Saporito *et al.* 2009).These characteristics make dendrobatids a good model to investigate phenotypic plasticity and resource allocation to aposematic signals versus growth during early development. We reared *D. auratus* larvae on either a relatively low or a higher food supply until metamorphosis was complete, whereupon we measured morphology and the spectral reflectance of skin in order to assess colouration and conspicuousness to predators. We hypothesised that *D. auratus* froglets would show developmental plasticity, and resource allocation to growth and aposematic signals would be modulated by oxidative stress. We predicted that under high-food provision froglets would grow relatively large, and they would reduce investment in warning signalling compared to low-food froglets.

We also predicted that there may be a negative correlation between warning signal expression and levels of oxidative stress in low-food froglets that were resource constrained, whereas no such apparent trade-off would be evident in high-food froglets.

3.3. Methods

3.3.1. Capture and Breeding of Adults

Adult *D. auratus* were collected during April and May 2010, at a shade organic coffee plantation in Santa Fe, Veraguas province, central Panama (8°31' N 81°03'W) (**Figure 1.1**). Near the plantation, individuals were randomly paired in glass terrariums (26 x 50 x 35 cm), using a simple shelter open on all sides to permit natural daylight and temperatures. All terrariums were similarly furnished with *Sphagnum sp.* moss and bromeliads (*Catopsis wangerini* Mez & Wercklé 1904) collected from the study site. Water and food were provided ad libitum in Petri dishes (diameter: 10 cm), replenished twice daily (07:30 h and 17:00 h). Moisture levels inside each terrarium were checked at that time to ensure ~90% relative humidity controlled by misting with filtered reverse osmosis water. Food consisted of soldierless live termites (*Nasutitermes nigriceps* Haldeman 1853) collected in the field. We used termites because they are part of the natural diet of D. auratus (Taigen & Pough 1983; Caldwell 1996) , could be collected in sufficient

quantities at our study site, and their nutritional value is comparable to an ant/mite diet (Huey & Pianka 1981; Redford & Dorea 1984). Termites are not a source of alkaloids for dendrobatids (Daly *et al.* 1992), but it seems unlikely that a diet of exclusively termites would have affected skin levels of alkaloids of the breeding frogs since skin alkaloids are known to persist for years in captive *D. auratus* (Daly *et al.* 1992, 1994b). Temperature and humidity in the terrariums, based on daily readings throughout the study, were 24.60 ± 0.22°C (mean ± SE) and 91.04 ± 0.99%, respectively. Another Petri dish containing a small volume of reverse osmosis water, and covered by an upturned black plastic flowerpot, served as a site for egg deposition.

3.3.2. Larval rearing and diet manipulation

In total 19 breeding pairs of adults produced fertile clutches, with an average clutch size of 5 ± 0.57 eggs and a latency to lay of 12 ± 2.36 days (mean ± SE). A total of 120 eggs were laid, and clutches were transferred individually to a similar empty glass terrarium where they were monitored daily. Some 30 out of 120 eggs (25%) showed signs of mould infection and were carefully removed using a sterile plastic pipette and discarded; all remaining eggs hatched (90 eggs). Immediately after hatching, larvae were carefully transferred to a 700 ml plastic tub containing 100 ml reverse osmosis water covered with mosquito net, and by using a split-brood design they were randomly assigned to a food supply (treatment) group. Thus, at the start of the rearing period the sample sizes in each group were n = 34

individuals from n = 13 families in the low-food group, and n = 28 individuals from n = 9 families in the high-food group. Larvae were fed daily with King British cichlid fish flakes (Fish and Fins Ltd., East Sussex, UK). To standardize presentation only red flakes were used, since lab analysis showed that total concentrations of carotenoids differed between red and brown flakes (our unpublished data). The quantity of food provided was recalculated weekly using the average body mass of low-food larvae as a reference (low-food, 8% body mass (w/w); high-food, 15% body mass (w/w)). The same or similar levels of food have previously been employed to yield differences in growth rates of frog larvae without causing starvation (Alford & Harris 1988; LaFiandra & Babbitt 2004). Before providing fresh food, any uneaten food was removed. Once each week, 50% of the water in each plastic tub was replaced. At the onset of metamorphic climax (developmental stage 43-46; Gosner 1960) feeding ceases and nutritional needs are instead met by tail resorption and catabolism of other body tissues (Lötters *et al.* 2007). Therefore we ceased food provisioning at this point. The two food supply groups had a similar duration of the tail resorption period (General Linear Mixed Model (GLMM); with food as a fixed factor and family as a random factor; high-food: 6.32 ± 0.21 days; low-food: 6.01 ± 0.28 days; food, $F_{1,8} = 1.22$, $P = 0.30$).

3.3.3. Morphometric measurements

At weekly intervals larvae were carefully removed from their tub and blotted dry with filter paper before being placed in a Petri dish (diameter: 5 cm) containing a

known volume of reverse osmosis water, and weighed to the nearest 0.001g using an Ohaus Scout Pro balance (Ohaus Europe GmbH, Switzerland). The dorsum of each individual was digitally photographed under standardized conditions with a Canon Power Shot G6 (7.1 megapixel) camera (Canon Inc. Japan). A metal ruler was included to provide a scale for the image. Snout-vent length (SVL) was measured in each individual. SVL is considered an anatomical character that shows plasticity under different environmental conditions in anuran larvae (Vences *et al.* 2002; Vonesh & Warkentin 2006). Larvae were photographed daily from the first day they climbed out of the water to monitor tail length. When tail length stopped decreasing, metamorphic climax was determined to have been reached (see Caldwell & de Araújo 2004). All measurements (0.001 mm precision) were carried out using ImageJ 1.43q (Rasband 1997).

3.3.4. Analyses of aposematic signals and background spectra

To be discernible to a predator's eye, prey must contrast against the background where they are normally perceived in terms of colour and luminance (perceived brightness based on photoreceptor outputs) (Stevens & Ruxton 2012). We therefore measured the spectral reflectance of the skin of metamorphosed froglets on the day of metamorphic climax using a USB2000 spectrometer (Ocean Optics Inc. FL, USA) with a bifurcated 400 µm UV/VIS optic fibre probe connected to a pulse xenon lamp (PX-2); at an angle of 45° and corrected for lamp drift using a white diffuse spectral standard (WS-1) (Maan & Cummings 2008). Measurements

were taken from four body regions in duplicate (head, dorsum, left and right flanks) and then averaged for subsequent analyses (**Figure A3.1**). We did not measure the reflectance of the ventral skin which is unlikely to function as an aposematic signal in poison frogs (Savage 2002; Maan & Cummings 2008; Wang & Shaffer 2008). The spectral reflectance of 136 samples of background leaves collected from the forest floor was also measured in triplicate and averaged following the methodology described above (**Figure A3.2**). The light that reaches the eye is a product of the reflectance spectra of the object observed and the irradiance of ambient light (i.e. radiance spectra; Endler 1990), therefore measures of ambient light irradiance $I(\lambda)$ were obtained at several locations in the field where adult frogs were captured for use as breeders (see above); n = 90 measurements on a sunny day and n = 85 measurements on a cloudy day (**Figure A3.3**), using a cosine corrected irradiance probe (CC-3-UV-T) with 180° field view, connected to an USB2000 spectrometer by means of a 400µm UV/VIS optic fibre following the method described in (Endler 1993). In all cases spectral reflectance data were collected between 300 - 750 nm and averaged to 1 nm intervals for analyses.

3.3.5. Modelling predator vision and froglet conspicuousness

Birds are visual oriented predators of many aposematic species (Collins & Watson 1983; Lindström *et al.* 1999a; Exnerová *et al.* 2008) including poison frogs (Master 1999). Birds seem able to perceive conspicuousness in poison frogs (Maan & Cummings 2012), and domestic chickens (*Gallus domesticus*) have attacked

poison frogs in experiments (Darst *et al.* 2006). Furthermore birds account for an appreciable amount of attacks on artificial aposematic prey in field experiments (e.g. Saporito *et al.* 2007; Noonan & Comeault 2009; Chouteau & Angers 2011). Colubrid snakes and spiders have also been reported to predate poison frogs (Summers 1999; Gray *et al.* 2010; Santos & Cannatella 2011) and Gray & Christy (2000) reported that the grapsid crab *Armases angustum* preys on *D. auratus* tadpoles. Therefore, our main results are based on psychophysical models of bird vision, but we replicated all analysis based on snake and crab vision following methods described in Maan & Cummings (2012), see **3.6 Appendices** section. We used the Vorobyev-Osorio visual model of colour discrimination (Vorobyev & Osorio 1998), which assumes that noise in the photoreceptors limits discrimination. Discrimination (conspicuousness) values are JNDs (just noticeable differences), with a value of 1 being the threshold for discrimination, and values of between 1 and 3 generally considered to mean that two objects could only be discriminated under ideal viewing conditions (rarely the case in the field) (Stevens *et al.* 2013). This model has been employed to calculate discrimination values in intraspecific (Maan & Cummings 2009; Ostrowski & Pröhl 2011; Wang 2011), and interspecific studies of poison frogs (Siddiqi *et al.* 2004; Darst *et al.* 2006).

Since the only bird documented to prey upon poison frogs (*Baryphthengus martii*) is a close relative to passerines (Livezey & Zusi 2007), we used cone sensitivity data for the blue tit (*Cyanistes caeruleus*), as a tetrachromatic visual model to calculate predicted photon catches for the different cone types (absorbance spectrum templates and oil droplets data from Hart *et al.* 2000). To model snake vision we used a trichromatic visual model from the coachwhip

colubrid snake *Masticophis flagellum*, with calculation of absorptance curves using a rhodopsin vitamin-A1 template according to Govardoskii *et al.* (2000). For crabs we used a dichromat visual model based on absorptance curves for the fiddler crab *Uca tangeri* with LW sensitivity curves manually digitized from Jordão, Cronin, & Oliveira (2007), and electrophysiological measures for the crab *Uca thayeri* with sensitivity to SW according to Horch, Salmon, & Forward (2002). Details of parameters used for the vision models are provided in **Table 3.1** and details of calculations are provided in **3.6 Appendices** section.

Table 3.1. Vision system parameters used in bird, snake and crab psychophysical models.

	Bird	Snake	Crab
Predator vision modelled	Blue tit (*Cyanistes caeruleus*)	Coachwhip snake (*Masticophis flagellum*)	Fiddler crab (composite of *Uca tangeri* and *Uca thayeri*)
	Tetrachromat version of the Vorobyev-Osorio model (Vorobyev & Osorio 1998)	Trichromat version of the Vorobyev-Osorio model (see Siddiqi *et al.* 2004)	Dichromat version of the Vorobyev-Osorio model (see Cummings *et al.* 2008)
Colour perception	Based on relative stimulation of visual cone photoreceptors with sensitivity to UV (λ_{max} = 371 nm), short (λ_{max} = 448	Based on relative stimulation of visual cone photoreceptors with sensitivity to UV (λ_{max} = 362 nm), short (λ_{max} =	Based on relative stimulation of visual cone photoreceptors with sensitivity to long (λ_{max} = 593 nm) and short (λ_{max}

	nm) medium (λ_{max} = 503 nm, and long (λ_{max} = 563 nm) wavelengths (Hart *et al.* 2000)	458 nm) and long (λ_{max} = 561 nm) wavelengths (Macedonia *et al.* 2009)	= 430 nm) wavelengths (Horch *et al.* 2002; Jordão *et al.* 2007)
Luminance perception	Based on double cone photoreceptors specialized in achromatic sensitivity (Kelber, Vorobyev, & Osorio 2003; Osorio & Vorobyev 2005).	Achromatic sensitivity based on absorbance of long wavelength cone pigments (Macedonia *et al.* 2009)	Achromatic sensitivity based on absorbance of long wavelength cone pigments (Jordão *et al.* 2007)
Weber fraction	0.05 (Siddiqi *et al.* 2004)	0.05 (Siddiqi *et al.* 2004)	0.12 (Hempel De Ibarra *et al.* 2000)
Relative number of photoreceptors	n_L = 1.00, n_M = 0.99, n_S = 0.71, n_{UV} = 0.37, n_D = 1.00	n_L = 0.85, n_S = 0.10, n_{UV} = 0.05 (Sillman *et al.*	n_L = 1.0, n_S = 1.0 (Cummings *et al.* 2008)

	(Hart *et al.* 2000)	1997)	
Prey detection system	Mostly based on vision (Martin 1999; Fernández-Juricic & Tran 2007)	Mostly olfactory, but visual cues may be involved (Brodie & Tumbarello 1978; Stuart-Fox, Moussalli, & Whiting 2008; Macedonia *et al.* 2009)	The use of visual cues for prey discrimination is unknown.
Predation events on poison frogs reported	One published report of predation in the wild (Master 1999); predation experiments using captive domestic hens (e.g. Darst *et al.* 2006; Darst & Cummings 2006),	Several accounts of predation in the wild (Santos & Cannatella 2011 supporting information); predation experiments using captive snakes (Brodie &	One published report of predation of tadpoles in the wild (Gray & Christy 2000), apparent use of colour discrimination mostly for intraspecific communication (Detto

predation on artificial prey in the wild (e.g. Saporito *et al.* 2007; Noonan & Comeault 2009; Chouteau & Angers 2011) and psychophysical models of bird vision (e.g. Siddiqi *et al.* 2004; Wang 2011; Maan & Cummings 2012) suggest that birds are predators of poison frogs.	Tumbarello 1978) suggest their likely importance as predators.	2007; Cummings *et al.* 2008; Baldwin & Johnsen 2012).

Note. Further details of modelling and formulas are provided in **3.6 Appendices**.

3.3.6. Antioxidant activity and oxidative damage

On the day of metamorphic climax 25 froglets from the high-food group and 25 from the low-food group were sampled at random and euthanized by stepped hypothermia and then stored at -80 ℃ until biochemical analyses. Remaining individuals were released into the wild. Samples were thawed, finely chopped using scissors, and then weighed to the nearest 0.0001g and 50 mM HEPES (N-2 hydroxyethylpiperazine-N´-2-ethanesulfonic acid) buffer solution was added on a 20% w/v basis. Samples were then homogenized ready for the following assays. We used whole frog homogenates to provide an organismal level overview of antioxidants and oxidative damage, because individual tissues are likely to differ in antioxidant levels and oxidative damage (López-Torres *et al.* 1993). Assessment of oxidative stress requires a range of assays that include measures of enzymatic and non-enzymatic antioxidants since components of antioxidant defences do not act in isolation, but react and compensate for each another. In addition, it is imperative to assay oxidative damage since this reflects the outcome of oxidative stress (reviewed in Selman *et al.* 2012). Therefore, we assayed oxidative damage in terms of levels of malondialdehyde (MDA), which is formed by the β-scission of peroxidised polyunsaturated fatty acids (Agarwal & Chase 2002), and has been reported as a physiological marker of oxidative stress during larvae metamorphosis (Mahapatra, Mohanty-Hejmadi, & Chainy 2001; Menon & Rozman 2007). In addition, we assayed superoxide dismutase

(SOD), a metalloenzyme that catalyses the dismutation of superoxide into oxygen and hydrogen peroxide and form a crucial part of intracellular antioxidant defence, for example, during early stages of development in amphibians (Montesano *et al.* 1989; Menon & Rozman 2007). Finally, we assayed total antioxidant capacity (TAC), which measures low-molecular weight non-enzymatic antioxidants such as vitamin E, carotenoids and flavonoids (reviewed in Selman *et al.* 2012).

Analysis of MDA was performed in duplicate using polypropylene screw cap tubes as described previously (Nussey *et al.* 2009) with some modifications. To 10 µl frog homogenate we added 10 µl butylated hydroxytoluene solution (BHT; .05% in ethanol), 80 µl phosphoric acid solution (0.44 M), and 20 µl thiobarbituric acid (TBA) solution (42 mM). Tubes were capped, vortexed for 5 s, then heated for 1 h at 100°C on a dry bath incubator. Tubes were then cooled on ice for 5 min. To extract the MDA-TBA complex, 80 µl of *n*-butanol (HPLC grade) was added to each tube, vortexed for 20 s then centrifuged at 15000 x g for 3 min at 4 °C to separate the two phases. A 60 µl aliquot of the upper, butanol phase, was carefully removed and transferred to an HPLC vial ready for HPLC analysis. Samples (40 µl) were injected into a Dionex HPLC system (Dionex Co., Camberley, Surrey, UK) fitted with a Hewlett-Packard Hypersil 5 µ ODS 100 x 4.6 mm column with a 5 µ ODS guard column. The column temperature was set at 37°C. The mobile phase (isocratic, flow rate: 1.0 ml/min) consisted of a 40:60 (v/v) mixture of methanol (HPLC grade) and buffer, the buffer being 50 mM potassium monobasic phosphate (anhydrous) solution, pH adjusted to 6.8 using 5 M potassium hydroxide solution. Fluorescence detection

utilized a RF2000 detector (Dionex Co.) set at 515 nm excitation and 553 nm emissions. Results are expressed as nmol MDA per g frog tissue. 49 samples were assayed in duplicate and repeatability calculated according to Lessells & Boag (1987) was high ($F_{48,49}$ = 8.66, P < 0.001; r = 0.79).

Total SOD was assayed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine (Cat. No. 706002; Cayman Chemical Co., USA). Following kit instructions, we mixed 100 µl of frog homogenate (see above) with 100 µl of Buffer Solution (containing 50mM of HEPES, 2mM EGTA, 420 mM D-mannitol and 140 mM sucrose, pH adjusted to 7.2 by adding 5 M potassium hydroxide). After vortexing for 10 s, samples were centrifuged at 1500 x g for 5 min at 4°C, and the supernatant was collected and diluted 1:20 v/v in kit Sample Buffer, which had itself been previously diluted 1:10 v/v in HPLC grade water. From this step onwards kit instructions were followed exactly. Absorbances were read at 440 nm using a Spectramax M2 plate reader (Molecular Devices Corp., USA). Results are expressed as units of SOD activity per mg frog tissue. 50 samples were assayed in duplicate and repeatability calculated according to Lessells & Boag (1987) was high ($F_{49,50}$ = 5.42, P < 0.001; r = 0.69).

TAC was assayed in terms of the capacity of the antioxidants in the sample to inhibit oxidation of ABTS® (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) (Cat No. 70901; Cayman Chemical Co., USA). Frog homogenates (see above) were diluted 1:1 (v/v) in 50mM HEPES buffer solution, vortexed for 10 s, and then centrifuged at 1500 x g for 5 min at 4°C. From this step onwards

kit instructions were followed. Absorbances were read at 750 nm using a Spectramax M2 plate reader (Molecular Devices Corp., USA). Results are expressed as nmol of Trolox equivalents per g of frog tissue. 50 samples were assayed in duplicate and repeatability calculated according to Lessells & Boag (1987) was high ($F_{49,50} = 25.15$, $P < 0.001$; $r = 0.92$).

3.3.7. Statistical analyses

Maximum SVL growth rate (SVLGR) was calculated by fitting a logistic curve to the data based on weekly changes in SVL, then calculating the first derivative at the point of inflection as described in Cavallini (1993). Principal component analysis (PCA) was conducted on biomarkers of oxidative balance using a varimax rotation. This approach is well suited when there is an *a priori* expectation of distinct groups of variables, for example as in markers of oxidative balance (Hõrak & Cohen 2010). The first principal component explained 41% of the total variance and was associated with variation in MDA and TAC (hereafter, $PC_{MDA\&TAC}$), while the second principal component explained 32% of the total variance and was associated with variation in SOD (hereafter, PC_{SOD}). Factor loadings for the first principal component were: -0.683, -0.549 and -0.481, whereas for the second principal component they were: 0.024, 0.642 and -0.767 for MDA, TAC and SOD respectively. Since MDA is a marker of oxidative damage higher values of $PC_{MDA\&TAC}$ indicate higher levels of oxidative stress. Similar PCAs were conducted on the relative photon catches of

single cones derived from psychophysical models for bird, snake and crab, respectively; this removes absolute variation that would otherwise result in the first principal component corresponding to overall variation in photon catch values (i.e. brightness variation) (Endler & Mielke 2005). For the bird vision model the first principal component captured 72% of the variance of single cones and was associated with variation in MW and LW versus SW and UV wavelengths, so this was used to represent variation in the type of colour (hereafter PC_{COL}). Factor loadings were: 0.534 and 0.533, for MW and LW and -0.444 and -0.484, for UV and SW, respectively. For the snake vision model the first principal component captured 67% of the variance of single cones and was associated with variation in UV and SW versus LW; with the following factor loadings: 0.581, 0.409 and -0.704, for UV, SW and LW, respectively. For the crab vision model the first principal component captured 100% of the variance and had the factor loadings: 0.707 and -0.707, for SW and LW, respectively.

GLMMs were fitted including food supply (treatment) as a fixed factor and family as a random factor unless otherwise stated. In certain analyses, SVL, SVLGR, $PC_{MDA\&TAC}$ or PC_{SOD} were included as covariates, in which case the interaction with food supply was also tested. In order to meet parametric assumptions JND colour based on bird vision was transformed as $(1/\text{JND colour})^2$ and signal luminance and JND colour were log transformed when based on the snake and crab vision models. Sample sizes differed slightly between analyses because a small number of reflectance spectra showed erratic readings (i.e. consistent negative values) or bad calibration and these values were therefore excluded from analyses. $P < 0.05$ was considered statistically

significant, and models were simplified by backward elimination starting with the interaction term(s) where appropriate. There were no significant effects of latency to lay or laying date on any of the above response terms (including all possible interactions) (all $F < 3.43$ and $P > 0.13$). Therefore, latency to lay and laying date are not considered further. Analyses were conducted using R v.2.12.1 (R Development Core Team 2010). All values reported in the Results are predicted means (± SE) from the statistical models, unless otherwise indicated.

3.4. Results

3.4.1. Survival, development time and growth rate

Some 28 of 90 larvae died before they reached metamorphosis, but the proportion of individuals that survived was similar in both food supply groups (GLMM with binomial errors: $\chi^2 = 0.007$, df = 1, $P = 0.93$). Compared to low-food individuals, high-food individuals reached metamorphosis sooner and were larger in terms of SVL and body mass (**Table 3.2**; **Table A3.1**); while relative body mass (i.e. mass controlling for size) was similar in both treatments (food, $F_{1,8} = 7.16$, $P = 0.028$; SVL, $F_{1,38} = 173.30$, $P < 0.001$; food x SVL, $F_{1,37} = 0.51$, $P = 0.48$). Hereafter, we report results based on body size variation, although conclusions were qualitatively the same based on body mass variation. High-

food individuals grew faster in terms of body size and body mass (**Table 3.2**; **Table A3.1**).

Table 3.2. Effects of food supply on development time, body size and mass, and growth rates in juveniles of the two food supply groups.

	df	*F*	*P*
Development time	1,8	26.79	**< 0.001**
Body size (SVL)	1,8	29.34	**0.004**
Body mass	1,8	48.72	**< 0.001**
SVL growth rate	1,8	23.21	**0.001**
Body mass growth rate	1,8	29.25	**< 0.001**

3.4.3. Luminance, colour and conspicuousness

We found no statistically significant effect of food supply on overall luminance, JND luminance, colour (PCcol) or JND colour modelled based on bird vision (**Table 3.3A**, **Table A3.1**). Similar results were found for models based on snake and crab vision (**Table 3.3A**).

3.4.4. Body size, growth and aposematic signals

In the high-food group, relatively large individuals at metamorphosis had lower luminance based on the bird visual model, while the inverse (small individuals, greater luminance) occurred in the low-food group (**Table 3.3B**; **Figure 3.1**). A similar statistical interaction was found for JND luminance (i.e. luminance conspicuousness) (**Table 3.3B**). A marginally non-significant interaction between food supply and body size was found for models based on snake and crab vision (**Table 3.3B**). In contrast, colour (PC_{COL}) and JND colour conspicuousness were not significantly related to body size (**Table 3.3B**). Similar results were found for snake and crab vision (**Table 3.3B**). Thus, while low-food individuals simultaneously maximised their investment in body size and signal luminance, high-food individuals that were larger had reduced luminance and conspicuousness (**Figure 3.1**). However, signal luminance and JND luminance contrast as perceived by birds, snakes and crabs was not predicted by growth rate in terms of SVLGR (**Table 3.3C**).

