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Warning colouration predicts behavioural and morphological differences in the strawberry poison- dart frog



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Abstract

Behaviours allow animals to adaptively adjust to short-term changes in the environment in accordance with their own physiological and morphological state. Consequently behaviour of animals may in part influence what selective pressures they experience and therefore the direction of evolutionary changes. However, the role of behaviour in influencing the evolution of other traits is relatively unexplored. There are, for example, few studies of behavioural traits across populations experiencing different selective regimes. We investigated the relationship between two behavioural traits that are usually considered key traits in studies of animal personalities (intraspecific aggression and boldness to explore novel habitats), and two morphological traits known to influence fitness (aposematic colouration and body size), across eight populations of the poison-dart frog *Dendrobates pumilio*. The study populations were located in the Bocas del Toro archipelago region, northwest Panama, which is the centre of phenotypic diversity of this species. We predicted that the behaviours of more conspicuous aposematically coloured individuals would be less constrained by predation risks than less conspicuous individuals. Therefore, the more conspicuous individuals should be more aggressive and bolder as they have a stronger warning signal to potential predators than less conspicuous individuals. In addition, we predicted that these differences would be reinforced by body size, as larger bodies would send a stronger warning signal. We found that males belonging to the more conspicuously coloured populations were significantly more aggressive and bolder than those belonging to less conspicuous populations. Furthermore, males belonging to more conspicuous populations were significantly larger than less conspicuous males. Taken together our results suggest that co-evolution occurs between risk-taking behaviour, aposematic colouration and body size across multiple natural populations of *D.pumilio*. We suggest that adaptive behavioural adjustments reinforce rapid divergence in aposematic colouration across populations of the same species, which may in turn reflect incipient speciation.

Keywords: animal behaviour; warning colouration; co-adapted traits; poison-dart frogs

Cover photographs kindly provided by Andreas Rudh

Contents

Abstract	1
Contents	2
Introduction	3
Aims and hypothesis	6
Methods	7
Study species	7
Study area	7
Behavioural assay	8
Body size measurements	10
Conspicuousness measurements	10
Statistical methods	10
Results	12
Body size	12
Exploration trials	13
Aggression trials	14
Covariation of traits	15
Discussion	16
Acknowledgements	19
References	20

Introduction

Behaviour of individuals interacts with the environment in which they occur and their and genetic constitution (Duckworth, 2008), and thus can have ecological and evolutionary consequences. Aggression and exploration are two risk-taking behavioural traits that have been well studied in various taxa for many years. They have been found to have fitness implications, be heritable and, at the level of individuals, show consistencies across contexts (e.g. aggression: Stamps & Krishnan, 1997; Dugatkin, 2004; Duckworth, 2006; exploration: Archer, 1973; Dingemanse *et al.*, 2002; Dingemanse *et al.*, 2004; van Oers, *et al.*, 2004; Brown *et al.*, 2005; van Oers, *et al.*, 2005; Brown *et al.*, 2007; and for review: Drent *et al.*, 2003; Dingemanse & Réale, 2005; Duckworth & Badyaev, 2007; Réale *et al.*, 2007; Wolf *et al.*, 2007; Duckworth, 2008). For example, organisms that explore novel habitats more thoroughly may have better dispersal capabilities (see Réale *et al.*, 2007). More aggressive organisms may have better competitive abilities to secure food, territories and mates (Armitage, 1986; Dugatkin, 2004; Duckworth, 2006; Duckworth & Badyaev, 2007).

Although there has been some recent interest in studying “animal personalities” (*i.e.* suites of co-adapted behavioural traits) (Réale *et al.*, 2007; Wolf *et al.*, 2007), the co-evolution between behavioural and morphological and/ or physiological traits has received less empirical attention. This is unfortunate since behavioural adjustments may facilitate both survival in novel environments and genetic evolution in novel directions (e.g. Price *et al.* 2003). Studies of behavioural traits across populations of the same species which are experiencing different selective regimes, may provide important insights into early stages of the speciation process.

Speciation is sometimes referred to as an “adaptive peak shift” (e.g. Hadany, 2003). In general, specific combinations of phenotypic traits result in high fitness in a specific environment. When phenotypic traits are genetically linked they are referred to as coadaptive gene complexes (Wallace, 1968; Ohta, 1980; Maticola & Templeton, 1999). Alternatively, part of the presented phenotype (often a behavioural trait) may be a plastic response to a particular environment (Price *et al.*, 2003). A way to visualise combinations of traits is an adaptive landscape, or fitness landscape, where the fitness values of combinations of traits are represented (Figure 1). There can be single or multiple “peaks” across populations (Wright, 1932). Between these peaks are areas of low fitness that are not likely inherited, but there can be differing fitness levels between peaks leaving some more fit than others. Selection drives the genetic linkage of these traits, by way of genetic assimilation (Waddington, 1961), where selection initially favours extreme phenotypes and, once differentiated, crossing of two different gene complexes, or a mutational change to the complexes, may lead to reduced fitness (Price *et al.*, 2003). These maladapted genotypes are selected against, maintaining combinations of traits that maximise fitness (Price *et al.*, 2003).

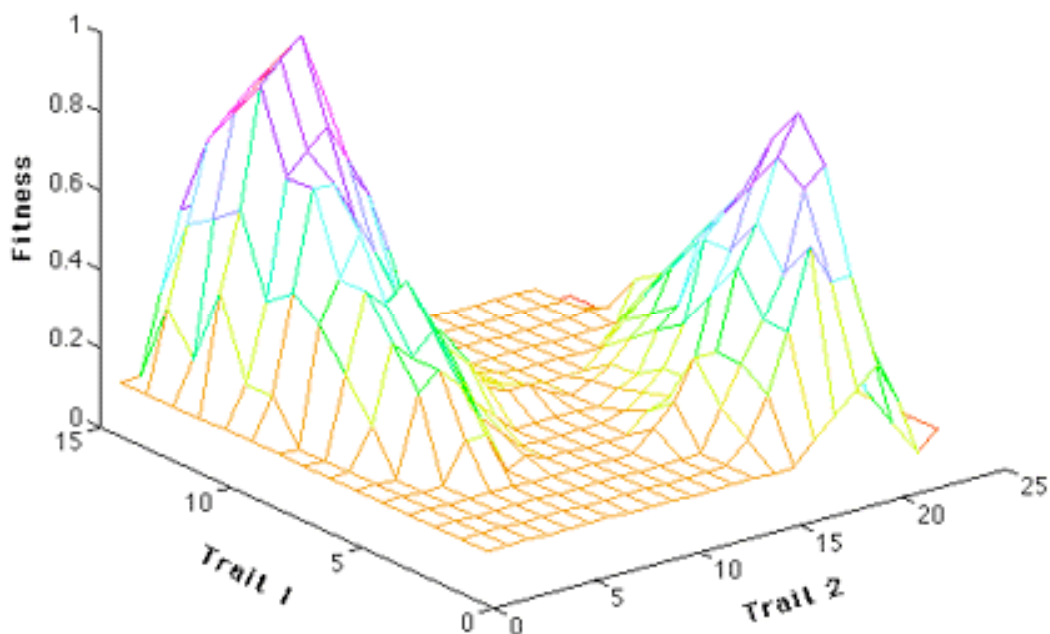


Figure 1. A schematic view of an adaptive landscape with two fitness peaks made by the combination of two traits. The left peak is higher than the right, and a valley of very low fitness separates the peaks.

In the present study we investigate the relationship between two key behavioural traits and two morphological traits across genetically diverged populations of the same species. The two selected morphological traits are in turn expected to co-evolve because one of them, body size, influences the fitness effects of the other, aposematic colouration.

Animals that have a warning signal that is associated with their unprofitability as prey are described as being aposematic (Mappes *et al.*, 2005). The signaller receives the benefit of predator avoidance but incurs costs in the production of this signal and of increased conspicuousness. Here, conspicuousness is defined as the colour difference of the prey item(s) as compared to its background from the perspective of likely predators (Osorio *et al.*, 2004). This increased conspicuousness increases risks of harm or death to the signaller if a predator is naïve, immune or has forgotten the association between the signal and unprofitability (Mappes *et al.*, 2005). The effectiveness of this signal, as mentioned earlier, is greater with larger signal size (e.g. larger body size; aggregated individuals) (Gamberale & Tullberg, 1996; Gamberale & Tullberg, 1998). By extension, we may expect body size (or aggregating behaviour) to coevolve with warning colouration and toxicity.