Table 3.3. Effects of food supply, body size and growth rates on signal luminance and conspicuousness based on models of bird, snake and crab vision.

	Bird vision			Snake vision			Crab vision		
Source of variation	*df*	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
(A) Warning signalling									
and food supply									
Luminance									
food	1,8	0.43	0.53	1,8	0.35	0.57	1,8	0.04	0.84
JND luminance									

	food	1,8	1.11	0.32	1,8	0.85	0.38	1,8	0.30	0.60
PC_{COL}										
	food	1,8	0.40	0.54	1,8	0.07	0.80	1,8	0.36	0.56
JND color										
	food	1,8	1.07	0.33	1,8	0.20	0.67	1,8	0.62	0.45
(B) Warning signalling and body size										
Luminance										
	food	1,8	0.32	0.58	1,8	0.35	0.57	1,8	0.04	0.84
	SVL	1,38	0.85	0.36	1,38	0.04	0.83	1,39	0.07	0.79
	food x SVL	1,38	5.20	**0.028**	1,37	3.48	0.07	1,38	3.60	0.06
JND luminance										

	food	1,8	1.11	0.32	1,8	0.85	0.38	1,8	0.30	0.60
	SVL	1,38	1.02	0.32	1,38	0.00	0.93	1,39	0.01	0.93
	food x SVL	1,38	4.36	**0.043**	1,37	2.93	0.09	1,38	3.08	0.08
Colour (PC_{COL})										
	food	1,8	0.40	0.54	1,8	0.07	0.80	1,8	0.36	0.56
	SVL	1,39	0.15	0.70	1,38	0.10	0.75	1,39	0.24	0.63
	food x SVL	1,38	0.86	0.36	1,37	1.43	0.24	1,38	0.05	0.81
JND colour										
	food	1,8	1.07	0.33	1,8	0.20	0.67	1,8	0.63	0.45
	SVL	1,39	0.48	0.49	1,38	0.19	0.66	1,39	0.01	0.93
	food x SVL	1,38	0.09	0.76	1,37	0.07	0.79	1,38	1.16	0.29

(C) Warning signalling and									
growth rate									
Luminance									
	food	1,8	0.43	0.53	1,8	0.35	0.57	1,8	0.04	0.84
	SVLGR	1,39	0.33	0.57	1,38	0.01	0.92	1,39	0.00	0.97
	food x SVLGR	1,38	0.47	0.49	1,37	0.74	0.40	1,38	0.77	0.39
JND luminance										
	food	1,8	1.11	0.32	1,8	0.85	0.38	1,8	0.30	0.60
	SVLGR	1,39	0.12	0.73	1,38	0.00	0.99	1,39	0.00	0.94
	food x SVLGR	1,38	0.54	0.47	1,37	0.99	0.33	1,38	0.90	0.35

Note. General Linear Mixed Models with food as a fixed factor and family as a random factor, boldface indicates significant values. For the bird model colour contrast was transformed as $(1/\text{JND colour})^2$ and for the snake and crab visual models luminance, and JND colour were log transformed in order to meet parametric assumptions.

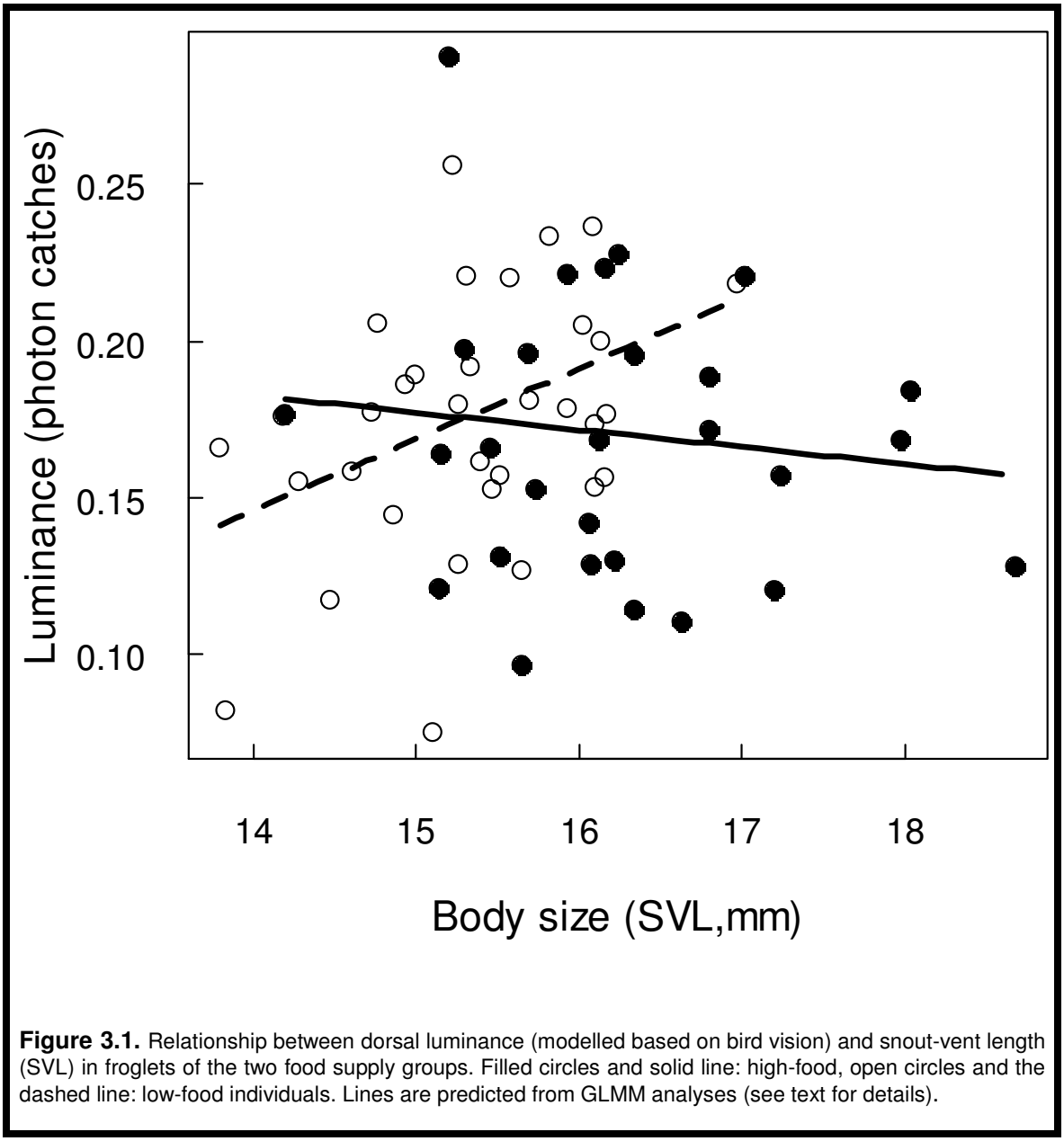

Figure 3.1. Relationship between dorsal luminance (modelled based on bird vision) and snout-vent length (SVL) in froglets of the two food supply groups. Filled circles and solid line: high-food, open circles and the dashed line: low-food individuals. Lines are predicted from GLMM analyses (see text for details).

3.4.5. Oxidative stress and aposematic signals

Although growth rates were higher in the high-food group, markers of oxidative balance were similar at the treatment level in both food supply groups (**Table 3.4).** We also examined whether oxidative balance was significantly associated with variation in growth rates, and whether this differed between food supply groups; however, we found no such relationships (**Table A3.2**). Yet, when oxidative stress levels were greater (high $PC_{MDA\&TAC}$) in the high-food treatment, signal luminance as perceived by bird, snake and crab predators was also higher, while conversely, high oxidative stress levels in the low-food group were associated with lower levels of signal luminance (**Table A3.3A**; **Figure 3.2**). A similar result was found in terms of JND luminance conspicuousness modelled based on bird, snake and crab vision (**Table A3.3A**). There were no significant associations between signal colour (PC_{COL}) or JND colour conspicuousness modelled based on bird, snake and crab vision and levels of oxidative stress (**Table A3.3A**). Levels of enzymatic antioxidant activity (PC_{SOD}) were independent of signal luminance, JND luminance, colour (PC_{COL}) or JND colour conspicuousness in both food groups (**Table A3.3B**).

Table 3.4. Markers of oxidative balance (mean ± SE) in body homogenates of juveniles of the two food supply groups.

	Food supply	
	High	Low
MDA (nmol g^{-1})	54.64 ± 2.41	52.13 ± 3.41
SOD (U SOD mg^{-1})	540.23 ± 66.20	545.15 ± 93.62
TAC (µmol Trolox g^{-1})	0.068 ± 0.005	0.060 ± 0.007

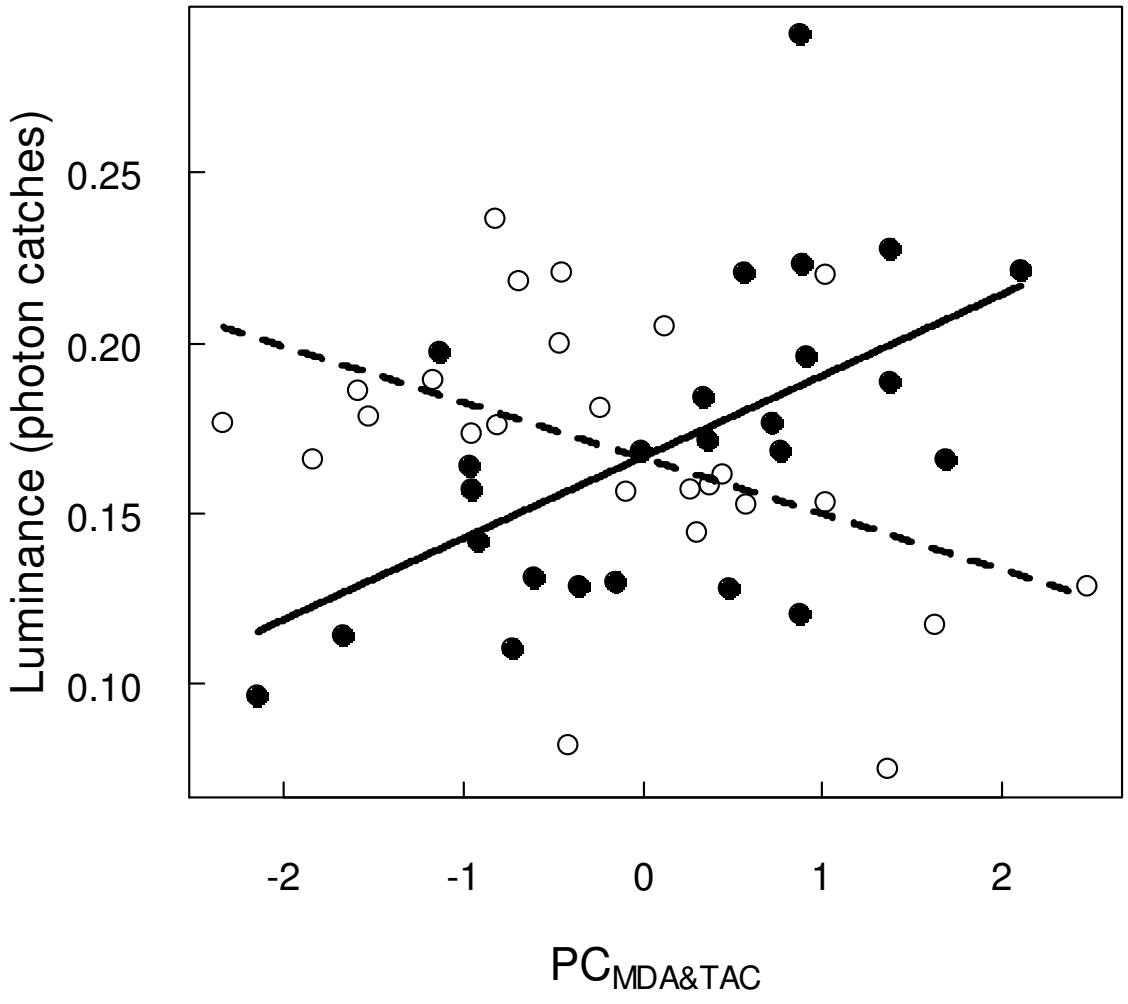

Figure 3.2 Relationship between dorsal luminance (modelled based on bird vision) and levels of oxidative stress ($PC_{MDA\&TAC}$) in body homogenates of juveniles of the two food supply groups. Filled circles and solid line: high-food, open circles and dashed line: low-food individuals. Values on the x-axis were multiplied by -1 to facilitate interpretation therefore positive values denote higher values of $PC_{MDA\&TAC}$. Lines are predicted from GLMM analyses (see text for details).

3.5. Discussion

We found that the nutritional environment experienced during early development had important consequences for body size and aposematic signal expression in green and black poison frogs at metamorphic completion. Where food was

abundant frogs grew larger, and investment in signalling and hence conspicuousness diminished. Signal luminance and levels of oxidative stress were positively correlated in high-food froglets, but were negatively correlated in low-food froglets suggesting a resource allocation trade-off when food availability is relatively low. Resource-limited froglets appeared to simultaneously maximise investment in growth and signalling within the limits of what they could attain, as constrained by oxidative stress.

Luminance and JND luminance (i.e. conspicuousness) of metamorphic *D. auratus* were affected by early nutrition and its interaction with body size. There was a positive correlation between body size and warning signal luminance in the low-food group, whereas in the high-food group where froglets were relatively large on average, body size and signal luminance were negatively correlated (**Figure 3.1**; **Table 3.2B**). In general models based on snake and crab vision generated qualitatively similar results to those based on bird vision, with the exception that the interaction between body size and food supply in the models which considered effects on signal luminance and JND luminance (i.e. conspicuousness) was marginally non-significant. This may be explained by the fact that, unlike birds, snakes and crabs do not possess cone cells specialized in luminance sensitivity (i.e. double cones; Osorio & Vorobyev 2008), and therefore snakes and crabs may be less sensitive to differences in brightness. Some snakes in the family Colubridae have evolved resistance to amphibian chemical defences (Brodie & Brodie 1999), in particular poison frogs (Brodie & Tumbarello 1978; Santos & Cannatella 2011). Therefore, such species would not be expected to discriminate amongst prey based on visual cues which

advertise the level of chemical defence. Yet, while diurnal crabs seem to be able to discriminate conspicuousness in adult *Oophaga* [*Dendrobates*] *pumilio* (Maan & Cummings 2012), their sensitivity to detect changes in integument luminance contrast may be constrained by their limited visual sensitivity in comparison to birds. Crabs have been reported to prey on *D. auratus* tadpoles (Gray & Christy 2000), which are cryptically coloured, but whether they prey on frogs post-metamorphosis is not known. Furthermore, behavioural and experimental evidence suggest that in crabs apparent colour discrimination is mainly devoted to intraspecific communication (Detto 2007; Cummings *et al.* 2008; Baldwin & Johnsen 2012). In general, birds seem to be better than snakes and crabs at decoding information about levels of defences based on the expression of aposematic signals in *Oophaga* [*Dendrobates*] *pumilio* (Maan & Cummings 2012). Whether this finding can be generalised to other species of dendrobatids awaits verification. Birds and snakes are candidate predators which may impose selection for diversification of defensive strategies in this group (Toledo, Ribeiro, & Haddad 2007). However, more work is needed in order to clarify the actual predators, their vision capabilities and their role in shaping colour variation in poison frogs.

As expected, when food was non-limiting (high-food group) larvae grew faster and they were larger at metamorphosis. As in other dendrobatids, *D. auratus* undergoes its larval stage inside water-filled pools, where the amount of water varies depending on rainfall (Caldwell & de Araújo 2004). Rapid development may enable individuals to avoid desiccation and predation during this vulnerable stage of life when chemical defences are lacking (Caldwell 1993).

Larger froglets are likely to have greater energy in the first few, critical days following metamorphic climax, when they must begin to forage to acquire chemical defences. In addition, if body size differences persist to sexual maturity, there could be important implications for reproductive success. In particular, larger males are better able to compete for mates (Summers 1989), and larger females may benefit in terms of higher fecundity and mate guarding (Wells 1977; Summers & Earn 1999). The likelihood of surviving until maturity is probably dependent upon rapidly obtaining toxic substances after metamorphosis (Daly *et al.* 1994b; Saporito *et al.* 2010). A larger froglet may have a greater foraging capacity when searching for toxic prey on the forest floor (Santos & Cannatella 2011).

While selection appears to have favoured large body size in adult poison frogs (Hagman & Forsman 2003), it is unclear how selection might shape developmental strategies in terms of relative allocations of resources to growth versus signal expression in immature, palatable *D. auratus*. Indeed, we found no significant effects of food supply on signal luminance, colour or corresponding conspicuousness at the treatment level. In visual oriented predators, texture and shape discrimination of small objects seems to be mediated by luminance contrast (Jones & Osorio 2004), and thus luminance is thought to be used by birds in motion detection of prey (Osorio & Vorobyev 2005). Distance may also influence how a signal is perceived by potential predators; for example, when viewed from afar a small target may be camouflaged yet when viewed in near proximity it may be readily discriminable because of its colouration (Tullberg, Merilaita, & Wiklund 2005; Bohlin, Tullberg, & Merilaita 2008). During close-up

inspection a highly conspicuous signal could deter predators and thus enable individuals to forage in the open and facilitate the acquisition of toxins from the diet (Speed *et al.* 2010).

Following detection the colour contrast of a prey may influence predator wariness and ultimately guide its decision whether to attack (Guilford 1986; Lindström *et al.* 1999; Osorio & Vorobyev 2005; reviewed in Stevens & Ruxton 2012). Indeed, larger body size is likely to amplify the aversive effect of the colour component of warning signals (Forsman & Merilaita 1999). This could explain why high-food froglets did not reduce investment in the colour of their warning signal. Once detected froglets may rely on automimicry (i.e. resemblance of adults in terms of colouration) to deter predators (Brower, Brower, & Corvino 1967; Speed, Ruxton, & Broom 2006). Toxic adults will have already educated predators to avoid individuals with similar appearance (Speed *et al.* 2012), thus allowing automimics to coexist in a population (Darst *et al.* 2006). It is interesting that high-food froglets did not have greater colouration than low-food froglets. This could be explained by the fact that predators are wary of novel coloured prey (Mappes *et al.* 2005), and empirical evidence suggests there is strong selection against rare phenotypes in natural populations (Noonan & Comeault 2009; Wennersten & Forsman 2009; Chouteau & Angers 2011). Alternatively, there may be stabilizing selection on colouration because of its importance in intraspecific signalling at adulthood. In dendrobatids mate selection, intraspecific competition and territorial defence are known to be influenced by variation in skin coloration (Maan & Cummings 2008; Ostrowski & Pröhl 2011; Crothers, Gering, & Cummings 2011). Indeed, uniformity in dorsal

skin colour and pattern within populations of the strawberry poison frog (*Oophaga* [*Dendrobates*] *pumilio*) has been attributed in part to sexual selection (Summers *et al.* 1999; Siddiqi *et al.* 2004; Summers, Cronin, & Kennedy 2004; Reynolds & Fitzpatrick 2007; Maan & Cummings 2008).

Whether aposematic signals provide fine-scale, honest information about defensive capacity remains a matter of controversy (Stevens & Ruxton 2012). Theoretical models have predicted that more toxic prey should invest less in signalling, because they have better chances of surviving attacks and should therefore avoid the conspicuousness costs of signals (Leimar *et al.* 1986; Speed & Ruxton 2005a, 2007). However recent resource competition models have suggested that aposematic signals and defences should correlate positively under conditions where such traits utilize a shared resource that is in limited supply (Blount *et al.* 2009; Lee *et al.* 2011). One such resource could be antioxidants, which have been suggested to be necessary both to produce signals and to prevent oxidative stress caused by the production or storage of toxic chemicals (Blount *et al.* 2009). Nevertheless, these models do not take into account the fact that many aposematic species undergo a process of colour change during development (e.g. Grant 2007; Tullberg *et al.* 2008) and may have little or no secondary defence at this time (Nylin *et al.* 2001; Nishida 2002; Saporito *et al.* 2010). Therefore, an interesting direction for future studies will be to investigate whether signal colour and luminance in froglets persists, and how aposematic signals correlate with levels of defensive alkaloids at adulthood.

One intriguing possibility is that well-nourished larger froglets, which we found to have reduced signal luminance, are better foragers and thus become most toxic as adults. If so, this could result in a negative signal-defence correlation as some theoretical studies have predicted (Leimar *et al.* 1986; Speed & Ruxton 2007; Blount *et al.* 2009), and as observed in some empirical studies (Darst *et al.* 2006; Wang 2011; Blount *et al.* 2012). In captivity at least, considerable sequestration of dietary alkaloids is readily achieved before adulthood in *D. auratus* (Daly *et al.* 1994b). Rates of toxin accumulation may be lower in the wild, but this requires investigation. Moreover, in resource-limited environments if toxin sequestration causes oxidative stress and this trades against signal production or maintenance, as hypothesised (Blount *et al.* 2009), then positive signal-defence correlations may be expected at adulthood. Several recent studies have reported such correlations either within, or across, aposematic species (e.g. Summers & Clough 2001; Bezzerides *et al.* 2007; Cortesi & Cheney 2010; Blount *et al.* 2012). However, the importance of resource availability in determining the sign of signal-defence correlations has only recently begun to be studied (Blount *et al.* 2012) and further empirical data are needed.

Metamorphosis in amphibians is characterized by increased ROS production (Inoue *et al.* 2004). Indeed significantly higher levels of lipid peroxidation and antioxidants have been found during this period in amphibians (Menon & Rozman 2007), and similarly, a positive correlation between levels of TAC and MDA has been reported previously in a study of European greenfinches *Carduelis chloris* (Hõrak *et al.* 2007). Therefore high levels of

$PC_{MDA\&TAC}$ suggests that non-enzymatic antioxidants were accumulated and/or released in response to oxidation of cellular lipids during metamorphosis; in contrast, SOD functions largely within cells (Stead & Park 2000) therefore its direct role in defence against lipid oxidation is unlikely. Growth is accompanied by formation of ROS as by-products of metabolism (Sies 1997), and rapid growth in particular has been linked to elevated oxidative damage or depleted antioxidant capacity in various taxa (e.g. Alonso-Alvarez *et al.* 2007; Nussey *et al.* 2009). However, we did not find any association between growth rate and levels of oxidative damage in tissue homogenates in either food supply group. Thus individuals may have optimized their growth in relation to their antioxidant defence capability, and the dietary availability of antioxidants was sufficient in both food supply groups to enable all individuals to cope with ROS resulting from growth.

In high-food froglets, size at metamorphosis correlated negatively with luminance (Figure 3.1). This reduction in luminance seems likely to have been facultative, rather than the consequence of a resource allocation trade-off, because there was no correlation between growth rate and luminance in either food group. Moreover, any trade-off in the allocation of resources to warning signals versus body size would be expected to apply equally or to a greater extent in the low-food group. On the contrary, body size and luminance were positively correlated in low-food froglets. In the high-food group higher levels of oxidative stress were associated with high luminance, whereas in the low-food group relatively high levels of oxidative stress were associated with low luminance. We think a likely explanation for this finding is that ROS-induced

oxidative stress constrained the ability to produce bright signals in the low-food group. Non-enzymatic antioxidant pigments are commonly responsible for skin pigmentation in poison frogs (Fox 1976), and oxidative stress may have depleted antioxidant pigments in low-food individuals, which in turn impaired signal production. Aposematic signal expression in particular can trade-off with growth, development time and body size (e.g. Grill & Moore 1998; Ojala *et al.* 2007; Lindstedt *et al.* 2010). However, our results suggest that resource-limited individuals need not always trade-off investment in somatic versus aposematic traits; instead it may be beneficial to become as big and bright as possible within the constraints of resource supply.