The study of the ecological role of aposematic colouration is primarily conducted using birds as the model predator, since they are primarily visual predators and easily accessible in a naïve state, and insects as the model prey (e.g. Gamberale & Tullberg, 1998; Exnerová *et al.*, 2006). The behavioural responses of likely predators to aposematic prey items have been studied extensively (e.g. Mappes *et al.*, 2005; Exnerová *et al.*, 2006), with aposematic colouration decreasing the probability of attack by predators (Roper & Cook, 1989; Gamberale & Tullberg, 1998) and increasing the learning efficiency of avoidance (Gittleman & Harvey, 1980; Sillén-Tullberg 1985; Roper & Redston, 1987; Roper, 1994). By contrast, there are few studies that have investigated traits that coevolve with aposematic colouration.

An appropriate study species for investigating the adaptive links between aposematic colouration, body size and risky behaviour is the strawberry poison-dart frog, *Dendrobates pumilio*, since it has both cryptic and aposematic morphs and displays significant variation of body size across populations (Siddiqi *et al.*, 2004; Hagemann & Pröhl, 2007; Rudh *et al.*, 2007).

D. pumilio males, and to a lesser extent females, are intrasexually competitive. Pröhl and Berke (2001) determined that females choose their territories in accordance with the presence of tadpole rearing sites (small water bodies in leaf axils), and males in accordance with the presence of females. Males that have secured a territory will guard it ferociously, and frequently exhibit aggressive behaviour towards conspecific males (e.g. territorial calling, fighting) (Baugh & Forester, 1994; Pröhl & Berke, 2001; Gardener & Graves, 2005; Pröhl, 2005). More aggressive males win more battles, and thereby maximise their chances of securing higher quality territories. It is assumed that aggressive territoriality comes with costs, such as an increased predation risk, increased energy consumption and/or increased injury risk. Males that do not possess such a territory, and are sexually mature, must make one of three decisions to reproduce. They can either: (1) explore the surrounding potentially suboptimal habitat until they find a free territory worth defending. This would be beneficial as they would avoid the costs of challenging signallers, but they would incur the costs of this avoidance since exploration of novel habitats introduces risks such as increased predation risk, fewer and only poor territories to choose from and the energy costs of finding unattended territories; (2) they could challenge a territory holding male for his territory, but there is a low chance of success (Baugh & Forester, 1994); (3) or they could wait until a territory becomes available, which would occur when the holder dies or moves on. There is no data on the role subordinate or 'sneaking' males play in the reproductive system of *D. pumilio*, despite these strategies being prevalent in other taxa (Birkhead & Møller, 1998), but with small fiercely protected territories and male egg guarding it would be assumed that the role of these strategies is minor in *D. pumilio*.

D. pumilio has a high degree of population differentiation (Hedemann & Pröhl, 2007; Rudh *et al.*, 2007). Rudh *et al.* (2007) used amplified fragment length polymorphisms to show that within the Bocas del Toro Archipelago region, 14 *D. pumilio* populations differed genetically from each other and there was significant "isolation by distance". Hagemann and Pröhl (2007) produced further evidence for the genetically differentiated state of the Bocas del Toro Archipelago region populations, and surprisingly produced evidence for the absence of monophyly for this species.

Variation of phenotypic traits is also significant between populations of *D. pumilio* (Summers & Clough, 2001; Pröhl *et al.*, 2007; Rudh *et al.*, 2007). Within the Bocas del Toro Archipelago region Rudh *et al.* (2007) found an array of morphological traits (e.g. body size, brightness, spot size, hue, saturation and coverage) differed considerably between populations. Of these traits, only differences in body size were significantly correlated with genetic distance. No investigation of the variation in conspicuousness was made in this study. In an area that encompassed most of the geographic range of *D. pumilio*, Pröhl *et al.* (2007) found that the general pattern of body size variation was that populations with bigger individuals were located to the northwest and populations of smaller individuals to the southeast. Call parameters varied between populations along this northwest-southeast gradient, with higher call rates, shorter calls, lower duty cycles and higher pulse rates found in the populations in the southeast (Pröhl *et al.*, 2007).

In much of its range *D. pumilio* is aposematically coloured and all colour morphs, both cryptic and conspicuous, investigated thus far, accumulate a chemical defence that includes a variety of skin alkaloids (Daly & Myers, 1967; Saporito *et al.*, 2007a, b). The centre of colour diversity of this species is the Bocas del Toro Archipelago region of Panama, where conspicuous red, orange, green and white

morphs occur as well as yellow/green, brown and blue/black cryptic morphs (Siddiqi *et al.*, 2004; Hagemann & Pröhl, 2007; Rudh *et al.*, 2007). *D. pumilio* in Costa Rica is relatively monomorphic, with populations having a red body and red, blue or black limbs (Hagemann & Pröhl, 2007). The Costa Rican colour morph has been used for the vast majority of *D. pumilio* behavioural and ecological studies, and these findings have been used to make species-wide generalisations despite the absence of the majority of phenotypic variation (e.g. Donnelly 1989 a, b; Forester *et al.*, 1993; Pröhl & Berke, 2001; Haase & Pröhl, 2002; Gardener & Graves, 2005; Graves *et al.*, 2005).

Investigations of single behavioural traits have already been performed among relatively few colour morphs, but giving relevant background information (e.g. Calling: Forester *et al.*, 1993; Bee, 2003a; Pröhl, 2003; Gardener & Graves, 2005; Graves *et al.*, 2005. Aggression: Forester *et al.*, 1993; Baugh & Forester, 1994; Bee, 2003a; Gardener & Graves, 2005. Territoriality: Donnelly, 1989 a, b; Forester *et al.*, 1993; Baugh & Forester, 1994; Bee, 2003b). No published investigations into *D. pumilio* behavioural syndromes and coadaptive gene complexes have been performed to date. It is well known that behavioural traits do differ at the population scale in many taxa (Foster & Endler, 1999), but this has not been thoroughly studied in *D. pumilio*. To date, only aggression has been thoroughly investigated in this species (e.g. Baugh & Forester, 1994). Therefore, exploration tests are required to be formulated, providing the impetus to investigate the roles conspicuousness and body size have with regard to the behaviour of *D. pumilio*.

Aims and hypothesis

The aims of the present study were to determine the variation of *D. pumilio* (1) male-male aggression, and (2) male boldness to explore novel habitats. We gathered information about the frog's conspicuousness and body size to use as predictor variables.

The hypothesis that we tested was that conspicuousness has coevolved with the risk-taking behaviours, aggression and exploration, since (1) the aposematic morphs are not likely to exhibit strong predator avoidance behaviour due to little need for it; (2) males are fiercely intrasexually competitive in securing an optimal territory, since this territory is likely to enable females to judge male quality; and (3) more aggressive males win the territory battles (Forester *et al.*, 1993; Baugh & Forester, 1994). As a consequence of this it is predicted that aposematic males would have the freedom to spend more energy on territory security and discovery. If the above hypothesis is supported, conspicuousness should link with aggression and boldness with more conspicuous frogs being more aggressive and bolder than those that are less conspicuous. In addition, body size should be tightly linked to conspicuousness as this provides a more effective warning signal to predators, with the outcome that larger frogs should also be more aggressive and bolder.

Methods

Study species

Dendrobates pumilio is indigenous to the eastern lowlands of Nicaragua, Costa Rica and northwestern Panama (Figure 2). It is a small diurnal neotropical species that is locally very abundant (IUCN, www.iucnredlist.org). Both sexes of this species are polygamous and courtship is complex. During courtship, females choose males that call on elevated sites in their territory. The male leads the female around his territory presenting potential oviposition sites in the leaf litter (e.g. covered leaves). If the female accepts the male, gametes are released simultaneously onto the oviposition site. Males guard and tend to the eggs. After approximately seven days the eggs hatch. The female later returns and the tadpoles wriggle up onto her back and are transported to individual water-filled leaf axils in the female's territory. The female tends the tadpoles, feeding them unfertilised eggs, and does not engage in courtship whilst rearing (Brust, 1993). Colour morphs are distinguished by conspecifics (Summers *et al.*, 1999; Siddiqi *et al.*, 2004), with females preferring their colour phenotype in laboratory experiments (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007). *D. pumilio* males are well known to be very territorially aggressive towards other male conspecifics while they defend their territory (Prohl, 2005). There is a strong prior residence affect that determines the winner of these contests regardless of the intruder's size (Baugh & Forester, 1994).