3.5.1. Conclusion

In conclusion, this study highlights the influence of developmental nutrition and oxidative stress on resource allocation to growth and aposematic signals. In particular, we found that when resources were abundant, individuals grew relatively large but reduced investment in signal luminance. These results generate predictions as to the likely importance of body size and aposematic signal expression as determinants of survival in wild froglets. Data to test these predictions are currently lacking and this is an important topic for further work.

3.6. Appendices: Supplementary Methods and Results

3.6.1. Description of bird vision model

Calculations were done based on a set of functions in Matlab R2009a (The MathWorks Inc, USA). The model starts by calculating the cone quantum catches (*qi*) for each photoreceptor class for froglets and ambient radiance spectra as

$$qi_{bird} = \int_{\lambda=300}^{750} R(\lambda) I(\lambda) A_{\mathrm{i}}(\lambda) d\lambda,$$

Equation 3.1 Calculation of cone quantum catches of photoreceptors of the bird vision model.

here $A_i(\lambda)$ represents the absorptance spectrum for each of the four photoreceptor cone classes of the bird integrated over 1 nm intervals from 300 to 750 nm. $R(\lambda)$ and $I(\lambda)$ represent the reflectance spectrum of the froglet´s skin and the irradiance spectra measured in the field respectively. Resulting photon quantum catches were standardized in order to account for variation in light conditions, using the von Kries transformation adaptation coefficient. This method assumes that photoreceptors adjust their sensitivity in proportion to the background light environment

$$Ki_{bird} = \left(\int_{\lambda=300}^{750} Ai(\lambda) I(\lambda) d\lambda \right)^{-1},$$

Equation 3.2 Von Kries transformation adaptation coefficient of photoreceptors of the bird vision model

This procedure ensures that colour perception relies on colour constancy, whereby the visual system removes variation in ambient light so that colours look similar under variable light conditions (Cuthill 2006). Therefore, the adjusted quantum catch data for each photoreceptor class was calculated as

$$q_a = Ki_{bird}\, x\, qi_{bird},$$

Equation 3.3 Adjusted quantum catches of each photoreceptor of the bird vision model.

Values of relative single cone quantum catches were then included in a Principal Component Analysis (see **3.3.7. Statistical analyses**).

Following calculations of quantum catches, the model assumes that the signal of each cone channel is proportional to the logarithm of the adjusted quantum catches; as such the contrast between a pair of stimuli was calculated as the quotient of adjusted photon quantum catches

$$\Delta q_a = log \frac{[q_{a1}(frog\ spectra)]}{[q_{a2}(background\ spectra)]}$$

Equation 3.4 Calculation of contrast between a pair of stimuli of each photoreceptor of the bird vision model.

The Vorobyev-Osorio colour discrimination model is based on evidence that colour discrimination is determined by noise arising in the photoreceptors and is independent of light intensity. Noise in each photoreceptor channel (e_i) was calculated as

$$e_i = \frac{\omega_i}{\sqrt{n_i}}$$

Equation 3.5 Calculation of noise in each photoreceptor of the bird vision model.

Where ω_i was taken as 0.05 and represents the Weber fraction of the most abundant cone type (Siddiqi *et al.* 2004) and n_i is the relative number of receptor types in the retina of the blue tit (Hart *et al.* 2000) ($n_L = 1.00$, $n_M = 0.99$, $n_S = 0.71$, $n_{UV} = 0.37$). Colour (chromatic) discrimination in the tetrachromatic visual model was calculated as JND values using the following equation

$$JND\ bird\ color = \sqrt[2]{\frac{\begin{matrix}(e_{UV}e_S)^2(\Delta q_L - \Delta q_M)^2 + (e_{UV}e_M)^2(\Delta q_L - \Delta q_S)^2 + (e_{UV}e_L)^2(\Delta q_M - \Delta q_S)^2 \\ +(e_Se_M)^2(\Delta q_L - \Delta q_{UV})^2 + (e_Se_L)^2(\Delta q_M - \Delta q_{UV})^2 + (e_Me_L)^2(\Delta q_S - \Delta q_{UV})^2\end{matrix}}{(e_{UV}e_Se_M)^2 + (e_{UV}e_Se_L)^2 + (e_{UV}e_Me_L)^2 + (e_Se_Me_L)^2}}$$

Equation 3.6 Just noticeable differences (JND´s) for colour discrimination of the bird vision model.

Since in our model, overall perceived luminance is considered to arise from stimulation of double cone photoreceptors, luminance discrimination was evaluated as

$$JND\ bird\ luminance = \left(\frac{\Delta q_D}{e_D}\right)$$

Equation 3.7 Just noticeable differences (JND´s) for luminance discrimination of the bird vision model.

Overall variation in colour was based on values from single cone photon catch scores (and a Principal Component Analysis of them) and variation in overall luminance was based on double cone photon catch scores (Δq_D). Colour and luminance discrimination (conspicuousness) was based on JND values.

3.6.2. Description of snake vision model

Calculations were done based on a set of functions in Matlab R2009a (The MathWorks Inc, USA). The model starts by calculating the cone quantum catches (*qi*) for each photoreceptor class for froglets and ambient radiance spectra as

$$qi_{snake} = \int_{\lambda=300}^{750} R(\lambda) I(\lambda) A_i(\lambda) d\lambda,$$

Equation 3.8 Calculation of cone quantum catches of photoreceptors of the snake vision model

here $A_i(\lambda)$ represents the absorptance spectrum for each of the three photoreceptor cone classes (i.e. UV, SW and LW) integrated over 1 nm intervals from 300 to 750 nm. $R(\lambda)$ and $I(\lambda)$ represent the reflectance spectrum of the froglet´s skin and the irradiance spectra measured in the field respectively. Resulting photon quantum catches were standardized in order to account for variation in light conditions, using the von Kries transformation adaptation coefficient. This method assumes that photoreceptors adjust their sensitivity in proportion to the background light environment

$$Ki_{snake} = \left(\int_{\lambda=300}^{750} A_i(\lambda) I(\lambda) d\lambda \right)^{-1},$$

Equation 3.9 Von Kries transformation adaptation coefficient of photoreceptors of the snake vision model

This procedure ensures that colour perception relies on colour constancy, whereby the visual system removes variation in ambient light so that colours look similar under variable light conditions (Cuthill 2006). Therefore, the adjusted photon quantum catch data for each photoreceptor class was calculated as

$$q_a = Ki_{snake}\, x\, qi_{snake},$$

Equation 3.10 Adjusted quantum catches of each photoreceptor of the snake vision model.

Values of the relative single cone quantum catches were then included in a Principal Component Analysis (see **3.3.7. Statistical analyses**).

Following calculations of quantum catches, the model assumes that the signal of each cone channel is proportional to the logarithm of the adjusted quantum catches; as such the contrast between a pair of stimuli was calculated as the quotient of adjusted quantum catches

$$\Delta q_a = log \frac{[q_{a1}(frog\ spectra)]}{[q_{a2}(background\ spectra)]}$$

Equation 3.11 Calculation of contrast between a pair of stimuli of each photoreceptor of the snake vision model.

The Vorobyev-Osorio visual discrimination model is based on evidence that color discrimination is determined by noise arising in the photoreceptors and is independent of light intensity. Noise in each photoreceptor channel (e_i) was calculated as

$$e_i = \frac{\omega_i}{\sqrt{n_i}}$$

Equation 3.12 Calculation of noise in each photoreceptor of the snake vision model.

Where ω_i was taken as 0.05 according to Siddiqi *et al.* (2004) and represents the Weber fraction of the most abundant cone type and n_i is the relative number of receptor types in the retina of the snake (data for *Thamnophis sirtalis*, Sillman *et al.* 1997) $(n_L = 0.85,\ n_S = 0.10,\ n_{UV} = 0.05)$. Colour (chromatic) discrimination in the trichromatic visual model was calculated as JND values using the following equation

$$JND\ snake\ colour = \sqrt[2]{\frac{(e_{UV})^2(\Delta q_L - \Delta q_S)^2 + (e_S)^2(\Delta q_L - \Delta q_{UV})^2 + (e_L)^2(\Delta q_{UV} - \Delta q_S)^2}{(e_{UV}e_S)^2 + (e_{UV}e_L)^2 + (e_S e_L)^2}}$$

Equation 3.13 Just noticeable differences (JND´s) for colour discrimination of the snake vision model.

We considered overall perceived luminance in snakes as in other vertebrates to arise from stimulation of LW cone photoreceptors with sensitivity in the long wavelength part of the spectra; therefore luminance discrimination was evaluated as

$$JND\ snake\ luminance = \left(\frac{\Delta q_L}{e_L}\right)$$

Equation 3.14 Just noticeable differences (JND´s) for luminance discrimination of the snake vision model.

Overall variation in colour was based on values from single cone photon catch scores (and a Principal Component Analysis of them) and variation in overall luminance was based on LW cone photon catch scores (Δq_L). Colour and luminance discrimination (conspicuousness) was based on JND values.

3.6.3. Description of crab vision model

Similar calculations were done based on a set of functions in Matlab R2009a (The MathWorks Inc, USA). The model starts by calculating the cone quantum catches (*qi*) for each photoreceptor class for froglets and ambient radiance spectra as

$$qi_{crab} = \int_{\lambda=300}^{750} R(\lambda)I(\lambda)A_{\mathrm{i}}(\lambda)d\lambda,$$

Equation 3.15 Calculation of cone quantum catches of photoreceptors of the crab vision model.

here $A_i(\lambda)$ represents the absorptance spectrum for each of the two photoreceptor cone classes (i.e. SW and LW) integrated over 1 nm intervals from 300 to 750 nm. Resulting photon quantum catches were standardized in order to account for variation in light conditions, using the von Kries transformation adaptation coefficient. This method assumes that photoreceptors adjust their sensitivity in proportion to the background light environment

$$Ki_{crab} = \left(\int_{\lambda=300}^{750} A_i(\lambda)I(\lambda)d\lambda\right)^{-1},$$

Equation 3.16 Von Kries transformation adaptation coefficient of photoreceptors of the crab vision model.

This procedure ensures that colour perception relies on colour constancy, whereby the visual system removes variation in ambient light so that colours look similar under variable light conditions (Cuthill 2006). Therefore, the adjusted photon quantum catch data for each photoreceptor class was calculated as

$$q_a = Ki_{crab}\ x\ qi_{crab},$$

Equation 3.17 Adjusted quantum catches of each photoreceptor of the crab vision model.

Values of the relative single cone quantum catches were then included in a Principal Component Analysis (see **3.3.7. Statistical analyses**).

Following calculations of quantum catches, the model assumes that the signal of each cone channel is proportional to the logarithm of the adjusted quantum catches; as such the contrast between a pair of stimuli was calculated as the quotient of adjusted quantum catches

$$\Delta q_a = log\,\frac{[q_{a1}(frog\ spectra)]}{[q_{a2}(background\ spectra)]}$$

Equation 3.18 Calculation of contrast between a pair of stimuli of each photoreceptor of the crab vision model.

The Vorobyev-Osorio visual discrimination model is based on evidence that colour discrimination is determined by noise arising in the photoreceptors

and is independent of light intensity. Noise in each photoreceptor channel (e_i) was calculated as

$$e_i = \frac{\omega_i}{\sqrt{n_i}}$$

Equation 3.19 Calculation of noise in each photoreceptor of the crab vision model.

Where ω_i was taken as 0.12 based on physiological receptor noise data according to Hempel De Ibarra *et al.* (2000) for honeybees *Apis mellifera* and n_i is the relative number of receptor types that for our purposes were set as ($n_L = 0.5,\ n_S = 0.5$) following (Cummings *et al.* 2008). Colour (chromatic) discrimination in the dichromatic visual model was calculated as JND values using the following equation

$$JNDcrab\ colour = \sqrt[2]{\frac{(\Delta q_S - \Delta q_L)^2}{(e_S)^2 + (e_L)^2}}$$

Equation 3.20 Just noticeable differences (JND´s) for colour discrimination of the crab vision model.

We considered overall perceived luminance in crab to arise from stimulation of LW cone photoreceptors with sensitivity in the long wavelength part of the spectra, luminance discrimination was evaluated as

$$JNDcrab\ luminance = \left(\frac{\Delta q_L}{e_L}\right)$$

Equation 3.21 Just noticeable differences (JND´s) for luminance discrimination of the crab vision model.

Overall variation in colour was based on values from single cone photon catch scores (and a Principal Component Analysis of them) and variation in overall luminance was based on LW cone photon catch scores (Δq_L). Colour and luminance discrimination (conspicuousness) was based on JND values.

Table A3.1. Development time, growth and growth rates (mean ± SE) in juveniles of the two food supply groups.

	Food supply	
	High	Low
Development time (days)	59.40 ± 1.81	63.93 ± 0.87
Body size (SVL, mm)	16.389 ± 0.229	15.5 ± 0.164
Body mass (mg)	491 ± 18	408 ± 12
SVL growth rate (mm day^{-1})	0.258 ± 0.011	0.219 ± 0.008
Body mass growth rate (mg day^{-1})	23.53 ± 1.16	18.99 ± 0.81

Note. Development time was calculated from hatching until metamorphosis was completed.

Table A3.2. Effects of food supply and growth rate on oxidative balance.

Source of variation	*df*	*F*	*P*
$PC_{MDA\&TAC}$			
food	1,8	1.23	0.30
SVLGR	1,29	0.18	0.67
food x SVLGR	1,28	0.75	0.39
PC_{SOD}			
food	1,8	0.09	0.77
SVLGR	1,28	0.04	0.83
food x SVLGR	1,27	0.49	0.49

Note. General Linear Mixed Models with food as a fixed factor and family as a random factor; boldface indicates significant value.

Table A3.3. Effects of food supply and oxidative balance on aposematic signals.

	Bird vision			Snake vision			Crab vision		
Source of variation	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*
(A) Warning signalling and oxidative balance ($PC_{MDA\&TAC}$)									
Luminance									
food	1,8	0.43	0.53	1,8	0.18	0.68	1,8	0.81	0.39
$PC_{MDA\&TAC}$	1,28	0.03	0.86	1,27	0.11	0.74	1,28	0.23	0.63
food x $PC_{MDA\&TAC}$	1,28	18.47	**<0.001**	1,27	19.57	**<0.001**	1,28	22.15	**<0.001**
JND luminance									
food	1,8	0.25	0.63	1,8	0.06	0.81	1,8	0.07	0.80

$PC_{MDA\&TAC}$	1,28	0.85	0.36	1,27	0.73	0.40	1,28	0.87	0.36
food x $PC_{MDA\&TAC}$	1,28	24.64	**<0.001**	1,27	17.58	**0.003**	1,28	20.55	**<0.001**
Colour (PC_{COL})									
food	1,8	0.40	0.54	1,8	0.07	0.80	1,8	0.36	0.56
$PC_{MDA\&TAC}$	1,29	0.00	0.94	1,28	0.00	0.98	1,29	0.10	0.76
food x $PC_{MDA\&TAC}$	1,28	0.01	0.92	1,27	0.29	0.60	1,28	0.08	0.77
JND colour									
food	1,8	1.07	0.33	1,8	0.20	0.67	1,8	0.62	0.45
$PC_{MDA\&TAC}$	1,29	0.49	0.49	1,28	0.02	0.88	1,29	0.19	0.66
food x $PC_{MDA\&TAC}$	1,28	0.10	0.75	1,27	2.26	0.14	1,28	0.31	0.58
(B) Warning signalling and									

oxidative balance (PC_{SOD})									
Luminance									
food	1,8	0.43	0.53	1,8	0.35	0.57	1,8	0.04	0.84
PC_{SOD}	1,29	0.48	0.49	1,28	0.02	0.90	1,29	0.16	0.69
food x PC_{SOD}	1,28	2.01	0.17	1,27	2.91	0.10	1,28	3.67	0.06
JND luminance									
food	1,8	1.11	0.32	1,8	0.85	0.38	1,8	0.30	0.60
PC_{SOD}	1,29	1.29	0.26	1,28	0.88	0.35	1,29	1.10	0.30
food x PC_{SOD}	1,28	2.39	0.13	1,27	2.15	0.15	1,28	3.04	0.09
Colour (PC_{COL})									
food	1,8	0.40	0.54	1,8	0.07	0.80	1,8	0.36	0.56

	PC_{SOD}	1,29	1.90	0.18	1,28	1.51	0.23	1,29	0.93	0.34
	food x PC_{SOD}	1,28	0.03	0.87	1,27	0.05	0.82	1,28	0.31	0.58
JND colour										
	food	1,8	1.07	0.33	1,8	0.20	0.67	1,8	0.62	0.45
	PC_{SOD}	1,29	0.00	0.95	1,28	0.08	0.78	1,29	0.06	0.80
	food x PC_{SOD}	1,28	2.11	0.16	1,27	1.32	0.26	1,28	1.75	0.20

Note. General Linear Mixed Models with food as a fixed factor and family as a random factor; boldface indicates significant value. For the snake and crab visual models, luminance and JND colour were log transformed to meet parametric assumptions.

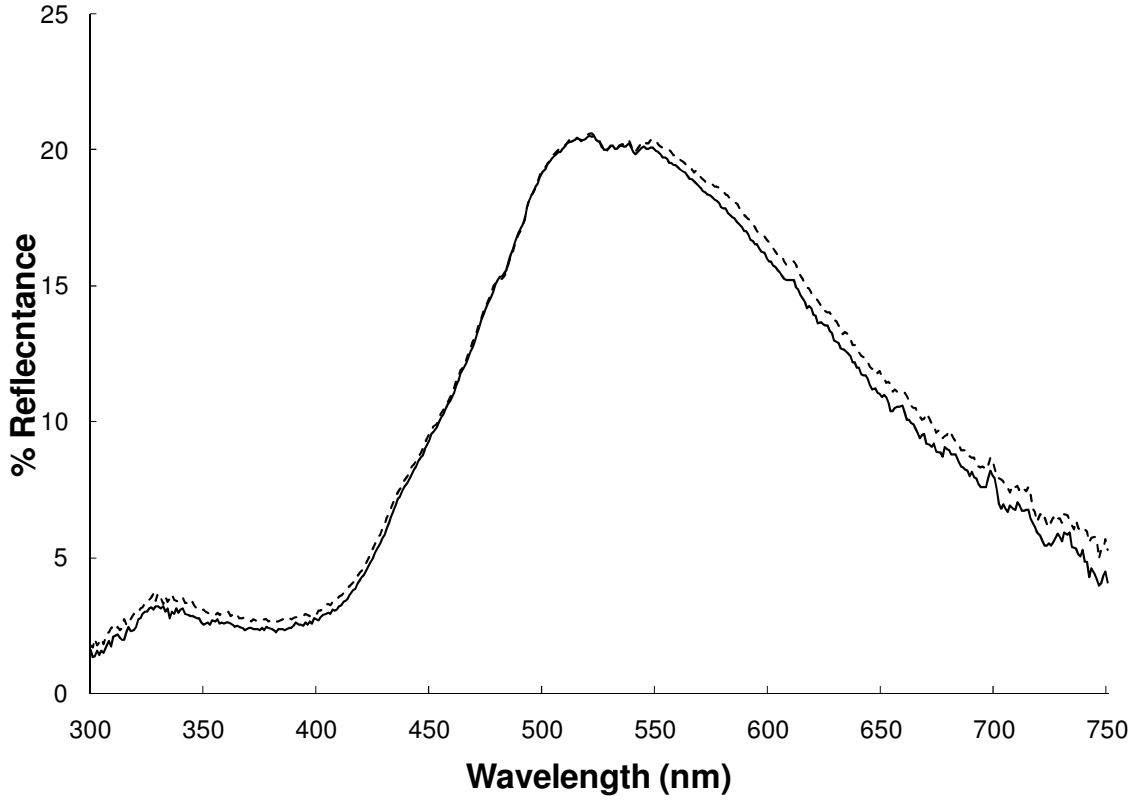

Figure A3.1 Mean reflectance spectra of froglets on the day of metamorphic climax in high (solid line; n=28 individuals) and low food groups (dashed line; n=34 individuals). Reflectance spectra are average measurements taken from four body regions (head, dorsum, left and right flanks).

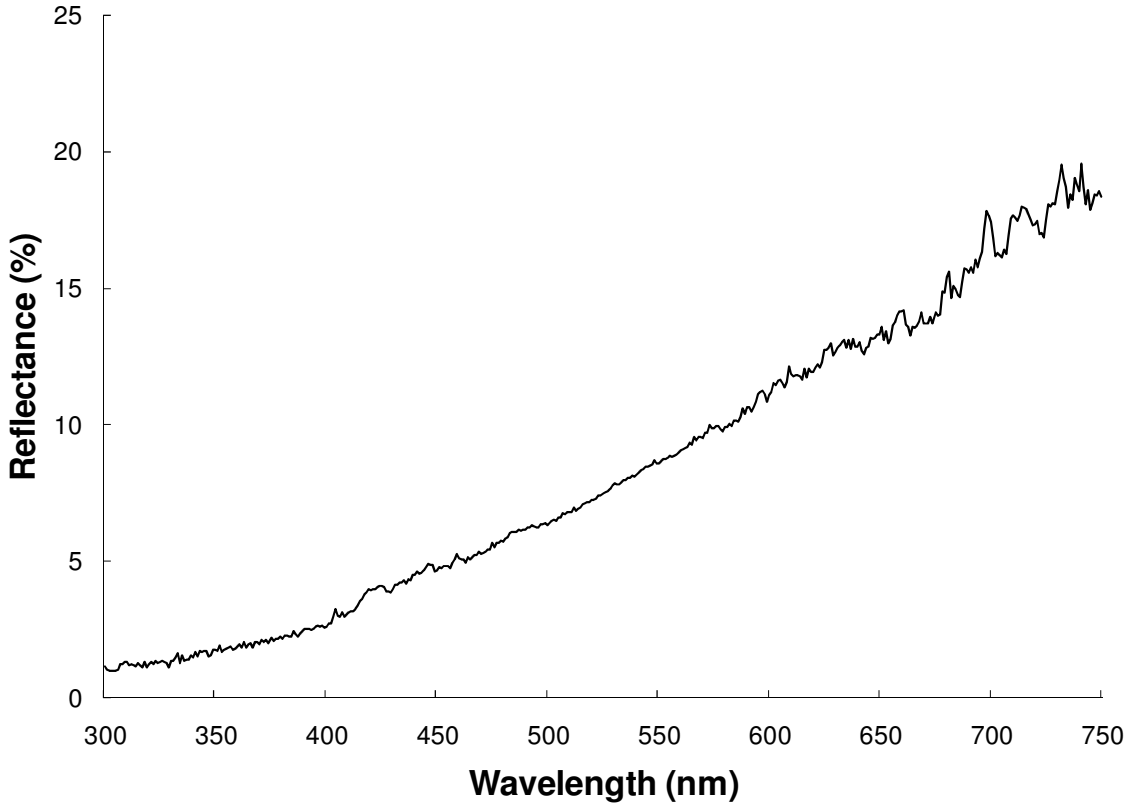

Figure A3.2 Mean reflectance spectra of natural background samples (n = 136). Samples were leaf litter collected from the sites where adult frogs used for breeding were captured.

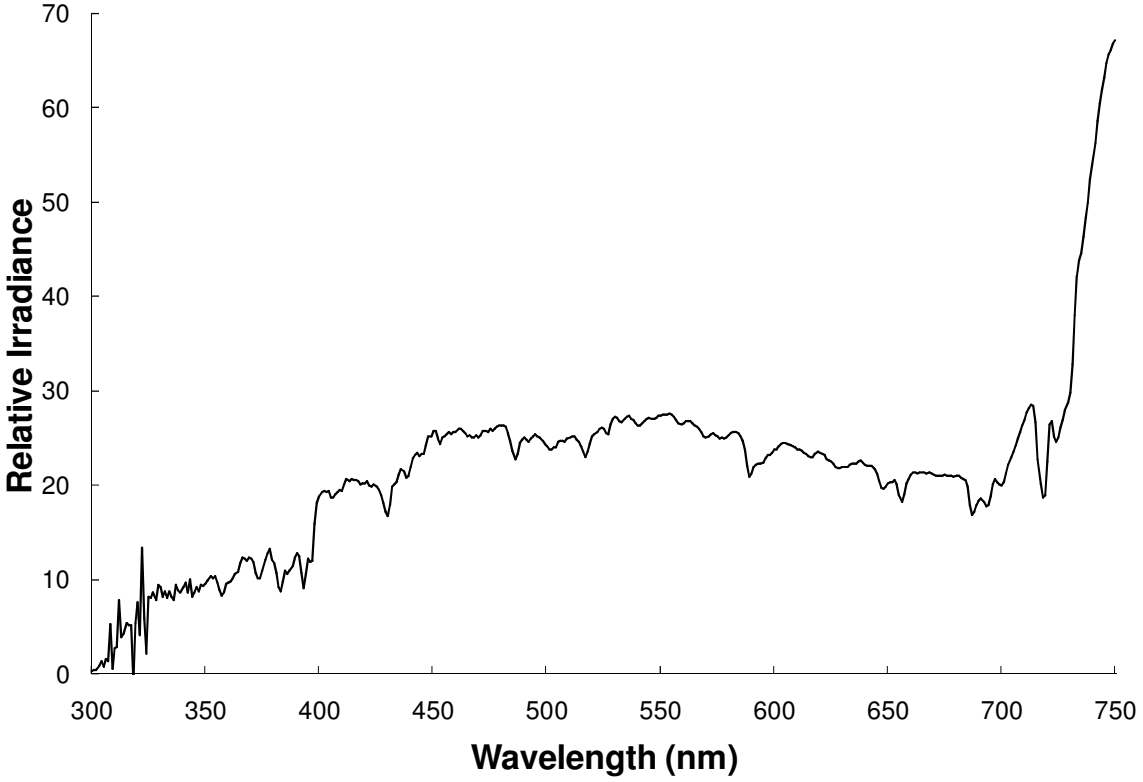

Figure A3.3 Mean irradiance spectra of ambient light at the study site (n = 90 measurements on a sunny day and n = 85 measurements on a cloudy day).

Chapter 4. Body size but not warning signal luminance influences predation risk in recently metamorphosed poison frogs[3]

4.1. Abstract

During early development many species have bright and conspicuous warning appearance, but have yet to acquire chemical defences, a phenotypic state which presumably makes them vulnerable to predation. Body size, and signal luminance, are known to be sensitive to variation in early nutrition. However, the relative importance of these traits as determinants of predation risk in juveniles is not known. To address this question we utilised computer-assisted design (CAD) and information on putative predator visual sensitivities to produce artificial stimuli of post-metamorphic froglets that varied in terms of body size and signal luminance. We then deployed artificial prey in the field and measured rates of attack by birds and unknown predators. Our results indicate that body size was a significant predictor of artificial prey survival. Rates of attack by bird predators were significantly higher on smaller models. However, predation by birds did not differ between artificial prey of varying signal luminance. This suggests that at the completion of metamorphosis smaller froglets are at a selective disadvantage, possibly because predators can discern they have

[3] This chapter has been submitted as: Flores, E. E., Stevens, M., Moore, A. J., Rowland, H. M., Vasquez, L., De La Cruz, J., Croxford, G., and Blount, J. D. Body size but not warning signal luminance influences predation risk in recently metamorphosed poison frogs. *Animal Behaviour.*

relatively low levels of chemical defence compared to larger froglets. There is likely to be a premium on efficient foraging, giving rise to rapid growth and the acquisition of toxins from dietary sources in juvenile poison frogs.

4.2. Introduction

Aposematic species are distasteful or otherwise unprofitable, and signal this property to predators with conspicuous colouration. Aposematism exploits the innate aversion of visually oriented predators towards conspicuous or novel colours, which results in increased predator wariness, enhanced avoidance learning rates, and thus reduced predation risk for the prey (Guilford 1986; Ruxton *et al.* 2004). Body size, colour and brightness contrast are key components of warning signals with the potential to influence predators' learning and avoidance (Ruxton *et al.* 2004; Stevens & Ruxton 2012). Visual oriented predators in particular are known to avoid large body size and large pattern elements of warning signals (Gamberale & Tullberg 1998; Gamberale-Stille 2000; Lindstedt, Lindström, & Mappes 2008). Indeed, larvae of some aposematic insects aggregate as a strategy to increase aversion in predators because in this way the signal size is enhanced (Gamberale & Tullberg 1998; Gamberale-Stille 2000; Riipi *et al.* 2001). Furthermore, colour and brightness contrast are known to facilitate detection, rejection and learning about warning signals in predators (Gamberale-Stille 2001; Ham *et al.* 2006; Aronsson & Gamberale-Stille 2012). Since predators can vary in their visual sensitivity

(Aidala *et al.* 2012; Moore *et al.* 2012), and how the cognitive and learning processes associated with visual stimuli take place (Kelber *et al.* 2003; Endler & Mappes 2004; Osorio & Vorobyev 2005; Stevens, Stoddard, & Higham 2009), any variation in the components of aposematic signals may be of critical importance for the survival of juveniles. For example, predators may attack novel aposematic prey more often (Noonan & Comeault 2009), monomorphism in warning signalling can result from anti-apostatic selection (Allen & Greenwood 1988) or polymorphic signal design may be selected when the community of predators is variable (Endler & Mappes 2004). However, predators can monitor the level of toxins they ingest rather than avoid toxic prey completely (Skelhorn & Rowe 2006). The propensity for some predators to attack prey despite the presence of warning colouration may impose a particular selective pressure on immature aposematic organisms, in which chemical defences have not yet been developed or acquired, thus exposing them to high predation risk (see Gray & Christy 2000; Sime et al. 2000; Nylin et al. 2001). In particular bird predators have been shown to taste-reject aposematic prey based on their level of chemical defences despite their similar warning appearance (Skelhorn & Rowe 2006).

Empirical evidence suggests that birds are important predators of aposematic species (e.g. Benson 1972; Exnerová et al. 2008) including poison frogs. Master (1999) reported that rufous motmots (*Baryphthengus martii*) prey upon poison frogs, while domestic hens (*Gallus domesticus*) have been shown to distinguish differences in conspicuousness and toxicity in poison frogs during predation experiments (Darst *et al.* 2006; Darst & Cummings 2006).

Psychophysical models of bird vision have confirmed that birds can discern differences in terms of colour and brightness of poison frogs (Maan & Cummings 2012), and thus variation in appearance may be important in influencing predation risk. In birds, aversion towards large and conspicuously coloured warning signals can be at least partly innate (Darst *et al.* 2006; Exnerová *et al.* 2007), and may result from dietary conservatism (Marples *et al.* 1998), and/or from previous learning experiences (Roper & Redston 1987; Roper 1994). Thus warning signal colour or brightness, and body size, can both affect aversion in birds (Forsman & Merilaita 1999; Jones & Osorio 2004). Nevertheless, the colour, brightness and size of a signal may independently influence the perceptual psychology of birds and therefore affect rates of attack (Schuler & Roper 1992; Gamberale-Stille & Tullberg 1999; Exnerová *et al.* 2010). Colour is generally thought to primarily guide the detection and classification/discrimination of large objects and should be relatively constant under variable ambient light conditions (Osorio *et al.* 1999; Osorio & Vorobyev 2005). Luminance ('lightness') information is used in encoding object boundaries and texture, and detection of small targets and movement, and will be more affected by changes in ambient light (Campenhausen & Kirschfeld 1998; Jones & Osorio 2004). Therefore, colour is likely to be important in learning of prey appearance and categorisation of prey types, whereas luminance and colour contrast may be important in initial detection and avoidance (Stevens & Ruxton 2012). There is some evidence that luminance contrast can also play a role in avoidance learning in praying mantids (Prudic *et al.* 2007). How important the role of luminance is for birds during avoidance learning under natural and

variable field conditions is not known. However, unlearnt avoidance of undefended prey in the field by wild birds is enhanced by greater luminance contrast levels (Stevens *et al.* 2007) and so luminance contrast may be important in deterring predators.

Determining the consequences of specific aspects of aposematic signals for predation risk is difficult, because predator-prey interactions involving aposematic prey are rarely observed in the wild (though see Finkbeiner et al. 2012) alternative experimental approaches that allow for the manipulation of aposematic phenotypes while at the same time measuring the responses of predators are more common. Artificial stimuli (models) made of plasticine or clay, for example, have been used to assess predation on aposematic amphibians (e.g. Saporito et al. 2007; Noonan & Comeault 2009; Chouteau & Angers 2011), reptiles (e.g. Brodie 1993; Wüster et al. 2004; Niskanen & Mappes 2005) and insects (e.g. Remmel & Tammaru 2009; Ihalainen & Lindstedt 2012). Observation of imprints left by predators (e.g. bites, beak marks) enables the identification of 'predation' at different spatial and temporal scales. Nevertheless, it can be challenging to run experiments using artificial prey, for example because of the need to correctly simulate prey coloration according to the visual sensitivities of putative predators. Visual systems are highly variable among taxa (Osorio & Vorobyev 2008), and thus it is important to consider which predator(s) the experiment will target, taking into consideration the ecological and evolutionary context.

During early development, resource allocation to growth and warning colouration can be constrained in aposematic prey, as affected by the quantity or quality of nutrition (e.g. Grill & Moore 1998; Ojala et al. 2005; Blount et al. 2012; Flores et al. 2013). Poison frogs are a group of aposematic animals that show high intraspecific variation in warning colouration (Lötters *et al.* 2007) but the consequences of variable signal design during immature stages is unclear. Indeed, we recently reported that availability of food during larval development in green and black poison frogs (*Dendrobates auratus*) can affect body size and signal luminance in post-metamorphic froglets (Flores *et al.* 2013). Specifically, froglets with access to relatively little food appeared to simultaneously maximise body size and luminance within the constraints of what they could attain. In contrast, froglets with access to greater amounts of food, which were larger on average, reduced their investment in signal luminance without changing signal colour as compared to smaller individuals. This suggests the possibility that having both large body size and high signal luminance may attract the attention of predators and thus raise predation risk. Such alternative developmental strategies as influenced by food supply seem likely to be adaptations to minimise predation risk (Ojala *et al.* 2007; Higginson & Ruxton 2009). Nevertheless the relative importance of body size and signal luminance as determinants of survival in juvenile froglets in the wild has not been tested before. More broadly, the role of achromatic contrast in warning signals in deterring predators, as opposed to colour, has rarely been explored, especially in a natural system.

Here, we present the results of a field study using clay models of *D. auratus* froglets. Artificial prey varied in either body size (Experiment 1), or signal luminance as perceived by birds (Experiment 2), in order to test the effects of these two traits on rates of attack by bird predators. Based on the fact that recent metamorphic froglets lack secondary defences, and therefore detectability risk impairs survival at this stage we predicted that (1) larger froglets would have lower survival than smaller froglets. We also predicted that (2) individuals with greater signal luminance should have lower survival.

4.3. Methods

4.3.1. Production of artificial stimuli

Artificial prey were designed to resemble recently metamorphosed juveniles of *D. auratus*, which were themselves derived from a field-based diet manipulation experiment carried out at Santa Fe, Veraguas province, during 2010 as described in Flores et al. (2013). Levels of body size (snout-vent length; SVL) and luminance (lightness sensitivity based on photon catches) of artificial prey were based on the results of an earlier diet manipulation experiment, in which dorsal luminance varied in relation to a statistical interaction between SVL and food supply. In essence, in froglets with a relatively high-food supply, above a certain body size we observed a reduction in signal luminance (Flores et al.

2013). Body contour and design of black dorsal pattern as seen from above were standardised, being measured using Image J 1.43q (Rasband 1997) based on a digital image of the dorsum of one randomly chosen metamorphic froglet collected at the field site. The image was taken with a Canon Power shot G6 (7.1 megapixel) digital camera (Canon Inc. Japan) and later scaled to the experimental SVL values (**Figure A4.1**). The proportion of the dorsum covered by black patterning was calculated using Image J 1.43q based on digital images of the dorsum of each experimental froglet in the high-food and the low-food supply groups, respectively. The proportion of the dorsum covered in black patterning did not differ significantly between food groups (General Linear Mixed Model (GLMM); with food as fixed factor and family as random factor; food: $F_{1,8}$ = 3.27, P = 0.11; mean ± SE = 0.58 ± 0.01 %, N = 62). This proportion was therefore used for all artificial prey. Dorsal signals are considered more important than ventral ones in warning signalling in dendrobatids (Wang & Shaffer 2008; Maan & Cummings 2012) and thus we included only a black dorsal pattern in artificial prey.

4.3.2. Experiment 1, effect of body size variation

Five prey phenotypes (S1 – S5) were designed to be equally spaced in increments of size (i.e. 0.846 mm) along the distribution of SVL values (**Table 4.1**). Since we were only interested in the effect of body size, we kept the values of single cone photon catches (colour) and double cone photon catches

(luminance) constant, according to the average of both food supply groups. In our design the prey phenotype 'S2' corresponds to the threshold value, as indicated in Flores et al. (2013), after which high-food supply froglets exhibited reduced signal luminance. To prepare the artificial prey, non-toxic, Sculpey III® clay (Polyform Products Co. IL, USA) and Fimo soft® clay (Staedtler Mars, GmbH & Co. Nürnberg, Germany) were manually mixed. Clay was weighed to the nearest 0.001g using an Ohaus Scout Pro balance (Ohaus Europe GmbH, Switzerland). Sculpey clay types were: 001 White, 042 Black and 1629 Granny Smith. Fimo clay types were: 26 Cherry Red and 37 Blue. To prepare 100 g of the average colour and luminance for Experiment 1, clay was mixed as follows: 63g of 1629 Granny Smith, 19g of 042 Black, 7g of 26 Cherry Red, 3.5g of 001 White, 3.5g of 37 Blue, 2.5g of 001 White and 2.5g of 042 Black.

Table 4.1. Artificial prey phenotypes in terms of snout-vent length (SVL) used for Experiment 1.

Artificial prey phenotype (SVL, mm)				
S1	S2	S3	S4	S5
14.45	15.30	16.14	16.99	17.84

4.3.3. Experiment 2, effect of signal luminance

To determine the effect of luminance variation, the median values of SVL in the upper (75-100%) interquartile range for the high and low food supply groups were calculated. We held body size constant, and calculated the upper median SVL values averaged across the two food supply groups (16.7 mm) for the artificial prey. The corresponding luminance values were then predicted from the results of a GLMM (Flores *et al.* 2013), thus:

Luminance (high food) = 0.26 – 0.005(SVL)

Equation 4.1. Modelled equation used to predict the luminance values based on snout-vent length (SVL) in froglets of the high-food treatment.

Luminance (low food) = - 0.17 + 0.023(SVL)

Equation 4.2. Modelled equation used to predict the luminance values based on snout-vent length (SVL) in froglets of the high-food treatment.

This design resulted in two levels of luminance: High = 0.21 and Low = 0.17, enabling us to test the effect of signal luminance on predation risk in large individuals. These levels represent luminance photon catches of real froglets, and span 4 JNDs in terms of contrast against a banana leaf background. Luminance values for the artificial prey were obtained by adding clay to 100 g of the average colour mixture of Experiment 1 artificial prey, as follows: 4 g of 001 White to obtain value High luminance, and 2g of 001 White to obtain value Low luminance.

4.3.4. Digital design of artificial prey and mould preparation

Artificial prey were digitally designed using SolidWorks 3D CAD 2011 SP 4.0 software (Dassault Systèmes SolidWorks Corp., Massachusetts, USA), simulating a *D. auratus* individual in a natural sitting posture. Digital files were exported in STL format ready for the manufacturing process and transferred to a Roland MDX 500 automatic milling machine (Roland DGA Corp., California, USA) by means of Mayka Expert 7.0 software (PicaSoft, France). Moulds for each specific size class were drilled in two steps to increase precision using a 3.0 mm ball nose on a 15.0 x 15.0 x 2.5 cm (L x W x H) block of resin board. Individual artificial prey were made by pressing the experimental mixed clay into the specific mould, carefully extruding the clay, and finishing by removing any excess of clay with a scalpel. The black dorsal pattern and eyes were manually applied using non-toxic black poster paint (Sargent Art Inc, Hazleton PA, USA). Front and hind legs were manually attached using the appropriate mixed clay for Experiment 1 and Experiment 2, respectively. In order to deploy the models they were glued to the blade of a standard shaped 15 x 10 cm piece of dry banana leaf, which is a typical substrate at our study site, using a small dab of Loctite Epoxi-mil epoxy adhesive (Henkel corporation, Düsseldorf, Germany).

4.3.5. Colour and luminance discrimination

In birds colour and luminance discrimination are likely based on the sensitivity of single and double cone cell photoreceptors, respectively (Osorio & Vorobyev 2005, 2008). We used a variation of the Vorobyev-Osorio (V-O) visual model of colour discrimination (Vorobyev & Osorio 1998), which has been employed to calculate discrimination values (i.e. just noticeable differences - JNDs) in intra- and interspecific studies of poison frogs (e.g. Wang 2011; Maan & Cummings 2012). A JND value of 1 is considered as the threshold for discrimination, and values between 1 and 3 mean that two objects can probably only be discriminated under good viewing conditions (Siddiqi *et al.* 2004). To calculate predicted photon catches for the single and double cones and discrimination of artificial prey against banana leaves as an ecologically realistic background, we measured the spectral reflectance of clay in triplicate using a portable Jaz spectrometer (Ocean Optics Inc. FL, USA) with a bifurcated 400 µm UV/VIS fibre optic probe connected to an internal Jaz PX pulsed short arc xenon lamp (Ocean Optics Inc. FL, USA). Measurements were made at an angle of 45°, and corrected for lamp drift using a white diffuse spectral standard (WS-1) (Maan & Cummings 2008). We measured the spectral reflectance of 12 dry banana leaves used as substrate for the artificial prey in triplicate and averaged them following the methodology described above (**Figure A4.2**). We also measured ambient light irradiance at several locations in the field during 2010, N = 90 measurements on a sunny day and N = 85 measurements on a cloudy day, using a cosine corrected irradiance probe (CC-3-UV-T) with 180° field view connected to an USB2000 spectrometer (Ocean Optics Inc. FL, USA) by means

of a 400µm UV/VIS fibre optic cable following the method described in Endler (1993) (**Figure A4.3**). The only known bird predator of *D. auratus* (i.e. *Baryphthengus martii*) is a close relative of higher passerine birds (Livezey & Zusi 2007), therefore we employed the UV-sensitive blue tit (*Cyanistes caeruleus*) as a tetrachromatic visual model (absorbance spectrum templates, oil droplets data and relative number of receptor types from Hart et al. 2000). Spectra were integrated over 1 nm intervals from 300 to 750 nm; details of calculations are provided in **4.6 Appendices** section.

4.3.6. Similarity between artificial prey and froglets

JND luminance and colour contrast did not differ significantly between the black pattern painted on artificial prey (N = 12) and the natural black pattern of randomly selected froglets (N = 10) derived from the experiment described in Flores et al. (2013) (JND luminance: GLM, $F_{1,20}$ = 0.01, P = 0.94; log(JND colour): GLM, $F_{1,20}$ = 1.71, P = 0.20; **Figure A4.4**). Similarly, JND luminance did not differ significantly between mixed clay and the same experimental froglets (JND clay ± SE = 4.51 ± 0.60, N = 10; JND frog ± SE = 5.34 ± 0.85, N = 10; JND luminance: GLM, $F_{1,18}$ = 0.51, P = 0.48). A qualitatively similar result was found for JND colour contrast (JND clay ± SE = 12.28 ± 0.68, N = 10; JND frog ± SE = 12.55 ± 0.96, N = 10; log(JND colour): GLM, $F_{1,18}$ = 0.24, P = 0.63) (**Figure A4.5**). Dorsal skin in dendrobatids mostly lacks UV reflectance (Summers *et al.* 2003; Noonan & Comeault 2009), and similarly experimental froglets did not

show appreciable levels of UV reflectance in their dorsal skin (Flores *et al.* 2013). Accordingly we found that the UV reflectance of our mixed clay was low (UV mixed clay ± SE: 0.077 ± 0.002, N =10), therefore it was unlikely to influence our results. JND colour discrimination was not significantly different among artificial prey ($F_{1,6}$ = 5.55, P = 0.06; **Table A4.1**). However JND luminance discrimination was significantly different among artificial prey ($F_{1,6}$ = 685.8, P < 0.001; **Table A4.1**). In general all JND values were discriminable to a bird predator (all JNDs > 3).

4.3.7. Deployment of models

Artificial prey were randomly deployed in the field between April - September 2011 at a shade organic coffee plantation in Santa Fe, Veraguas province, central Panama (8°31' N 81°03'W). For Experiment 1 we deployed a total of N = 600 models, and for Experiment 2 a total of N = 240 models. We used a randomized block design, in which each block (N = 6), contained either N = 100 models (20 of each phenotype for Experiment 1) or N = 40 models (20 of each phenotype for Experiment 2), deployed randomly along non-linear transects, maintaining an approximate minimal distance of 10 m among models and 50 m among blocks (Cuthill *et al.* 2005; Stevens, Hardman, & Stubbins 2008b; Rowland *et al.* 2008). Blocks were deployed one at a time, with all the models in a single block deployed the same day early in the morning. Monitoring of models was performed on a daily basis 24 h after deployment following the same order

and for a total of seven days. Experiment 2 started at the same study site two weeks after Experiment 1 had concluded, in order to minimize any possible effects of learning by predators.

4.3.8 Statistical analyses

Analyses were conducted using R v.2.12.1 (R Development Core Team 2010). Survival analysis was performed using Cox proportional-hazards regression (Cox 1972). This non-parametric survival analysis allows inclusion of censored records (i.e. non-avian predation) providing more information to the survival function (Cuthill *et al.* 2005). Models with U or V-shaped beak marks (Brodie 1993; Hegna *et al.* 2011) were classified as attacked by birds and were therefore removed, photographed, and recorded as dead. Models attacked by mammals, with unidentified marks, complete disappearances and those which were not attacked were recorded as censored. We were not interested in any effect of block *per se* and its inclusion as a factor did not qualitatively change the results reported here. In Experiment 1, when there was a significant effect of prey size on survival, planned comparisons based on the Wald statistic between pairs of prey were conducted and the hazard ratio with corresponding confidence intervals between pairs also reported. In Experiment 2, the effect of luminance on large prey was also tested using the Wald test. Here the hazard ratio represents the multiplicative average effect of one category of prey with respect to the other on the hazard related to the incidence of being killed or risk

of mortality. To test whether the probability of attack by birds differed between Experiments 1 and 2, we conducted a binomial logistic regression including the estimates of effects (i.e. odds ratio) (see Hegna et al. 2011). Here the odds ratio represents the ratio of the odds of attack in Experiment 1 to the odds in Experiment 2. $P < 0.05$ was considered statistically significant in all analyses.

4.4. Results

4.4.1. Experiment 1: effect of body size on predation risk

A total of 44 out of 597 artificial prey were attacked by birds (7%) (**Figure 4.1** & **Figure 4.2**) whereas 34 prey were attacked by unknown predators (6%), while three models could not be re-found and were classed as censored. Overall smaller prey survived less than larger prey (**Figure 4.2**; Cox regression; Wald $X^2_4 = 11.14$, $P = 0.025$). Survival of the smallest prey was not significantly different from the threshold sized prey (S1 vs S2; hazard ratio = 1.35, $CI_{95\%} = 0.64 - 2.86$, Wald $X^2_1 = 0.63$, $P = 0.43$), although the threshold sized prey survived significantly less well compared to the next size category (S2 vs S3; hazard ratio = 0.24, $CI_{95\%} = 0.08 - 0.71$, Wald $X^2_1 = 6.57$, $P = 0.01$). Survival of prey in category S3 was not significantly different from category S4 (hazard ratio = 1.52, $CI_{95\%} = 0.43 - 5.40$, Wald $X^2_1 = 0.43$, $P = 0.51$), and a similar result was found for categories S4 versus S5 (hazard ratio = 0.99, $CI_{95\%} = 0.32 - 3.07$,

Wald $X^2_1 = 0$, $P = 0.98$). Survival of models attacked by unknown predators occurred independently of size (Wald $X^2_4 = 5.96$, $P = 0.20$).