Study area

From the 1st to 29th of November 2007 we visited a total of eight localities within *D. pumilio*'s range in the Bocas del Toro archipelago region, Panama (Figure 2). Males of these populations had their exploration and aggression measured, as well as their conspicuousness and size. The choice of localities was based on the ease of access, the local abundance of frogs, their conspicuousness and their ease of observation and capture. The localities chosen were well known (Summers *et al.*, 2003; Siddiqi *et al.*, 2004; Rudh *et al.*, 2007) and encompassed many different colour morphs, including a range of conspicuousness levels according to the bird and frog chromatic systems (Siddiqi *et al.*, 2004).

To avoid sampling biases due to differences in diurnal activity of *D. pumilio*, the times of first and last trial were between 0915 and 1530 hours. This is after the morning peak activity, 0745 to 0915 hours, and before the late afternoon peak (Graves, 1999). Capture of the frogs was performed with the use of a transparent plastic cup and a stick or a gloved hand to knock the individuals into the cup. Once the frogs were in the cup, white mosquito netting was placed, and secured, over the opening. Sex of the frogs was determined either by observation of them calling, as only males call, or by visual inspection of their gular sac which is pigmented in males (Bunnell, 1973).

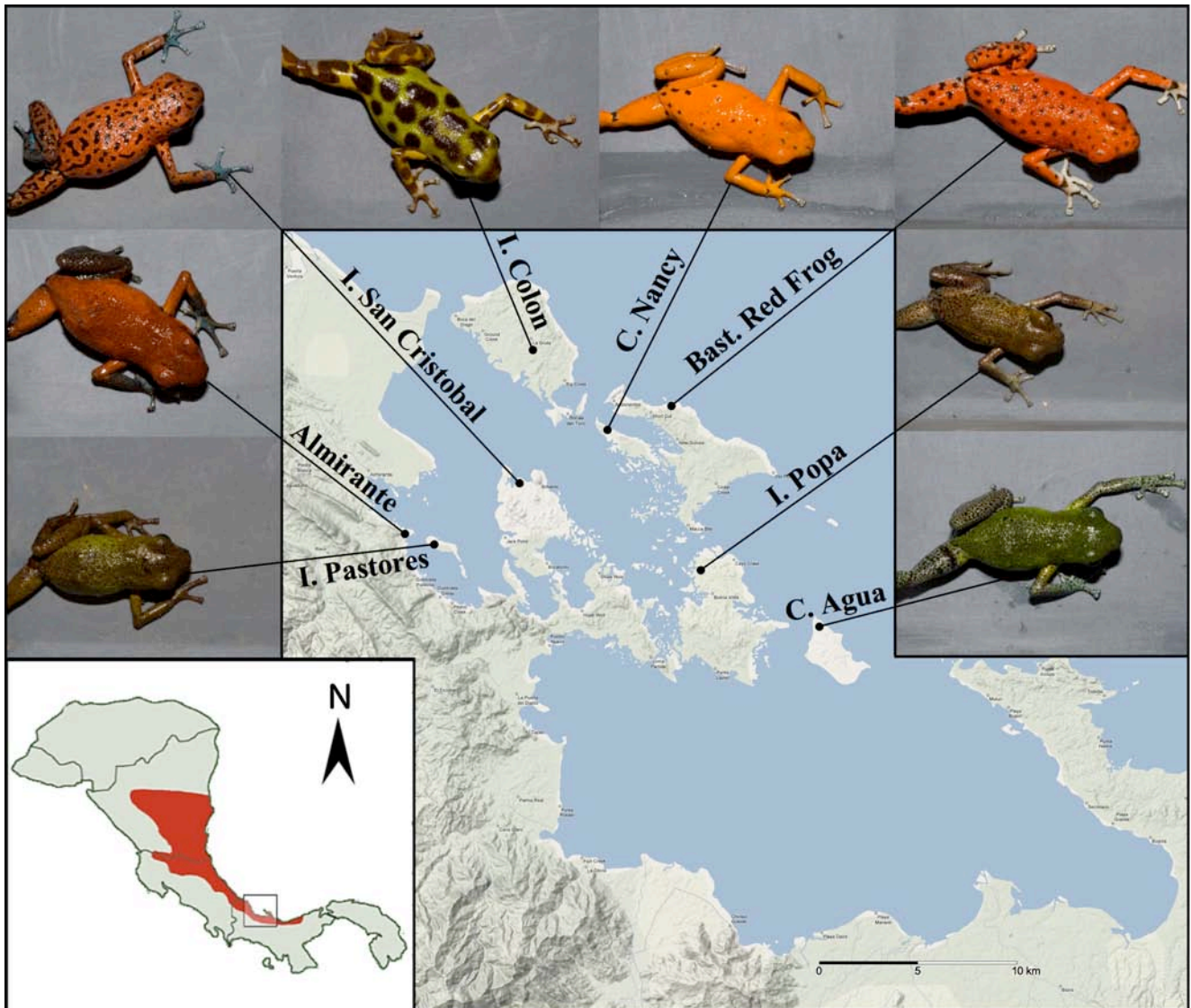


Figure 2. Approximate positions of the eight studied populations of *Dendrobates pumilio* in the Bocas del Toro archipelago region. Insert photographs represent an example of each of the studied population's morphology. The map to the left shows Central America, with the distribution of *D. pumilio* in Nicaragua, Costa Rica and Panama shown in red.

Behavioural assay

Approximately 5 to 10 minutes passed between the initial capture of the frog and the initiation of the aggression trial. During this waiting period the frog was kept as close to its perch as practical. Another 5 to 10 minutes passed between the end of the aggression trials and the beginning of the novel environment trial. After the novel environment trial the frogs were photographed, their spectral reflectance was measured, and they were released back at the site where they were caught.

For aggression trials a 0.5 mm thick, opaque plastic cylindrical arena measuring 36 cm in diameter and 25 cm in height was positioned as close as possible to the male frog's capture site which is likely to include part of the territory as most males were calling prior to capture. Pröhl and Berke (2001) estimated *D. pumilio* males had territory core areas of approximately 3m² and home ranges of approximately 20m², so by placing the arena as close to the male's calling site as possible we maximised our chances of being within their core area and invoking a natural aggressive response.