Figure 4.1 Juvenile *D. auratus* artificial prey on banana leaf substrate, with beak mark imprints. Inset showing an original intact model.

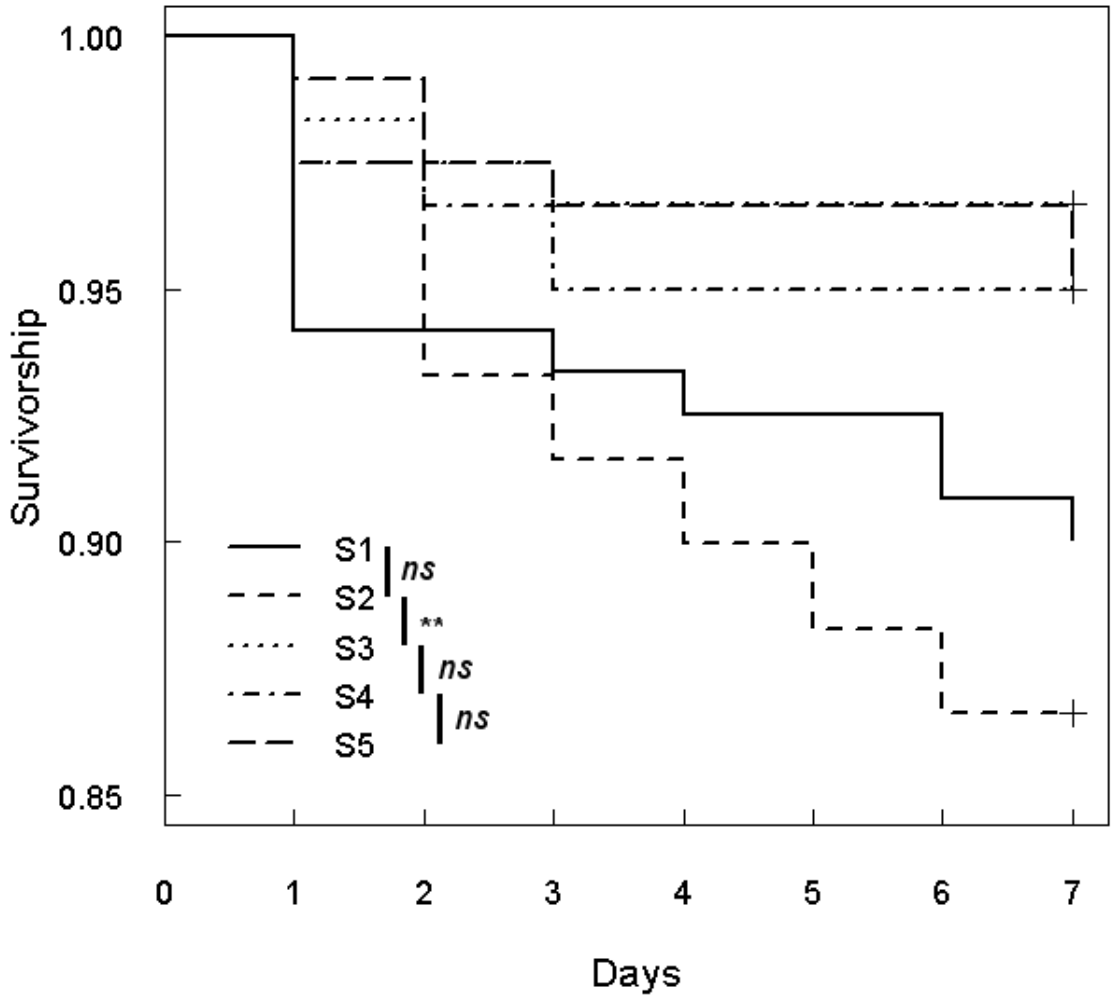

Figure 4.2 Cumulative survivorship curves for five categories of body size of artificial prey over 7 days. See Table 1 for details of size categories. Smaller artificial prey representing the threshold size (S2) survived significantly less compared to larger prey. Vertical bars in the legend represent the planned comparisons conducted between pairs of artificial prey categories; *ns*: no significant.

4.4.2. Experiment 2: effect of signal luminance on predation risk

There were a total of 8 out of 235 models attacked by birds (3%), and 21 models were attacked by unknown predators (9%), while 5 models could not be re-found and were classed as censored. Signal luminance was not a significant predictor of survival in larger artificial prey (High vs Low; hazard ratio = 2.96, $CI_{95\%}$ = 0.60 – 14.69; Wald X^2_1 = 1.77, P = 0.18). Similar results were found when attacks by unknown predators were considered (Wald X^2_1 = 0.58, P = 0.44).

The probability of attacks in Experiment 1 was two fold higher when compared to Experiment 2 (odds ratio = 2.16, $CI_{95\%}$ = 1.10 – 4.15; Wald X^2_1 = 5.07, P = 0.024), which may imply that the failure to detect any effect in luminance was due to lack of statistical power because of the lower predation rates in Experiment 2.

4.5. Discussion

We found that larger body size in artificial frogs resulted in greater survival compared to smaller models. It is known that both pattern element size and body size of prey enhance the effectiveness of warning signals, but this is to our knowledge the first evidence that body size *per se* affects signal efficiency. Our study suggests that post-metamorphic body size in *D. auratus* could be under selection pressure imposed by bird predators. Contrary to our predictions,

predation by birds did not differ between artificial prey varying in terms of signal luminance, which could result from relaxed selection on this aposematic trait during early life stages or lack of statistical power in our design. Use of artificial prey has proven to be a useful technique for understanding how predators respond to variation in warning signals (Benson 1972; Lindström 1999b; Chouteau & Angers 2011). Several previous studies have taken into account the visual system of the potential predator in the design of artificial prey (Stevens *et al.* 2007, 2008b; Rowland *et al.* 2008), although ours is the first study to have used this approach in poison frogs.

4.5.1. Effect of body size

We found that birds avoided attacking larger artificial prey. This is contrary to the prediction that larger prey would suffer greater predation because of increased detectability (Roberts, Taylor, & Uetz 2006; Lindstedt *et al.* 2008). Body size has been shown to be a predictor of detectability in early larval stages of the caterpillar *Orgyia antiqua* (e.g. Sandre et al. 2007). However, attack rates by bird predators have been found to be negatively correlated with body size in artificial prey of this species (Mänd, Tammaru, & Mappes 2007). This could be related to the increased effect of the warning signal in larger prey (Remmel & Tammaru 2011). One possible explanation for our results, therefore, is that larger artificial prey were more aversive to bird predators because predators have an innate wariness of large warning signals (Gamberale & Tullberg 1996,

1998). That is not to say that larger froglets benefit from reduced attack rates by birds because they truly resemble adults (i.e. automimicry, Speed et al. 2006). This seems an unlikely explanation, because it has been found that well-resourced froglets (relatively high food supply) grew large but reduced their investment in the luminance component of the warning signal (Flores *et al.* 2013). Such a reduction in warning signalling would not be expected if large juveniles were automimics of adults, this requires further examination.

Body size in particular seems to be under strong selection imposed by visual-hunting predators, in those species that acquire warning colouration early in life such as *D. auratus* (Gamberale & Tullberg 1996; Forsman & Merilaita 1999). Interestingly, artificial prey in the size category representing the smallest (S1 = 14.45 mm) and the threshold value (S2 = 15.30 mm) had lower survival. We found that the threshold value represented a shift point in our experiment after which survival increased with body size (see **Figure 4.2**). This result supports the idea of a perceptual size threshold beyond which survival increases or is maintained without further beneficial effects of increments in body size (e.g. Forsman & Herrström 2004). However the size category immediately after the threshold value represented an increment of 5.4%, which is not necessarily the minimal perceptual difference to which bird predators in the wild can effectively show aversion (see Swaddle 1999). Notably, the smallest and the threshold size categories in our experimental design were similar to that reported as an average SVL for recent metamorphic *D. auratus* froglets in the wild (range: 14.0 – 14.8 mm; Eaton 1941; Pope 1941). In poison frogs body size has been reported to correlate positively with the strength of warning signals (Hagman &

Forsman 2003; Santos & Cannatella 2011), suggesting an association between these phenotypic traits as one mechanism for the evolution of aposematism. This association has been strongly linked to diet specialization in terms of the acquisition of alkaloid-bearing arthropods (Santos & Cannatella 2011). Consequently we may expect small juveniles in the population to be more vulnerable than those with larger body size, due to a lower capacity to acquire and store secondary defences (Daly *et al.* 2002; Saporito *et al.* 2010). Since birds are capable of differentiating prey of different sizes (Gamberale & Tullberg 1996; Grieco 2002), and also seem to detect differences in alkaloid defence levels in poison frogs (Darst *et al.* 2006; Darst & Cummings 2006), it could be that birds at our study site selectively attack froglets that are smaller than a certain threshold, and therefore similar in body size to recent metamorphic, less defended froglets.

4.5.2. Effect of luminance

Although luminance contrast can be an effective warning signal alone (Prudic *et al.* 2007), our results show that luminance variation did not significantly explain differences in the survival of artificial prey. As demonstrated previously, conspicuous signalling does not necessarily reduce attack rates in small prey (Niskanen & Mappes 2005; Mänd *et al.* 2007). It could be that lack of mobility of the artificial prey impaired the perception of luminance by bird predators; however, levels of JND luminance of the two artificial prey phenotypes in

Experiment 2 against a banana leaf background were discriminable to the modelled bird vision system (i.e. both > 3.0) (see **Table A4.1**). One possibility is that the relatively small luminance differences amongst artificial prey did not reach the threshold at which birds can discern and respond in terms of different attack rates. This will require further experimentation. Luminance perception can be strongly affected by environmental light conditions (Osorio & Vorobyev 2005), especially in the tropical forest understory where gaps of light and shadows are common (Théry 2001). Therefore, the complex background environment of the forest floor may have rendered birds unable to discern differences in luminance, or it at least was not a reliable cue to be used in discrimination. It should also be noted that in complex habitats other factors can interact to influence the perception of prey, e.g. distance, shadows and countershading (Tullberg *et al.* 2005; Rowland *et al.* 2008); this requires further study. Another possibility is that selection imposed by birds at our study site on signal luminance is weak (see Ojala et al. 2007). Colour signals should be more stable under variable environmental conditions (Osorio & Vorobyev 2005); therefore, birds make use luminance contrast for initial detection while post-detection assessment of aposematic signals requires colour contrast. Birds seem to show innate wariness towards conspicuous colours that are generally associated with aposematic species (Schuler & Roper 1992; Lindström *et al.* 1999a; Exnerová *et al.* 2007). Thus, empirical studies have demonstrated that birds in particular seem to select against variation in signal colour and pattern in poison frogs in the wild (Noonan & Comeault 2009; Chouteau & Angers 2011; Hegna, Saporito,

& Donnelly 2012), although these studies did not specifically test for variation in luminance contrast while the colour of the signal was kept constant.

4.5.3. Conclusions

Size-dependent predation risk may impose selection pressures on anti-predator strategies employed during non-defended life stages in aposematic species. For example, it could be beneficial to remain small if size correlates positively with detectability (Higginson & Ruxton 2009), in particular where predators are naïve with respect to prey defences. However, we found that the smallest prey had the lowest survival prospects. It therefore seems likely that there could be a trade-off between time spent foraging and the risk of predation in juveniles of diurnal aposematic species such as *D. auratus*. In this species attaining larger body size at metamorphosis may facilitate the acquisition of toxic defences thereafter via foraging, because larger individuals may have a higher aerobic capacity (Santos & Cannatella 2011). It would be interesting to observe how investment in aposematic signalling may change as a functionally significant level of secondary defences is achieved during post-metamorphic development. Less conspicuous but more toxic juveniles would likely have reduced encounter rates with predators, but in the event of an attack they are more likely to survive (Leimar *et al.* 1986; Speed & Ruxton 2007).

Despite the relatively small differences in luminance amongst artificial prey used in this study, the failure to detect a significant effect of luminance on predation rates could be due to low statistical power. Future experimental designs may consider using a larger sample size and/or a longer elapsed time to measure predation rates in the wild.

In conclusion our results indicate that variation in body size as consequence of early nutrition (see Flores et al. 2013) has consequences for survival. The results highlight a potential selection pressure imposed by bird predators against small size in juvenile *D. auratus* at metamorphosis completion. Contrary to our expectations it seems that achieving larger body size at metamorphosis confers a selective advantage, possibly because birds associate larger body size with a likelihood of the presence of chemical defence (Maan & Cummings 2012). Nevertheless, since conspicuous appearance alone is not sufficient to confer complete protection against predators (Endler & Mappes 2004; Mappes *et al.* 2005), large juveniles may in principle face increased inspection and 'handling' by predators (Riipi *et al.* 2001; Mänd *et al.* 2007). This raises the possibility that larger post-metamorphic froglets in natural populations have, in fact, attained higher levels of secondary defences, a hypothesis that needs to be further explored.

4.6. Appendices: Supplementary Methods and Results

4.6.1. Modelling predator vision and artificial prey conspicuousness

Calculations were done based on a set of functions in Matlab R2009a (The MathWorks Inc, USA). In birds colour perception stems from the comparison of the relative stimulation of the different single cones sensitive to ultraviolet (UV), short (SW), medium (MW) and long (LW) wavelengths with opponent colour channels (Kelber *et al.* 2003). Meanwhile luminance sensitivity (achromatic) appears to be based on the stimulation of the double cone photoreceptors (Kelber *et al.* 2003; Osorio & Vorobyev 2005). We used the blue tit (*Cyanistes caeruleus*), which has an ultraviolet shifted ultrashortwave cone type, as a tetrachromatic visual model to calculate predicted photon catches for the different cone types. For luminance, we used an extension of the model using double cones (Siddiqi *et al.* 2004). The model starts by calculating the cone quantum catches (*qi*) for each photoreceptor class for froglets or clay and ambient radiance spectra as (**Equation 3.1**):

$$qi_{bird} = \int_{\lambda=300}^{750} R(\lambda) I(\lambda) A_i(\lambda) d\lambda,$$

here $A_i(\lambda)$ represents the absorptance spectrum for each of the four photoreceptor cone classes of the bird integrated over 1 nm intervals from 300 to 750 nm $R(\lambda)$ and $I(\lambda)$ represent the reflectance spectrum of the froglet´s skin or clay and the irradiance spectra measured in the field respectively. Resulting photon quantum catches were standardized in order to account for variation in light conditions, using the von Kries transformation adaptation coefficient. This method assumes that photoreceptors adjust their sensitivity in proportion to the background light environment (**Equation 3.2**):

$$Ki_{bird} = \left(\int_{\lambda=300}^{750} A_i(\lambda) I(\lambda) d\lambda \right)^{-1},$$

This procedure ensures that colour perception relies on colour constancy, whereby the visual system removes variation in ambient light so that colours look similar under variable light conditions (Cuthill 2006). Therefore, the adjusted quantum catch data for each photoreceptor class was calculated as (**Equation 3.3**):

$$q_a = Ki_{bird}\, x\, qi_{bird}$$

Following calculations of quantum catches, the model assumes that the signal of each cone channel is proportional to the logarithm of the adjusted quantum catches; as such the contrast between a pair of stimuli was calculated as the quotient of adjusted photon quantum catches (**Equation 3.4**):

$$\Delta q_a = log \frac{[q_{a1}(frog\ or\ clay\ spectra)]}{[q_{a2}(background\ spectra)]}$$

The Vorobyev-Osorio colour discrimination model is based on evidence that colour discrimination is determined by noise arising in the photoreceptors and is independent of light intensity. Noise in each photoreceptor channel (e_i) was calculated as (**Equation 3.5**):

$$e_i = \frac{\omega_i}{\sqrt{n_i}}$$

where ω_i was taken as 0.05 and represents the Weber fraction of the most abundant cone type (Siddiqi *et al.* 2004) and n_i is the relative number of receptor types in the retina of the blue tit (Hart *et al.* 2000) ($n_L = 1.00$, $n_M = 0.99$, $n_S = 0.71$, $n_{UV} = 0.37, n_D = 1.00$). Colour (chromatic) discrimination in the

tetrachromatic visual model was calculated as JND values using the following equation (**Equation 3.6**):

$$JND\ bird\ color = \sqrt{\frac{(e_{UV}e_S)^2(\Delta q_L - \Delta q_M)^2 + (e_{UV}e_M)^2(\Delta q_L - \Delta q_S)^2 + (e_{UV}e_L)^2(\Delta q_M - \Delta q_S)^2 + (e_S e_M)^2(\Delta q_L - \Delta q_{UV})^2 + (e_S e_L)^2(\Delta q_M - \Delta q_{UV})^2 + (e_M e_L)^2(\Delta q_S - \Delta q_{UV})^2}{(e_{UV}e_S e_M)^2 + (e_{UV}e_S e_L)^2 + (e_{UV}e_M e_L)^2 + (e_S e_M e_L)^2}}^2$$

Since in our model, overall perceived luminance is considered to arise from stimulation of double cone photoreceptors, luminance discrimination was evaluated as (**Equation 3.7**):

$$JND\ bird\ luminance = \left(\frac{\Delta q_D}{e_D}\right)$$

Colour and luminance discrimination (conspicuousness) was based on JND values.

Table A4.1. JNDs of artificial prey from Experiment 1 (effect of body size) and Experiment 2 (effect of signal luminance) against banana leaf background. JNDs were calculated as the discrimination between two spectral stimuli following the V-O model (see **4.7 Appendices** for details of vision model). Values are mean ± SE.

	N	JND luminance	JND colour
		Experiment 1	
	10	4.51 ± 0.60	12.28 ± 0.68
		Experiment 2	
LL	5	3.49 ± 0.10	8.50 ± 0.17
LH	3	7.81 ± 0.16	7.84 ± 0.28

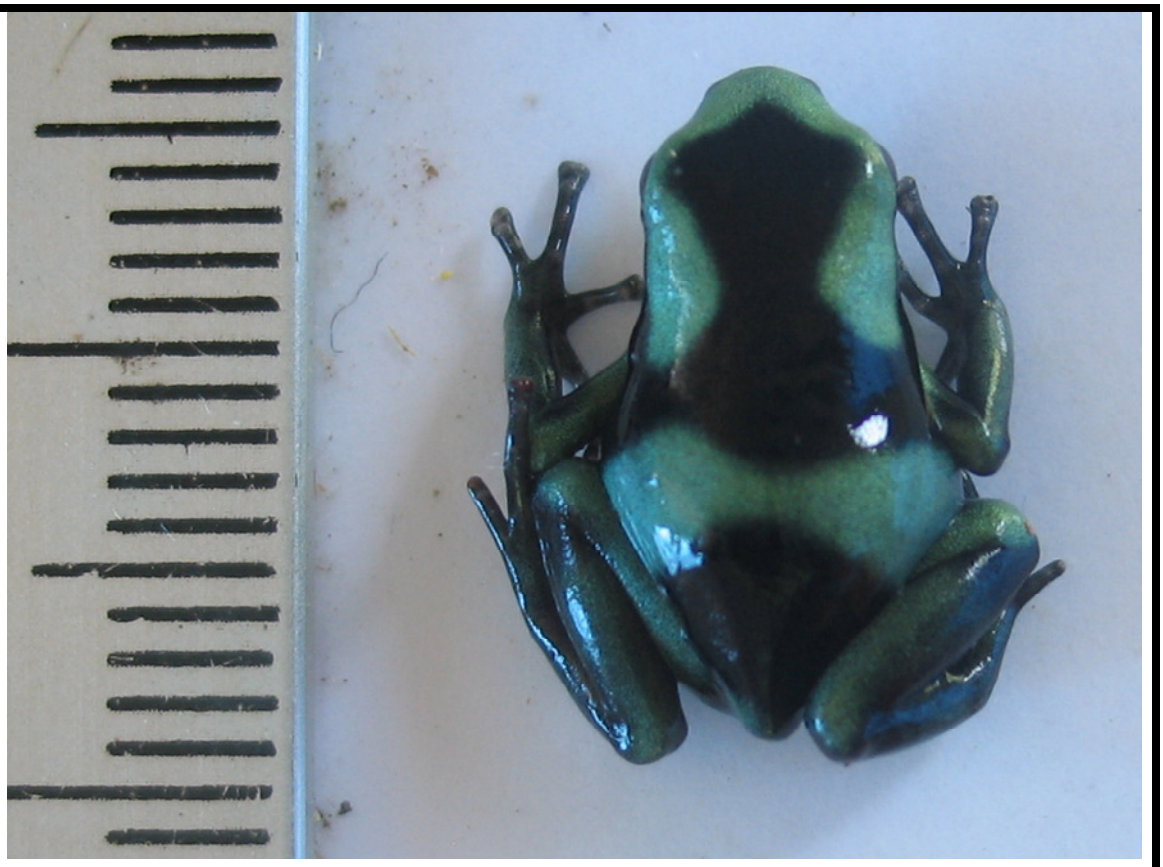

Figure A4.1 Dorsal view of the metamorphic juvenile used to design the contour and black dorsal pattern of 3D artificial prey. Each division in the scale represent 1 mm.

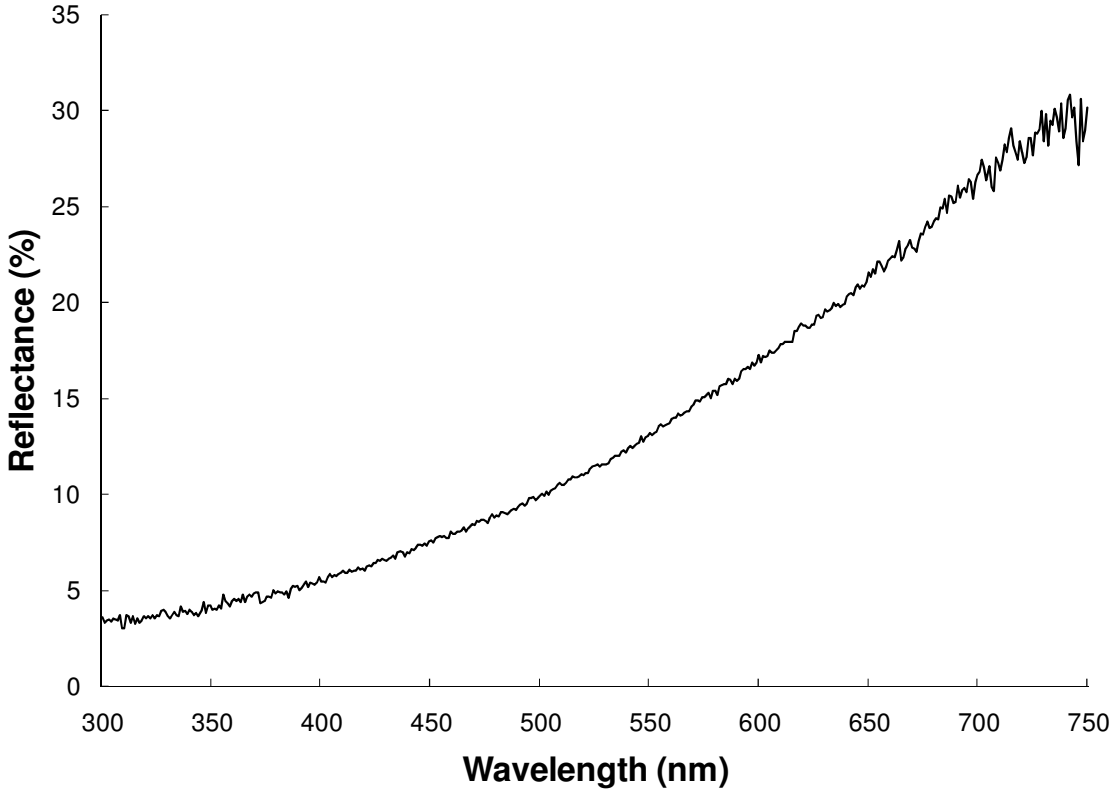

Figure A4.2 Mean reflectance spectra of banana leaves (sample $N = 12$) used as natural substrates for the artificial prey.

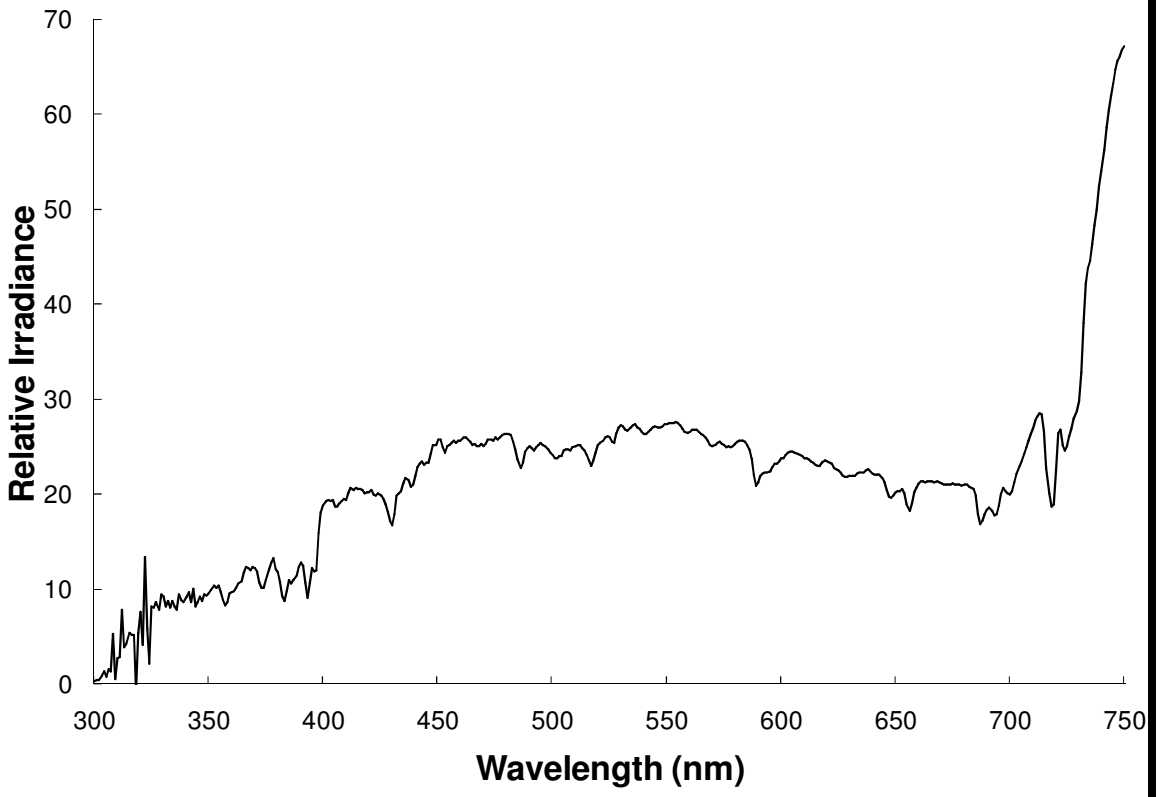

Figure A4.3 Mean irradiance spectra of ambient light at the study site. Values are those measured during fieldwork in 2010.

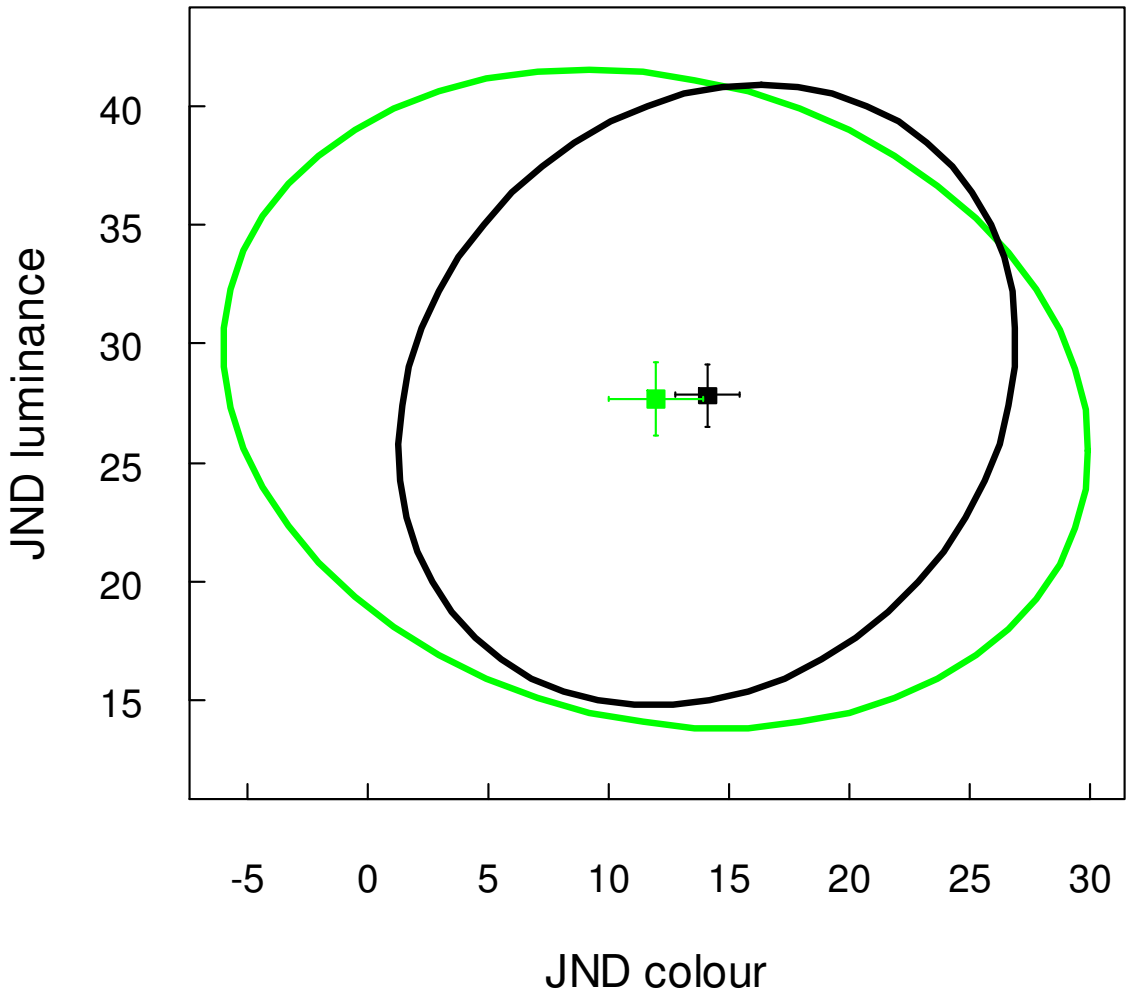

Figure A4.4 Comparison of overall conspicuousness of the black pattern painted on artificial prey (N = 12) and those of randomly selected froglets (N = 10) against a banana leaf substrate as viewed by a bird predator. The x-axis is JND colour contrast, and the y-axis JND luminance contrast. Ellipses show 95% confidence interval for artificial (black line) and experimental froglets (green line). Black and green squares are the mean ± SE for artificial prey and experimental froglets from Flores et al. (2013).

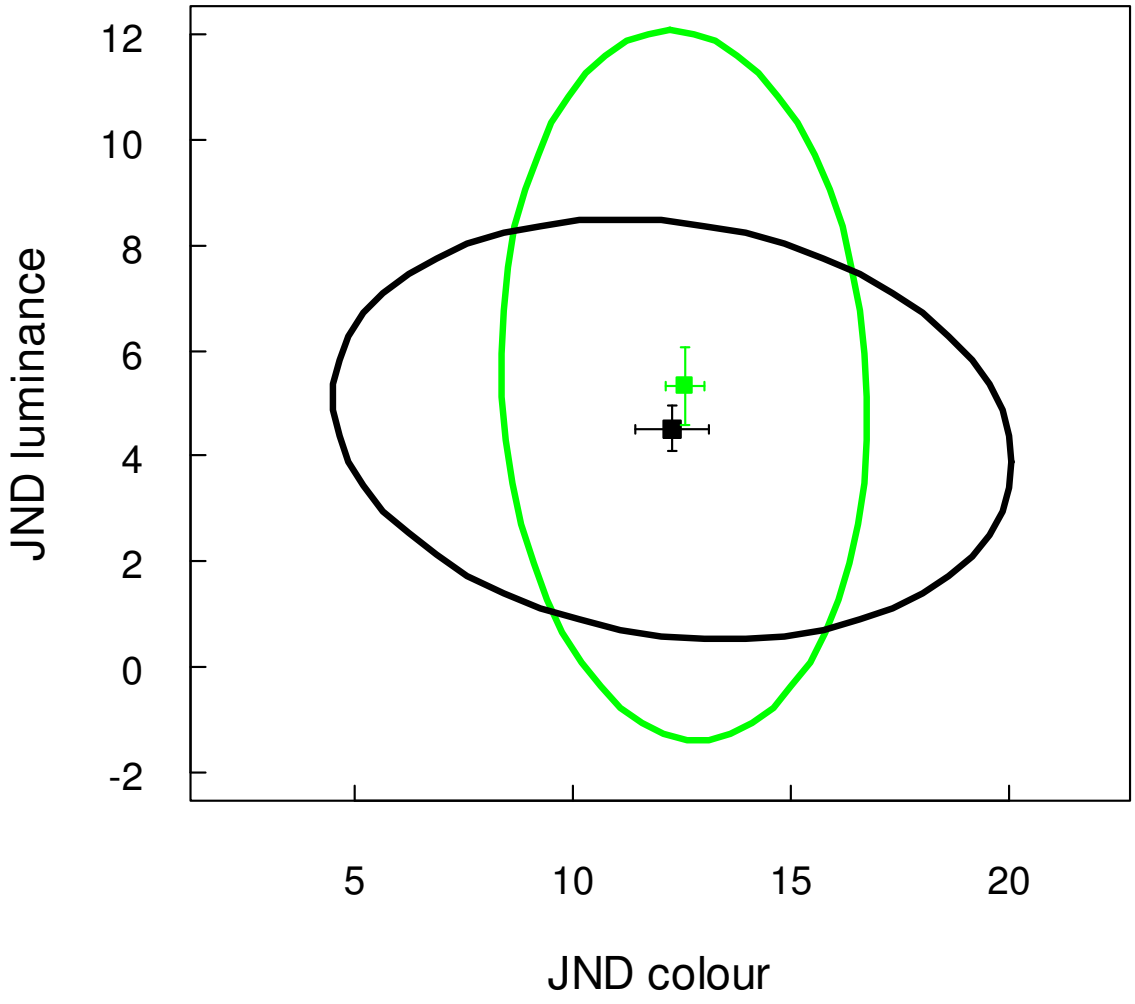

Figure A4.5 Comparison of overall conspicuousness of the mixed clay of artificial prey (Experiment 1) (N = 10) and those of randomly selected froglets (N = 10) against the banana leaves substrate as viewed by a bird predator. The x-axis is JND colour contrast, and the y-axis JND luminance contrast. Ellipses show 95% confidence interval for mixed clay (black line) and experimental froglets (green line). Black and green squares are the mean ± SE for mixed clay of artificial prey (Experiment 1) and experimental froglets from Flores et al. (2013).

Chapter 5. Relationships amongst aposematic signals, foraging capacity and toxic defences: behavioural observations of juveniles of a poison frog in the wild

5.1. Abstract

For immature aposematic individuals that have weak or absent chemical defences there might be a trade-off between time spent exposed while foraging and survival following encounters with predators. Phenotypic traits such as body size and the expression of aposematic signals might be expected to modulate the risk of attack by predators. Indeed, experiments using artificial prey juveniles of the green and black poison frog (*Dendrobates auratus*) has shown that smaller individuals suffer higher rates of attack by birds. *D. auratus* develops its chemical defences through the post-metamorphic diet, which suggests that larger, relatively well-fed juveniles are chemically better defended. I investigated this possibility in *D. auratus* in the wild. I found that aposematic colouration was independent of body size, while both feeding rates and levels of chemical defence were correlated with body size. Expression of aposematic signals and levels of chemical defences were not correlated. These results suggest that larger post-metamorphic froglets which have greater foraging efficiency may be at a selective advantage conferred by their superior levels of chemical defences.

5.2. Introduction

Evolution of the aposematic syndrome allows species to exploit a novel niche, as for example in the shift from nocturnal to diurnal patterns of activity (Santos *et al.* 2003; Merilaita & Tullberg 2005). Here, aposematic signals help to reduce predation risk during the acquisition of food (Speed *et al.* 2010). In anurans, for example, those species that have foraging widely often possess aposematic colouration and/or toxic defences, while more sedentary species lack chemical defences and behave cryptically (Pough & Taigen 1990). Nevertheless foraging behaviour can be risky if the link between conspicuous warning colours and toxic defences is decoupled. For example, during early stages of life aposematic species may lack secondary defences (e.g. Daly *et al.* 1994a; Nylin *et al.* 2001; Saporito *et al.* 2010) and their colourful external appearance may be incompletely developed (Grant 2007; Tullberg *et al.* 2008). Where toxic defences come from dietary sources (Speed et al. 2012) there must be a premium on efficient foraging in order to sequester secondary defences quickly, which may entail a trade-off between foraging and survival following encounters with predators. This trade-off may constrain the time animals devote to obtaining food (Kotler & Blaustein 1995). This is of critical importance because if metabolic demands are not properly met during early growth, animals may face fitness consequences at maturity (Metcalfe & Monaghan 2003).

The days following metamorphosis can be highly risky for immature individuals of aposematic species, especially if they lack secondary defences

(e.g. Roznik & Johnson 2009). Those individuals that achieve a larger body size at metamorphosis will likely experience higher survival rates in the wild (Berven 1990), because body size may correlate with physiological condition and hence foraging performance (Pough & Kamel 1984). Indeed, I have shown that larger post-metamorphic green and black poison frogs *Dendrobates auratus* have lower rates of attack by birds in an artificial prey experiment (**Chapter 4**). One possible explanation for this result is that predators associate larger body size with greater levels of diet-derived chemical defence. This seems possible because juvenile poison frogs are known to sequester chemical defences from dietary alkaloid-rich arthropods, mainly mites and ants (Donnelly 1991). However, whether larger juvenile *D. auratus* are better defended has not been studied before. An association between body size and levels of chemical defences is not inevitable. Diversity and concentration of alkaloids in *D. auratus* are widely variable among individuals (Daly, Myers, & Whittaker 1987; Daly *et al.* 1994a). This variability may be a consequence of variation in the abundance and kind of arthropods, individual differences in alkaloid sequestration ability (Saporito *et al.* 2006, 2007a, 2012), as well as individual variation in foraging.

Another potential reason why attack rates on relatively large juveniles are low (**Chapter 4**), is that the effect of warning signals on predator behaviour is enhanced by large body size (Mänd *et al.* 2007; Lindstedt *et al.* 2008). That is, a larger aposematic signals cause stronger stimulation of the perceptual system in visual-hunting predators making prey less acceptable (e.g. Mappes & Alatalo 1997; Riipi *et al.* 2001; Nilsson & Forsman 2003).

Warning signals are expected to be relatively invariable within species in order to facilitate predator learning and aversion (Beatty *et al.* 2004; Sherratt 2008). Nevertheless, it is becoming increasingly apparent that there is often considerable variation in warning signals, which has been shown to correlate with levels of chemical defences within and across aposematic species (Darst *et al.* 2006; Bezzerides *et al.* 2007; Cortesi & Cheney 2010; Wang 2011; Maan & Cummings 2012). A recent resource competition model has predicted levels of warning signal expression and chemical defence to be positively correlated within species (i.e. 'honest' signalling) when nutritional resources are limiting. On the other hand, where resources are abundant it should pay for individuals to maximise toxin levels while reducing investment in signalling (Blount *et al.* 2009; Lee *et al.* 2011). This is because signalling itself carries a conspicuousness cost, and highly defended individuals are likely to survive any attack unharmed (Blount *et al.* 2009; Lee *et al.* 2011). However, these 'resource competition models' ignore the fact that many aposematic species juveniles have incompletely developed defences while being coloured. We have found that individuals with a relatively high food supply which had large body size reduced their investment in the luminance component of warning signals (**Chapter 3**). Therefore, it is clear that development of aposematic signals is sensitive to variation in early nutrition (Grill 1999; Ojala *et al.* 2007; Blount *et al.* 2012). However, how warning signals and levels of chemical defences correlate in juveniles is unclear.

In the present study I conducted behavioural observations of foraging activity and exposure time of immature *D. auratus* in the wild, and related this to

body size, warning signalling and levels of chemical defences. Based on the results of earlier work (**Chapters 3 & 4)** I predicted that: (1) larger individuals will have reduced luminance contrast compared to smaller individuals; (2) larger juveniles will have higher levels of chemical defences and a greater foraging rate compared to smaller juveniles; and (3) larger, more toxic juveniles will spend more time exposed on the forest floor. I also examined whether there was any evidence of 'honest' (or 'dishonest') signalling in this sample of wild juveniles.

5.3. Methods

At the study site in Veraguas Province, Panama (**Figure 1.1**), 30 juveniles of *D. auratus* were monitored between 07:30 - 11:00 h, in the period August to October 2011. Juveniles were identified visually but also verifying that their body size had SVL less than the minimum size of a random sample of adults in the population (juveniles: n = 30, SVL = 16.05 ± 0.59 mm (range: 10.11 – 22.63 mm); adults: n = 30, SVL = 34.28 ± 0.41 mm (range: 29.91 – 37.62 mm)). Once a juvenile was discovered in the field it was observed from a distance to avoid disturbance (≥ 2 m) for exactly 1 h, using binoculars when needed. During this time feeding attempts (snapping behaviour) were counted using a tally counter. Snapping behaviour is generally used in anurans, including dendrobatids, as an indicator of prey capture (Jaeger, Hailman, & Jaeger 1976; Whitfield & Donnelly 2006; Meuche, Linsenmair, & Pröhl 2011). The time each animal spent visible

during the period (as opposed to the time it was in a burrow, under leaves or under cover) (see Jaeger and Hailman 1981) was also recorded using a chronometer. After observation the juveniles were captured and carefully transferred to the field lab for other measurements.

5.3.2. Morphometrics

At the field lab juveniles were weighed to the nearest 0.001g using an Ohaus Scout Pro balance (Ohaus Europe GmbH, Switzerland). The dorsum of each individual was digitally photographed under standardized conditions with a Canon Power Shot G6 (7.1 megapixel) camera (Canon Inc. Japan). A metal ruler was included to provide a scale for the image. Snout-vent length (SVL) was measured in each individual image using ImageJ 1.43q (Rasband 1997).

5.3.3. Analyses of aposematic signals and background spectra

Spectral reflectance $R(\lambda)$ of the skin of each juvenile was measured in triplicate soon after capture in four body regions: head, dorsum, and right and left flanks, using a Jaz spectrometer (Ocean Optics Inc. FL, USA) with a bifurcated 400 µm UV/VIS fibber optic probe connected to an internal Jaz PX pulsed short arc xenon lamp (Ocean Optics Inc. FL, USA) at an angle of 45° and corrected for

lamp drift using a white diffuse spectral standard (WS-1) (Maan & Cummings 2008) and then averaged for subsequent analyses (**Figure A5.1**). Additionally, spectral reflectance of leaf substrate samples (n= 3 per juvenile) collected along the foraging path of each focal juvenile was measured in triplicate and averaged following the same methodology described above (**Figure A5.2**). As a measure of ambient light irradiance $I(\lambda)$ data from 175 locations in the field (see **Chapter 3**) were collected using a cosine corrected irradiance probe (CC-3-UV-T) with 180° field view, connected to an USB2000 spectrometer by means of a 400µm UV/VIS optic fibre following the method described in Endler (1993) (**Figure A5.3**). In all cases spectral reflectance data between 300 - 750 nm were employed and averaged to 1 nm intervals prior to analyses.

5.3.4. Modelling predator vision and conspicuousness

Juveniles of *D. auratus* are active during the day, and therefore it is very likely that they may encounter visual predators during their foraging time. To characterize the conspicuousness of each juvenile a passerine bird vision model as a putative predator was employed (see **Chapter 3 § 3.3.5**). I used the tetrachromatic version of the Vorobyev-Osorio visual model of colour discrimination (Vorobyev & Osorio 1998), which assumes that noise in the photoreceptors limits discrimination. Discrimination (conspicuousness) values are JNDs (just noticeable differences), with a value of 1 being the threshold for discrimination, and values of between 1 and 3 generally considered to mean that

two objects can only be discriminated under ideal viewing conditions (rarely the case in the field). Calculations were done based on a set of functions in Matlab R2009a (The MathWorks Inc, USA).

In birds colour perception stems from the comparison of the relative stimulation of the different single cones sensitive to ultraviolet (UV), short (SW), medium (MW) and long (LW) wavelengths with opponent colour channels (Kelber *et al.* 2003). Meanwhile luminance sensitivity (achromatic) appears to be based on the stimulation of the double cone photoreceptors (Kelber *et al.* 2003; Osorio & Vorobyev 2005). Since the only bird documented to prey upon poison frogs, the Rufous Motmot (*Baryphthengus martii*), is a close relative of higher passerine birds (Livezey & Zusi 2007), the cone sensitivities of the passerine blue tit (*Cyanistes caeruleus*), which has an ultraviolet shifted ultrashortwave cone type, were used as a tetrachromatic visual model to calculate predicted photon catches for the different cone types (absorbance spectrum templates and oil droplet data from Hart *et al.* 2000). For luminance an extension of the model using double cones was used (Siddiqi *et al.* 2004). The model starts by calculating the cone quantum catches (*qi*) for each photoreceptor class for the juvenile and ambient radiance spectra as (**Equation 3.1**):

$$qi_{bird} = \int_{\lambda=300}^{750} R(\lambda)I(\lambda)A_{\mathrm{i}}(\lambda)d\lambda,$$

here $A_i(\lambda)$ represents the absorptance spectrum for each of the four photoreceptor cone classes of the bird integrated over 1 nm intervals from 300 to 750 nm. $R(\lambda)$ and $I(\lambda)$ represent the reflectance spectrum of the juvenile´s skin and the irradiance spectra measured in the field, respectively. Resulting photon quantum catches were standardized in order to account for variation in light conditions, using the von Kries transformation adaptation coefficient. This method assumes that photoreceptors adjust their sensitivity in proportion to the background light environment (**Equation 3.2**):

$$Ki_{bird} = \left(\int_{\lambda=300}^{750} A_i(\lambda) I(\lambda) d\lambda \right)^{-1},$$

This procedure ensures that colour perception relies on colour constancy, whereby the visual system removes variation in ambient light so that colours look similar under variable light conditions (Cuthill 2006). Therefore, the adjusted quantum catch data for each photoreceptor class was calculated as (**Equation 3.3**):

$$q_a = Ki_{bird}\, x\, qi_{bird},$$

Values of relative single cone quantum catches were then included in a Principal Component Analysis (PCA) (see **5.3.7 Statistical analyses**).