A digital video recorder was attached to a tripod and placed over the arena. A black umbrella was attached to the tripod to protect the camera from rain and to reduce the risk that the frog might perceive from the camera's lens. Before the frog was placed in the arena, a mirror, 10 cm high and 22.5 cm long, was placed on the side of the arena opposite the position of the observer. The netting was removed from the cup and the cup was placed back on the ground in the arena. If the frog did not hop to the ground voluntarily, taps and/ or gentle shakes were administered to encourage the frog to hop to the ground. The observer was required to be present during all trials since the frogs could climb and escape from the arenas. The observer sat as still and as quietly as possible within 1.5 metres of the arena. If the frogs were observed escaping, they were gently guided back into the arena using a long thin stick.

An adjustment of the protocol introduced by Baugh and Forester (1994) was used to score each frog as "aggressive" or "not aggressive". If the frogs did not move, if they attempted to escape or if they hid they were deemed "not aggressive". Those that called or tracked statically, called or tracked while advancing, charged and veered, or charged and contacted the mirror were defined as "aggressive" (see explanation of these behaviours in Baugh & Forester, 1994). The response during the first 5 seconds after the frog saw its reflection was the period observed. The frog was determined to have seen its reflection when it turned to face the mirror, or when it changed its path when its alignment was within 90 degrees of the mirror. This arrangement is assumed to elicit a natural reaction from the frog, as though an intruder of the same colour and size was found on his territory. During the first 5 seconds, the frog is assumed not to have had time to discover that it is unable to escape from the arena, and it was therefore not forced to attack since no other option was available. Playback of the video recordings was performed in QuickTime software.

A patch of habitat near our base was selected for the novel environment trials. This environment might, or might not, introduce a perception of risk to the frogs. An identical arena was used for this trial as for the aggression trials. The same release procedure was used here as for the aggression trials, except that the frogs were stored near, but not in, the arena before they were introduced to the arena. This was to ensure that the frogs were totally naïve to the environment when they were introduced. The same digital video camera arrangement was used. Playback of the video recordings was performed in QuickTime software.

Once the frog was introduced to the arena, its path was traced using the software ImageJ. This path was saved as an image file and imported into PowerPoint, where it was made transparent, adjusted for varying size, and overlaid on top of a 10 x 10 unit grid (Figure 3). A circle was placed on this grid to represent the arena. Each 1 x 1 unit ('a' in Figure 3) the frog's path covered was counted as 1, and the areas of the incomplete squares ('b' through 'e' in Figure 3) were calculated geometrically. The sum of the units was calculated regardless of whether the frog landed in the square or just jumped over it. Each 1 x 1 unit was only counted once, even if the frog entered it on more than one occasion. A circle with diameter x has an area 78.54% of that of a square with sides of length x . Thus the percentage of the arena that each frog covered was calculated by dividing the sum of the units the frog covered by 78.54×100 .