Following calculations of quantum catches, the model assumes that the signal of each cone channel is proportional to the logarithm of the adjusted quantum catches; as such the contrast between a pair of stimuli was calculated as the quotient of adjusted photon quantum catches (**Equation 3.4**):

$$\Delta q_a = log \frac{[q_{a1}(juvenile\ spectra)]}{[q_{a2}(background\ spectra)]}$$

The Vorobyev-Osorio colour discrimination model is based on evidence that colour discrimination is determined by noise arising in the photoreceptors and is independent of light intensity. Noise in each photoreceptor channel (e_i) was calculated as (**Equation 3.5**):

$$e_i = \frac{\omega_i}{\sqrt{n_i}}$$

where ω_i was taken as 0.05 and represents the Weber fraction of the most abundant cone type and n_i is the relative number of receptor types in the retina of the blue tit (Hart *et al.* 2000) ($n_L = 1.00$, $n_M = 0.99$, $n_S = 0.71$, $n_{UV} =$

$0.37, n_D = 1.00$). Colour (chromatic) discrimination in the tetrachromatic visual model was calculated as JND values using the following equation (**Equation 3.6**):

$$JND\ bird\ color = \sqrt{\frac{(e_{UV}e_S)^2(\Delta q_L - \Delta q_M)^2 + (e_{UV}e_M)^2(\Delta q_L - \Delta q_S)^2 + (e_{UV}e_L)^2(\Delta q_M - \Delta q_S)^2 + (e_S e_M)^2(\Delta q_L - \Delta q_{UV})^2 + (e_S e_L)^2(\Delta q_M - \Delta q_{UV})^2 + (e_M e_L)^2(\Delta q_S - \Delta q_{UV})^2}{(e_{UV}e_S e_M)^2 + (e_{UV}e_S e_L)^2 + (e_{UV}e_M e_L)^2 + (e_S e_M e_L)^2}}^2$$

Because overall perceived luminance is considered to arise from stimulation of double cone photoreceptors in our model, luminance discrimination was evaluated as (**Equation 3.7**):

$$JND\ bird\ luminance = \left(\frac{\Delta q_D}{e_D}\right)$$

Overall variation in colour was based on values from single cone photon catch scores (and a Principal Component Analysis of them) and variation in overall luminance was based on double cone photon catch scores (Δq_D). Colour and luminance discrimination (conspicuousness) were based on JND values.

5.3.5. Alkaloid extraction

On the day of capture each juvenile was euthanized by stepped hypothermia and then skinned. Skins were stored in 2 ml polypropylene screw cap vials at -80℃ until biochemical analyses. The protocol for extraction and analysis of alkaloid compounds followed that of Saporito et al. (2006) with some modifications. Individual skin samples were weighed and macerated x 3, each time 1:10 (w/v) with methanol (MeOH). The extract was diluted 1:1 (v/v) with distilled water. The final solution was extracted x 3, each time 1:1 (v/v) with chloroform ($CHCl_3$) vortexed for 20 s and then centrifuged at 1600 x g for 3 min at 4℃. To eliminate excess water anhydrous sodium sulphate (Na_2SO_4) from the combined extract was added in amount sufficient to allow formation of clumps that were then retained during filtration using Whatman cellulose filter paper (GE Healthcare Co., USA). The final extract was evaporated to dryness in a vacuum drier and then redissolved by adding 10 ml of n-hexane. The hexane-chloroform layer was extracted x 3, each time with 2 ml of 0.1N hydrochloric acid (HCl) vortexed for 20 s and centrifuged as described before, then the hexane layer (containing neutral materials such as fatty acid esters and other lipids) was carefully removed. The acid layer was adjusted to pH 9.0 with 5*M* potassium hydroxide (KOH) and then extracted x 3, each time with 3 ml of chloroform. Na_2SO_4 was added to the combined extract to remove excess water, which was filtered and evaporated in a vacuum as described before to yield the alkaloid

fractions. The concentrated alkaloid fraction was dissolved in methanol, vortexed for 20 s, so that 50 µl of this fraction corresponded to 50 mg of wet skin tissue.

5.3.6. Alkaloid identification and quantification

The alkaloid fractions were characterized by gas chromatography in combination with mass spectrometry (GC-MS). GC-MS analysis was performed on an Agilent 7890A GC system (Agilent Technologies, UK Limited) with an Agilent J&W capillary 30 m x 0.25 µm DH-5 (5% Phenyl)-methylpolysiloxane phase fused silica column (Agilent Technologies, UK Limited) connected to an Agilent 7000 triple quadrupole mass spectrometer with 70-eV electron impact ionization. The working temperature program of the oven during the analysis was 80°C held for 2 min and then 10°C min^{-1} up to 320°C and held for 1 min, over a total of 27 min, using helium as carrier gas at a constant flow rate of 1.2 ml min^{-1}. The sample of alkaloid fractions (2 µl) was injected into the column using the pulsed splitless mode. Mass spectra were obtained in scan mode from 40 to 600 amu. Resulting mass spectra were surveyed with AMDIS (Stein 1999) and confirmation of individual alkaloid peaks was done with the aid of the NIST MS Search 2.0 library and retention times and relative ion abundance compared with those data published for anuran alkaloids ((Daly, Spande, & Garraffo 2005). Alkaloids were quantified in relation to a calibration curve constructed by using known concentrations of a standard of the alkaloid decahydroquinoline (DHQ) 195A

(Sigma-Aldrich Co. Ltd., UK), and obtained under the same chromatographic conditions. DHQ is an alkaloid found in populations of *D. auratus* in Panama (Daly et al. 2000). Chemical defences were calculated as: total concentration of alkaloids (µg) per 50 mg of skin; total number of alkaloids; and total number of alkaloid classes. Eleven classes were identified: Izidine, 3,5-Disubstituted indolizidine, 5,8-Disubstituted indolizidine, Pyrrolizidine, Allopumiliotoxin, Decahydroquinoline, Histrionicotoxin, Pumiliotoxin, Pyrrolidine, Tryciclic and Unclassified. In addition, alkaloid class diversity was calculated using a Shannon diversity index (H) (Shannon & Weaver 1949) as:

$$H = -\sum_{i=1}^{n} pi \ln pi$$

Equation 5.1 Shannon – Weaver diversity index.

where n represents the total number of alkaloid classes and p_i is the proportion relative to n of a certain alkaloid i. The H index is a good indicator of diversity and represents here the evenness and abundance of the different alkaloid classes in each juvenile´s skin.

5.3.7. Statistical analyses

Analyses were conducted using R v.2.12.1. (R Development Core Team 2010). We conducted a PCA using a varimax rotation on the relative photon catches of single cones, as this removes absolute variation that would otherwise result in the first principal component corresponding to overall variation in photon catch values (i.e. brightness variation) (Endler & Mielke 2005). The first principal component explained 66% of the variance of single cones and was associated with variation in MW and LW versus SW and UV wavelengths, so this was used to represent sensitivity of colour perception (hereafter PC_{COL}). Factor loadings were: -0.511 and -0.526, for MW and LW and 0.413 and 0.539, for UV and SW, respectively.

We conducted Pearson´s correlations to test relationships amongst variables. To meet parametric assumptions feeding rate, alkaloid concentration and number of alkaloids were log transformed prior to analysis. General linear models (GLMs) were employed to test for the interactive effects of SVL and warning signals (signal luminance, PC_{COL}, JND luminance and JND colour contrasts) on levels of chemical defences. Non-linear relationships were modelled by including a quadratic term into the model to test hypothesis based on the resources competition model of Blount *et al.* (2009). $P < 0.05$ was considered statistically significant , and backward simplification was conducted on full models by continuous deletion of non-significant terms resulting in the most parsimonious model (Crawley 2007). All values reported in the Results are predicted means (± SE), unless otherwise indicated.

5.4. Results

5.4.1. Do larger juveniles have reduced signal luminance?

Body size was not related to signal luminance (Pearson´s correlation: $r = 0.18$, $n = 30$, $P = 0.32$), JND luminance contrast ($r = -0.05$, $n = 30$, $P = 0.77$), signal colour (PC_{COL}) ($r = -0.25$, $n = 30$, $P = 0.18$), or JND colour contrast ($r = 0.23$, $n = 30$, $P = 0.21$). Since the relationship between body size and signal expression may be non-linear we also fitted quadratic models, but did not find any significant relationships between body size and signal luminance (GLM: SVL, $F_{1,28} = 1.00$, $P = 0.32$; SVL^2, $F_{1,27} = 2.99$, $P = 0.09$), signal colour (PC_{COL}) (GLM: SVL, $F_{1,28} = 1.90$, $P = 0.18$; SVL^2, $F_{1,27} = 0.02$, $P = 0.89$), JND luminance contrast (GLM: SVL, $F_{1,28} = 0.08$, $P = 0.77$; SVL^2, $F_{1,27} = 0.12$, $P = 0.73$), or JND colour contrast (SVL, $F_{1,28} = 1.65$, $P = 0.21$; SVL^2, $F_{1,27} = 0.21$, $P = 0.65$).

5.4.2. Do larger juveniles have higher levels of chemical defences and greater feeding rates?

Body size was positively correlated with total alkaloid concentration ($r = 0.57$, $n = 30$, $P < 0.001$; **Figure 5.1a**), number of alkaloids ($r = 0.75$, $n = 30$, $P < 0.001$,

Figure 5.1b), alkaloid diversity (r = 0.70, n = 30, P < 0.001, **Figure 5.1c**) and feeding rate (r = 0.49, n = 30, P = 0.005). Feeding rate was not itself significantly correlated with alkaloid concentration (r = 0.21, n = 30, P = 0.27). However, feeding rate was positively correlated with number of alkaloids (r = 0.39, n = 30, P = 0.03) and alkaloid diversity (r = 0.37, n = 30, P = 0.04).

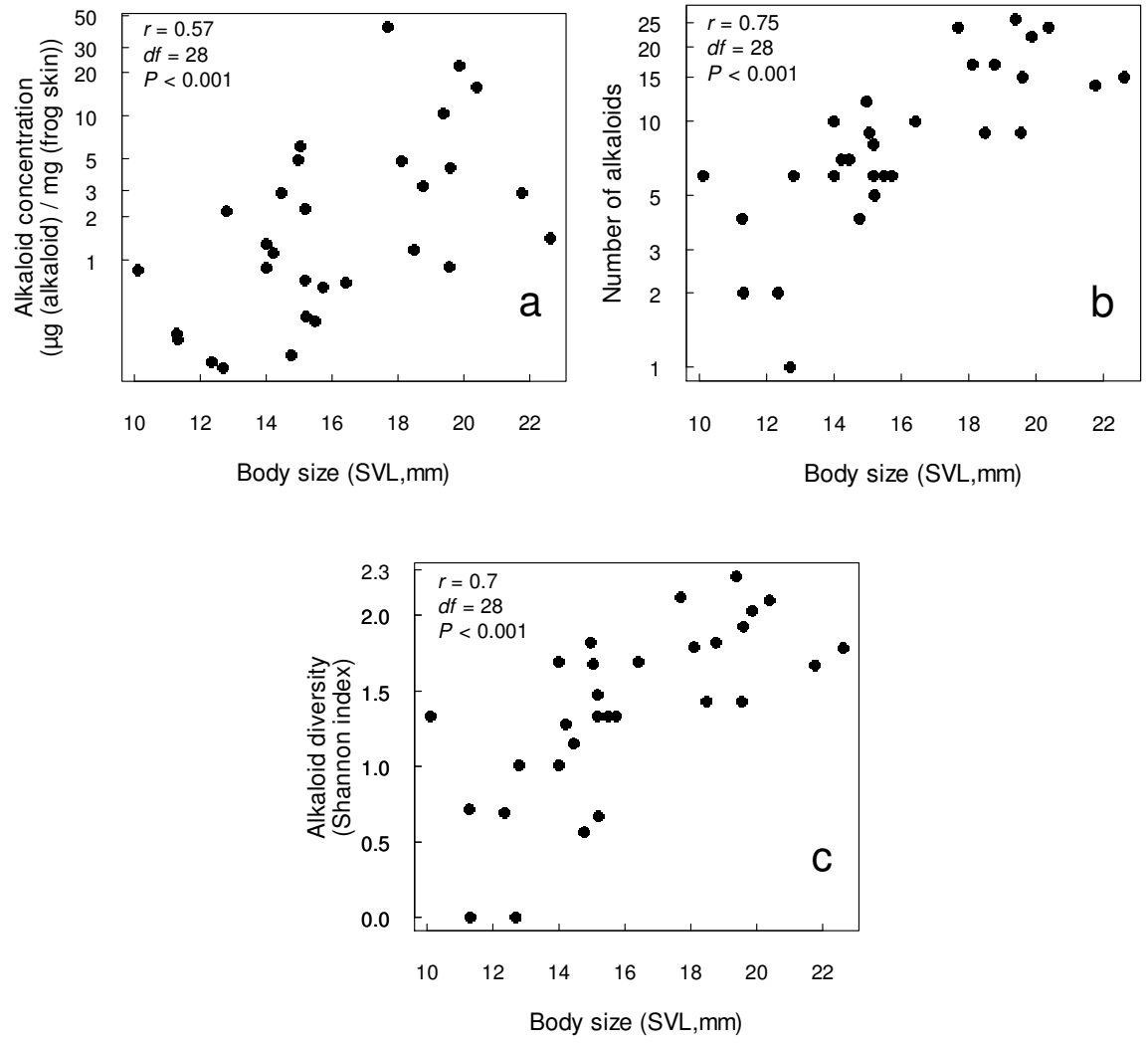

Figure 5.1 Chemical defences: (**a**) alkaloid concentration, (**b**) number of alkaloids and (**c**) diversity of alkaloids, were associated with body size (SVL); all P < 0.05.

5.4.3. Do larger and more chemically defended juveniles spend more time exposed?

On average juveniles spent 82 % (43.05 ± 2.46 min, range: 20.75 – 60 min) of their time exposed on the forest floor. Nevertheless, time spent exposed was not significantly correlated with body size (r = 0.23, n = 30, P = 0.22), alkaloid concentration (r = 0.14, n = 30, P = 0.46), alkaloid number (r = 0.20, n = 30, P = 0.28), alkaloid diversity (r = 0.15, n = 30, P = 0.41) or feeding rate (r = 0.03, n = 30, P = 0.86). Similarly, time spent exposed was not significantly correlated with signal colour (r = 0.28, n = 30, P = 0.13), signal luminance (r = -0.22, n = 30, P = 0.25), JND luminance contrast (r = -0.13, n = 30, P = 0.48), or JND colour contrast (r = -0.19, n = 30, P = 0.31).

5.4.4. Do juveniles signal honestly?

I assessed whether body size affected the correlation between chemical defences and warning signals using a GLM including body size as a covariate. In all cases the interaction term between signal expression and body size, and the main effect of signal expression, was not a significant predictor of chemical defence (**Table 5.1**).

Table 5.1. Relationship between warning signals and body size and levels of chemical defences.

	Source of variation	*df*	*F*	*P*
A) Alkaloid concentration				
	SVL	1,28	13.78	**< 0.001**
	PCcol	1,27	0.00	0.98
	SVL *x* PCcol	1,26	1.55	0.22
	SVL	1,28	13.78	**< 0.001**
	luminance	1,27	0.30	0.60
	SVL *x* luminance	1,26	0.21	0.65
	SVL	1,28	13.78	**< 0.001**
	JND luminance	1,27	0.20	0.66
	SVL *x* JND luminance	1,26	0.29	0.59
	SVL	1,28	13.78	**< 0.001**
	JND colour	1,27	0.00	0.95
	SVL *x* JND colour	1,26	0.13	0.72
B) Number of alkaloids				

SVL	1,28	37.91	**< 0.001**
PCcol	1,27	0.35	0.56
SVL *x* PCcol	1,26	1.51	0.23
SVL	1,28	37.91	**< 0.001**
luminance	1,27	0.60	0.44
SVL *x* luminance	1,26	0.67	0.42
SVL	1,28	37.91	**< 0.001**
JND luminance	1,27	0.00	0.94
SVL *x* JND luminance	1,26	0.12	0.73
SVL	1,28	37.91	**< 0.001**
JND colour	1,27	0.21	0.65
SVL *x* JND colour	1,26	0.04	0.84
C) Alkaloid diversity			
SVL	1,28	27.09	**< 0.001**
PCcol	1,27	0.45	0.51
SVL *x* PCcol	1,26	0.68	0.42
SVL	1,28	27.09	**< 0.001**

luminance	1,27	0.35	0.56
SVL *x* luminance	1,26	1.24	0.28
SVL	1,28	27.09	**< 0.001**
JND luminance	1,27	0.04	0.84
SVL *x* JND luminance	1,26	0.83	0.37
SVL	1,28	27.09	**< 0.001**
JND colour	1,27	0.56	0.46
SVL *x* JND colour	1,26	0.03	0.86

Note. General Linear Models of the effect of warning signals and body size on levels of chemical defences, boldface indicates significant value. Alkaloid concentration and number of alkaloids were log transformed to meet parametric assumptions. Signal expression and their interaction with body size (SVL) were not significant predictors of alkaloid concentration or alkaloid diversity in juveniles; all $P > 0.05$.

5.5. Discussion

Body size of juvenile *D. auratus* is positively correlated with feeding rate and levels of chemical defence. Surprisingly, there was no other relationship between body size and the expression of warning signals or between body size or warning signal and time spent exposed on the forest floor. Finally, warning signalling is independent of chemical defence.

As predicted body size was positively correlated with levels of chemical defence. This finding supports the suggestion that larger post-metamorphic froglets are at a selective advantage, because bird predators associate large body size with greater secondary defences (**Chapter 4).** Nevertheless during immature stages of life aposematic animals may have relatively weak secondary defences compared to adults (Daly *et al.* 2002; Nishida 2002). The diurnal and active behaviour of juveniles of *D. auratus* may expose them to predators such as spiders (see Summers 1999a; Santos & Cannatella 2011 supporting information) that do not necessarily rely on colour vision when hunting (Szelistowski 1985; Orlando & Schmid 2011). Such encounters can result in traumatic injuries (Gray, Ouellet, & Green 2002). Although these traumatic injuries may not be the result of predatory attempts, they may have fitness consequences (Gray & Christy 2000). It has been demonstrated that the presence of alkaloids in the skin of adult *D. auratus*, rather than their conspicuous colouration, is responsible for the survival of individuals following attack by the tarantula spider *Sericopelma rubronitens* (Gray *et al.* 2010) and by the garter snake *Thamnophis sirtailis* (Brodie & Tumbarello 1978). Similarly, ants and wandering spiders seem to be deterred by the chemical defences of the strawberry poison frog *Oophaga* [*Dendrobates*] *pumilio* (Fritz, Rand, & DePamphilis 1981; Szelistowski 1985). Therefore, there must be a premium on the rapid acquisition of chemical defences in juvenile poison frogs, to confer protection against both visual- and non-visual hunting predators.

Larger juveniles also had more individual types of alkaloids and a higher diversity of alkaloid classes than smaller juveniles. In dendrobatids ontogenetic

changes in feeding behaviour associated for example with body size, and intra- and inter-specific competition for food, can affect the amount and types of prey consumed (Lima & Magnusson 1998, 2000), which in turn may result in selective sequestration of alkaloids (Saporito *et al.* 2012). Variation in the profile of secondary defences may have no adaptive significance if all chemical compounds are equally toxic (Pasteels, Grégoire, & Rowell-Rahier 1983). However, this seems unlikely, at least in poison frogs. The vast majority of skin samples of juveniles had alkaloids belonging to the Izidine, Decahydroquinolines (DHQ), and Pumiliotoxin (PTX) classes (unpublished data). Pyrrolizidine alkaloids belong to the class Izidine and in general are effective against invertebrate predators like spiders and ants (Brown 1984; Eisner & Eisner 1991; Hare & Eisner 1993), but may be ineffective against birds (Yosef, Carrel, & Eisner 1996). Juveniles of *D. auratus* raised in captivity and fed on leaf-litter arthropods had Pyrrolizidines as the major alkaloid in their skins; however wild-caught adults showed minor or trace levels of this alkaloid (Daly *et al.* 1994a). DHQs have been shown to have low toxicity in vertebrates mainly via blocking of neuronal receptors (Daly, Garraffo, & Spande 1999). PTXs are highly toxic alkaloids which interrupt sodium-channel activity in vertebrates (Daly *et al.* 2005). Indeed *D. auratus* is able to metabolically transform PTX 251D from dietary sources, into the potentially more toxic compound Allopumiliotoxin (Daly *et al.* 2003). I lack evidence of the relative effectiveness of specific alkaloids against predators, but a possible explanation for high levels of individual variability in alkaloid profiles is that a complex mixture of components acts synergistically, or alternatively, possession of a large number of different compounds confers

better defence against a diverse array of predators (Ruxton *et al.* 2004; Skelhorn & Rowe 2005). By acquiring a more diverse array of alkaloids, juvenile aposematic organisms may enhance avoidance learning, especially if predators taste the prey before ingestion (Brower 1984; Nishida 2002). For example, birds are known to handle aposematic animals with care and to associate distastefulness with toxicity (Darst & Cummings 2006; Skelhorn & Rowe 2010).

Body size was positively correlated with feeding rate. Similarly, in poison frogs of the genus *Mantella*, body size (SVL) has been shown to be positively correlated with the amount of prey consumed (Clark *et al.* 2006). It is probable that larger (perhaps older) juveniles are more familiar with the environment, and consequently more efficient at detecting and capturing prey than smaller individuals (Donnelly 1991). In poison frogs a high capacity for aerobic exercise required during active foraging (Taigen & Pough 1983; Pough & Taigen 1990) has been linked to the evolution of fast metabolic rates (Santos 2012), which in turn would be balanced by high energy gain from increased acquisition of food (Jaeger & Barnard 1981). Consequently larger juveniles would be able to move greater distances and spend more time searching and capturing alkaloid-rich arthropods, especially if they are not evenly distributed in the environment (Pough & Taigen 1990). Diet specialization, in particular of alkaloid-rich arthropods, may require the ingestion of large quantities of prey items because of their low nutritional value (Huey & Pianka 1981). In addition, the increased acquisition of toxic alkaloids would demand higher energy intake for transport, storage and maintenance of secretory apparatus and detoxification mechanisms (Matsui *et al.* 2000; Dobler 2001; Angel *et al.* 2003). In instances when alkaloid

sources fluctuate temporarily and seasonally (Bower 1992; Saporito *et al.* 2006) individuals might have limited access to secondary defences, in which case selection will favour larger juveniles as they will be better competitors when searching for food.

Feeding rate was correlated with the number and diversity of alkaloids, but not with total alkaloid concentrations. This may reflect the high variability in alkaloid content of dietary sources (Bower 1992). Food acquisition reflects the necessity to meet metabolic demands during growth (Killen, Brown, & Gamperl 2007) in addition to sequestration of chemical defences. It may also be relevant that the exact age of the observed juveniles was unknown, given that feeding behaviour may change during the course of development. In aposematic animals the individual profile of diet-derived secondary defences may reflect genetic differences, spatial and temporal variation in prey availability, and variation in predation risk (reviewed in Speed *et al.* 2012).

It was previously found that larger, better nourished post-metamorphic *D. auratus* had reduced signal luminance (**Chapter 3**). In contrast, in the present study of wild juveniles there was no significant relationship between body size and signal luminance. This discrepancy cannot simply be attributed to a difference in the size range of juveniles in the two studies. Body size did not differ significantly between the wild juveniles (range: 10.11 – 22.63 mm) and the high-food experimental group of post-metamorphic froglets described in the earlier work (**Chapter 3**) (range: 14.20 – 18.68 mm) (Two sample t-test: t_{56} = 0.30, P = 0.38). Similarly, body size did not differ significantly between the

observed juveniles and the low-food group described in **Chapter 3** (range: 13.79 – 16.97 mm) (Two sample t-test: $t_{62} = -1.23$, $P = 0.89$). Therefore, the size variation of wild juveniles spanned that of the experimental froglets in the earlier study. Possibly, the discrepancy exists because the sample of wild juveniles spanned a relatively wide variation in ages, and colouration may change across seasons (Nylin *et al.* 2001; Tullberg *et al.* 2008), with ontogeny (Hoffman & Blouin 2000; Grant 2007), and in relation to variation in dietary intake of pigments (Fox 1976; Nijhout 1991; Bezzerides *et al.* 2007). This requires further study.

Contrary to my expectations levels of chemical defences and warning signals did not affect time the juveniles spent exposed on the forest floor. This could be because the need to feed in juveniles is so strong that it outweighs the risk of predation. Furthermore, there may be a learning component to the propensity to take risks by spending time exposed, which can only be gained through interactions with predators during development.

Larger juveniles had higher levels of chemical defences, but this was not related to warning signalling. Previous studies have found positive correlations (e.g. Summers & Clough 2001; Bezzerides *et al.* 2007; Cortesi & Cheney 2010) or negative correlations (e.g. Darst *et al.* 2006; Wang 2011) between these two aposematic traits within- or across species. The lack of any significant relationships between warning signalling and levels of chemical defences may reflect a high degree of heterogeneity in the chemical content of dietary sources, as previously reported in aposematic species (Bower 1992; Saporito *et al.* 2009).

Furthermore, it is likely that levels of chemical defences are lower in juveniles than adults (Daly *et al.* 1992, 2002), while it is also apparent that warning signal expression differs amongst age classes. Specifically, JND luminance contrast of the wild juveniles in this study (n = 30, JND = 26.57 ± 2.24 (mean ± SE)) was higher than that of a random sample of adult frogs from the same population (n = 30; 15 females and 15 males; JND = 12.76 ± 1.15) (GLM; $F_{1,58} = 29.98$, $P < 0.001$). JND colour contrast, on the other hand, did not differ significantly between the wild juveniles and adults (GLM; $F_{1,58} = 2.21$, $P = 0.14$).

Interestingly, it has recently been reported that brighter adult poison frogs (*Oophaga* [*Dendrobates*] *pumilio*) are more toxic, i.e. there is honest signalling based on brightness variation (Maan & Cummings 2012).We do not know whether brighter adult *D. auratus* are also more toxic, but an intriguing possibility is that, by having high levels of brightness on average, juvenile *D. auratus* are in effect automimics of the most toxic adults in the population. However, it also has been shown that polymorphic batesian poison frog mimics tend to mimic the less toxic model when they co-occur in sympatry with multiple potential models in a population (Darst & Cummings 2006).This warrants further study.

5.5.1. Conclusions

Aposematic appearance must ultimately allow prey to acquire food despite the risk of being detected by predators. I have shown that large body size in juvenile

D. auratus was related to higher feeding rates and greater accumulation of chemical defences. This finding supports the results from **Chapter 4,** in which larger individuals were found to be less likely to suffer attacks by bird predators.

5.6. Appendices: Supplementary information

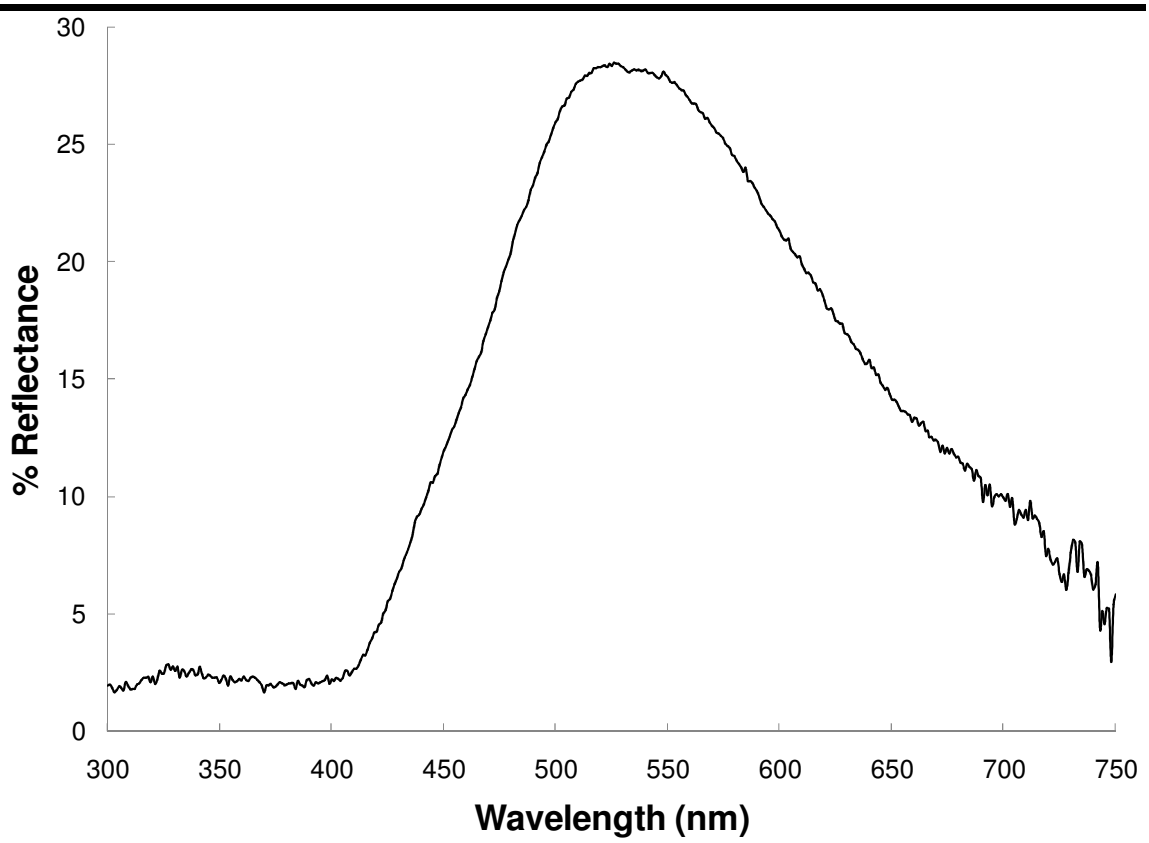

Figure A5.1 Mean reflectance spectra of the dorsal skin of juveniles observed in the field during the experiment (n = 30). Reflectance spectra are average measurements taken from four body regions (head, dorsum, left and right flanks).

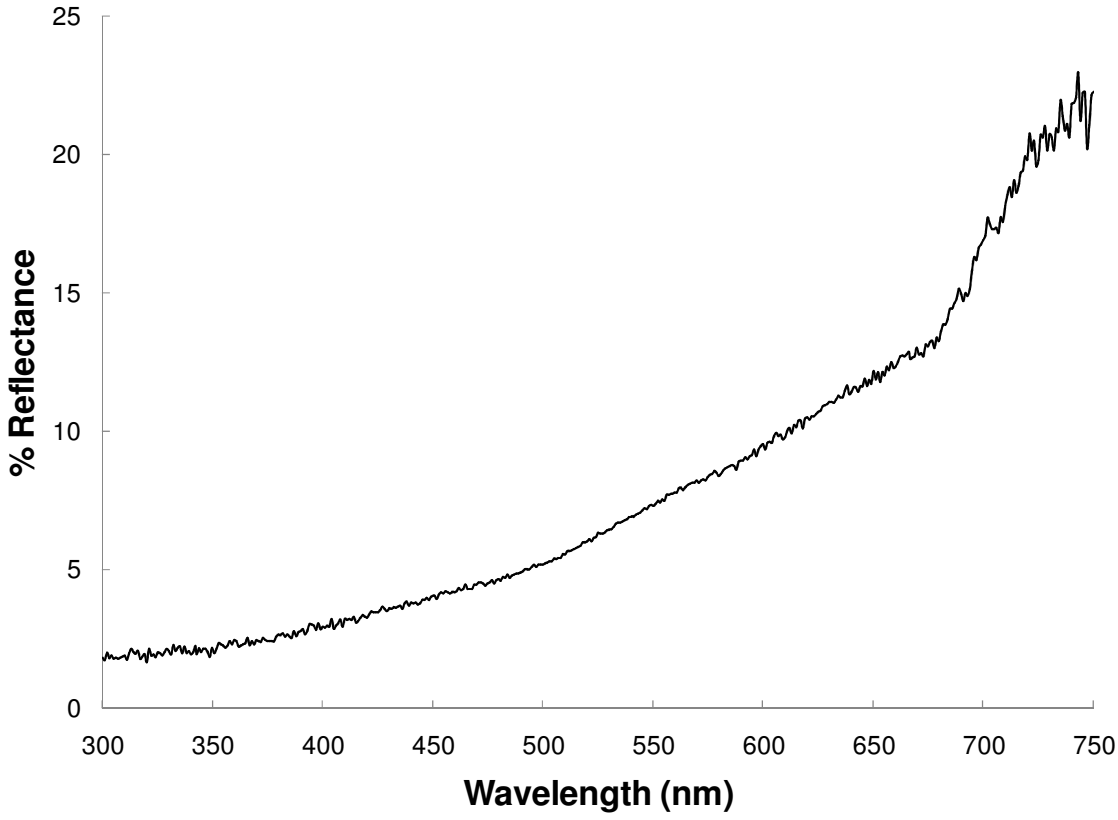

Figure A5.2 Mean reflectance spectra of natural background samples (n = 90). Three samples were collected along the foraging path of each observed juveniles at the study site.

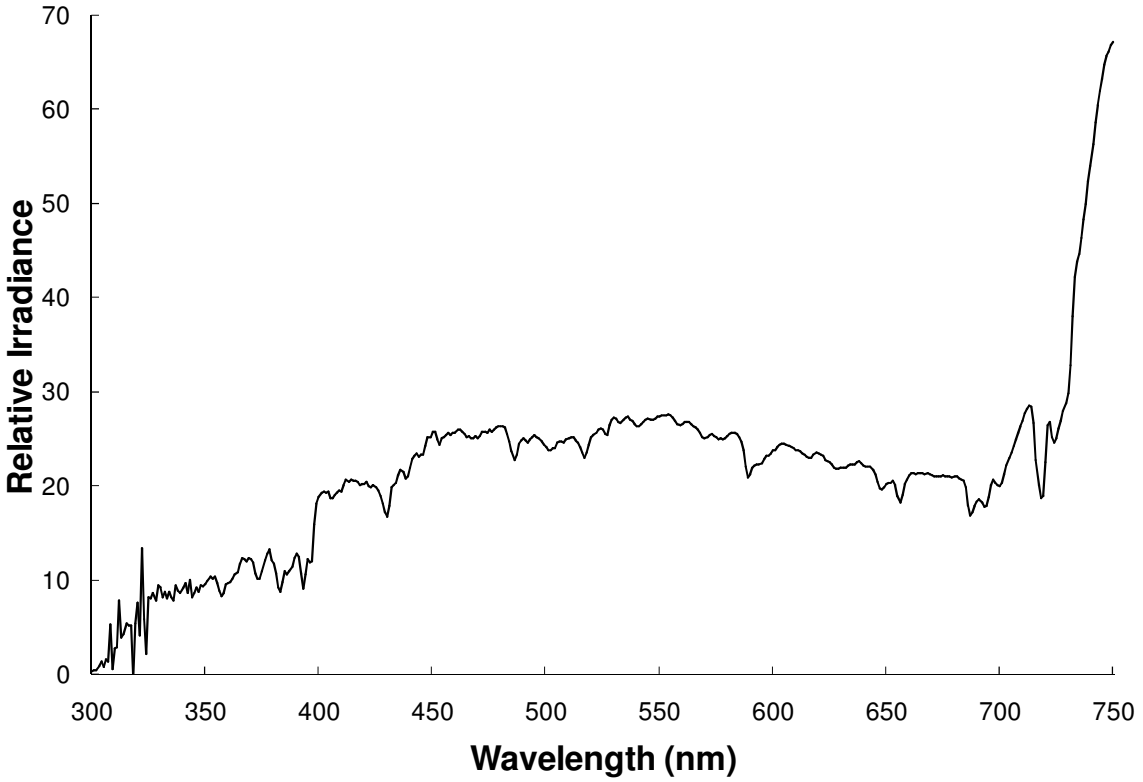

Figure A5.3 Mean irradiance spectra of ambient light at the study site (n = 90 measurements on a sunny day and n = 85 measurements on a cloudy day). Values are those measured during fieldwork in 2010 (see **Chapter 3**).

Chapter 6. General discussion

Aposematism was first defined more than a century ago (Wallace 1867), yet, today the causes and consequences of variation in external appearance of aposematic species are still topics of debate and research (Guilford & Dawkins 1993; Gamberale & Tullberg 1998; Lindström 1999b; Endler & Mappes 2004). Here I have described an ontogenetic change in egg and larvae pigmentation in *D. auratus* for the first time (**Chapter 2**) and examined the effects of environmental conditions (i.e. variation in food supply) during early development on investment in growth versus aposematic signal expression (**Chapter 3**) and the fitness consequences of such variation in terms of survival (predation risk) in the wild (**Chapter 4**). Finally, correlations among aposematic traits were explored jointly with the concept of honesty in immature *D. auratus* in the wild (**Chapter 5**). In this section I summarise the key findings of this thesis to examine the consequences of variation in signal design in terms of selection and propose additional explanations and new ideas for further study.

6.1. Phenotypic plasticity of aposematic traits early in life

Plasticity in traits that are associated with aposematism fuels adaptation to different biotic and abiotic conditions (e.g. Lindstedt *et al*. 2009; Mochida 2011; Michie *et al*. 2011; Rodríguez *et al*. 2012). As such these plastic phenotypes can be subject to selection by means of natural selection, sexual selection, or both

(e.g. Rudh *et al*. 2007; Reynolds & Fitzpatrick 2007; Brown *et al*. 2010; Santos 2012). Variance in integument colouration could have fitness consequences in aposematic species, not least because unfamiliar novel phenotypes in an aposematic population can result in biased predation on that unfamiliar phenotype (Mappes *et al.* 2005; Noonan & Comeault 2009; Wennersten & Forsman 2009). This is likely to be most problematic for immature stages when aposematism is not yet fully developed (Nylin *et al.* 2001; Grant 2007; Saporito *et al.* 2010). An example of atypical coloration is found in unusually pale eggs and tadpoles seen in some dendrobatid species, *Ranitomeya* [*Dendrobates*] *vanzolinii* and *Ranitomeya* [*Dendrobates*] *flavovitatta* (Brown *et al.* 2011) and *D. auratus* (Flores, Moore, & Blount 2012). Further study will be needed to examine whether this colour is associated with greater or lower predation rates. Taxonomy of the family *Dendrobatidae* has been the subject of continuous debate in regard to trait polymorphisms (Grant *et al.* 2006; Santos *et al.* 2009; Brown *et al.* 2011), such as oocyte and larval pigmentation, that are used to identify species (Grant *et al.* 2006; Köhler 2011). In **Chapter 2** we proposed hypotheses that may explain such variation and its adaptive significance. Adding to that discussion in particular, white pigmentation of eggs causes strong contrast (Cott 1940) against the leaf litter background where *D. auratus* eggs are normally deposited (Wells 1978), making them easier to detect. While it seems unlikely that this white colour is aposematic, we cannot entirely rule out this possibility because of the potential that maternally-derived toxins are transferred to the eggs. The aposematic bufonid *Atelopus chiriquiensis* lays pale yellow eggs in which mothers deposit highly toxic alkaloids (Pavelka *et al.* 1977),

perhaps as protective strategy against parasites or predators. This maternal allocation of initial secondary defence may be a plastic adaptation to seasonal or spatial patterns of predation and pathogenic risk. Although no chemical defence in eggs or tadpoles of *D. auratus* has been reported, maternal allocation of alkaloids in trophic eggs that are fed to tadpoles in *Oophaga* [*Dendrobates*] *pumilio* (Stynoski 2012) suggests that this could in theory also be found in other poison frogs. Perhaps females sequester more chemical defences during the breeding season to transfer defence to their eggs (Kellner & Dettner 1995). If the conspicuous appearance of pale eggs and larvae is accompanied by secondary defence and is shared among individuals in the population, reinforcing avoidance and reluctance to attack by predators, then this may lead to an evolutionary stable strategy (Leimar *et al.* 1986). These ideas are speculative and would require detailed examination.

While several causes might have produced abnormal pigmentation of the eggs (McGraw 2005; Griffith *et al.* 2006; McGraw 2008; Galván & Alonso-Alvarez 2009, 2010), and diet may influence pigmentation of immature stages (see **Chapter 1 § 1.2**), the immature phenotype at metamorphic completion is typical of adult external colour and pattern (**Chapters 2 & 3**). The mechanisms for this ontogenetic change may include the up-regulation of hormones and the activation of specific genes at more advanced stages of development (Hayes and Menendez 1999, Lee et al 2009). Nonetheless, integument colour and pattern at metamorphic completion seem to be under strong stabilising selection (**Chapters 2 & 3**). Colour and pattern are two important components of aposematic signals (Ruxton *et al.* 2004), that have been shown to have heritable,

plastic variation in response to environmental factors (e.g. Liebert & Brakefieldtt 1990; Hazel 2002; Sword 2002; Ojala, Lindström, & Mappes 2007). In **Chapter 3,** signal luminance was affected by early nutrition. Design of dorsal patterning is known to vary among populations of *D. auratus* (Lötters *et al.* 2007), but its effectiveness in enhancing predator aversion or reducing detection via disruption of the body contour (Stevens 2007) remains unknown.

Visually-oriented predators use luminance contrast (perceived level of brightness) as an initial detection mechanism of small targets (Osorio & Vorobyev 2005), but also as a reliable signal of the toxicity of prey (Prudic *et al.* 2007; Maan & Cummings 2012). The possibly strategic reduction in signal luminance contrast observed in larger post-metamorphic individuals (**Chapter 3**), in fact is apparently unimportant for selection (**Chapter 4**). This could be because information conveyed by signal luminance alone is less reliable than colour contrast for bird predators at this early stage. On the other hand, if predation pressure is relaxed in relation to this component of the aposematic signal (Endler & Mappes 2004), any variation in luminance contrast will be less important than variation in colour contrast, because colour makes individuals distinguishable from cryptic species (Holloway, Gilbert, & Brandt 2002; Sherratt & Beatty 2003; Rowe *et al.* 2004) resulting in weak selection for luminance contrast.

6.2. Honesty and automimicry of the aposematic signal

In the wild, larval stages of poison frogs may experience food limitation due for example to kin competition, and poor characteristics of breeding sites (Caldwell & de Araújo 1998, 2004; Summers & McKeon 2004). In a diet manipulation experiment we found that under limited food supply froglets maximised their investment in both body size and luminance contrast within the limits of what they could attain, resulting in a positive correlation between these traits (**Chapter 3**). This investment in warning signalling was associated with reduced body levels of non-enzymatic antioxidants. Such a reduction in antioxidant defence might be expected to have deleterious consequences for the capacity to store alkaloids post-metamorphosis. In poison frogs, alkaloids are harboured in glands located in the same skin tissue where antioxidant pigment granules, responsible for imparting integument colour, are also deposited (Angel *et al.* 2003; Saporito *et al.* 2010). It is likely that antioxidant pigments are required to reduce the somatic damage incurred through the accumulation of chemical defences (Ahmad 1992). Therefore, there is a possibility that investment in signalling impairs the increasing sequestration of chemical defences in those brighter juveniles after metamorphosis, although this requires explicit study. Nevertheless, the physiological pathways involving specific molecules in trade-offs between resource allocation to warning signalling and chemical defences, are as yet unknown in poison frogs (Maan & Cummings 2012). In addition, we cannot rule out the possibility that levels of food supply experienced by high-food *D. auratus* tadpoles (**Chapter 3**) were beyond the conditions which exist in nature. If so, it would be difficult to assess

the adaptive significance of the observed reduction in signal luminance contrast in larger, high-food individuals.

In this study I did not find evidence for or against honest signalling in immature individuals of *D. auratus*. Although variation in chemical defences correlated positively with body size in juveniles, there were no significant correlations between expression of aposematic signals and body size or chemical defences (**Chapter 5**). This is at odds with empirical evidence showing honest signalling (Bezzerides *et al.* 2007; Maan & Cummings 2012; Blount *et al.* 2012) and/or dishonest signalling (Wang 2011; Blount *et al.* 2012) in aposematic species. Juveniles signalled relatively strongly in terms of luminance contrast compared to adults in the population. By being brighter on average compared to adults, one intriguing possibility is that juveniles are automimics of the better defended, brightest adults in the populations. This is on the assumption that adults signal honestly about the strength of chemical defences based on variation in luminance contrast, a possibility which warrants study.

Despite the fact that genetic (Hazel 2002; Holloway *et al.* 2002; Tullberg *et al.* 2008) and environmental factors (Grill 1999; Lindstedt *et al.* 2009; Blount *et al.* 2012) may affect expression of specific aposematic traits during early life; in juveniles there was no significant variation in colour compared to aposematic adults in the population (**Chapters 2 & 3**). Hence, it seems possible that immature individuals might exploit the protection gained from adult forms in the population without investing in secondary defences (Brower *et al.* 1967; Ruxton *et al.* 2004; Speed *et al.* 2006). In an interesting example, the reversion to a

normal-pigmented phenotype at metamorphosis (**Chapter 2**) may be an indication that the best strategy for juveniles on reaching the terrestrial stage is to bear some resemblance to conspicuously coloured adults in the population (Darst *et al.* 2006) instead of turning into a dull cryptically coloured phenotype. This plastic ontogenetic colour change would appear to exemplify an automimicry strategy, because after metamorphosis juveniles do not have secondary defences.

Nonetheless, mimicking the external appearance of a defended form may reduce the fitness of automodels (truly aposematic prey) due to sampling errors by predators (Mappes & Alatalo 1997b). Automimics are expected to be able to coexist in a population if predators exert negative-frequency dependent selection, reducing the fitness of automimics compared to automodels (Speed *et al.* 2006, 2012). A peak in frequency of immature *D. auratus* can be predicted after the breeding season, but at any given moment in time they will be less abundant than adults. But if juveniles gain enough protection from automimicry, why do they need to forage for toxic substances? Two main reasons for this can be mentioned: 1) predators can vary in their sensory abilities and visual systems, and may not necessarily rely on visual cues alone when hunting; therefore, there is a need to sequester toxic substances quickly during immature stages (**Chapter 5**); and 2) if unreliable signallers increase in frequency in the population then apostatic selection will result in deterioration of the signalling system (Sherratt & Beatty 2003; Franks, Ruxton, & Sherratt 2009). In any case predators are expected to pay a high cost if mistakenly taking a defended individual (Dawkins & Guilford 1991), and at any given time the frequency of

adults (automodels) in the population will be higher than juveniles (automimics), and hence the reliability of the signal is expected to be maintained while juveniles develop the same diet as adults.

6.3. Concluding remarks

Here it has been shown that early environmental conditions can lead to plastic changes in the aposematic traits of *D. auratus* (**Chapters 2 & 3**). Although, previous research has found that life history traits may trade against expression of aposematic signals during early development (Grill & Moore 1998; Ojala *et al.* 2007; Lindstedt *et al.* 2010), the evidence presented in this thesis supports for the first time the suggestion that resource allocation to warning signalling may be physiologically constrained by oxidative damage (**Chapter 3**) .

It seems likely that integument colouration is an important cue for signal reliability as assessed by birds and other less visual-oriented predators such as snakes. Although juveniles do not resemble adults exactly, predators probably generalise to avoid warningly coloured individuals (Gamberale-Stille & Tullberg 1999; Darst & Cummings 2006; Ihalainen *et al.* 2008; Aronsson & Gamberale-Stille 2012). Thus, the best strategy for predators could be to rely on body size as an index of likely toxin levels, and by doing so select against those smaller juveniles who are less capable of acquiring toxic defences efficiently (**Chapters 4 & 5**).

The lack of relationship between aposematic colouration and toxicity in juveniles not only reflects decoupling between these traits at immature stages, but suggests that the best strategy for survival to maturity may be to resemble the most toxic individuals in the population (i.e. automimicry) (**Chapter 5**), especially if variability of dietary toxins and/or genetic factors impair the adequate sequestration of toxins in juveniles (Daly *et al.* 2003; Saporito *et al.* 2012). Larger, more active and hence more conspicuous prey are likely to experience higher detection rates (Roberts *et al.* 2006; Mänd *et al.* 2007; Lindstedt *et al.* 2008), but the concominant risk of predation will be diminished by an enhanced deterrence signal in large individuals (Lindström *et al.* 1999b; Niskanen & Mappes 2005; Mänd *et al.* 2007; Higginson & Ruxton 2009), which have greater capacity to rapidly acquire toxic defences **(Chapter 5**; Peters 1983). Although ontogenetic colour change has been reported from immature to mature stages in species of poison frogs (Hoffman & Blouin 2000), for *D. auratus* acquiring large body size after metamorphosis and resemblance of adult external appearance seem to be key determinants for survival.

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