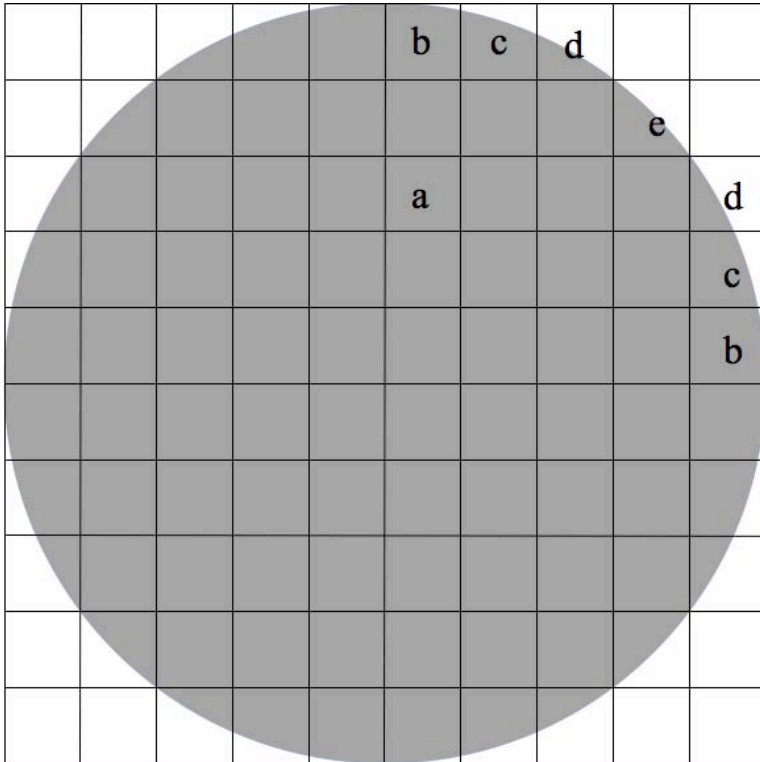


Figure 3. The template used to calculate the area explored by each frog. Each full square (a) has an area of 1 unit (n=60). The partially filled squares (b) have areas of 0.98 units (n=8), (c) has an area of 0.80 units (n=8), (d) has an area of 0.27 units (n=8), and (e) has an area of 0.55 units (n=4).

Body size measurements

Body size measurements were carried out by Andreas Rudh from Uppsala University. Each individual was digitally photographed at a standardised distance using a custom-made camera stand. The sampling was performed using a Canon 350D camera with an EFS 60 mm f/ 2.8 Macro USM Canon lens with built-in flash to normalise light conditions. Individuals were held on an L-shaped grey plastic device with size reference markings. We later measured body size in snout-vent length (SVL) with Adobe® Photoshop® CS3 Extended.

Conspicuousness measurements

Andreas Rudh, Uppsala University, carried out the spectral reflectance measurements and colour distance calculations. Spectral measurements were performed with a spectrophotometer, and colour distance (ΔS) was calculated according to Osorio *et al.* (2004). Colour distance incorporates the spectral reflectance of the frog from the perspective of a bird, the likely predator of *D. pumilio*, and compared it to the spectral reflectance of leaf litter, the most common background of *D. pumilio* (Rudh, *pers. comm.*).

The order of populations (except for a switch between the two most conspicuous populations) in the measure of ΔS was consistent over all light intensities and supported a classification of frog populations as either (1) low, (2) intermediate or (3) high conspicuousness (Table 1). This ordinal variable was used as a predictor in the statistical analyses.

Statistical methods

Variation at the population level was investigated with one-way analysis of variance (ANOVA) with type III sum of squares on the exploration and body size variables. Though the exploration variable

was not strictly a continuous variable, it was deemed appropriate to treat it as continuous since no individual had a value of 0 or 100%. Population was treated as a random predictor variable. For all ANOVAs, the assumption of homogeneity of variance was tested with Levene's test and the assumption of normality of errors was tested with Shapiro-Wilk's test. Tukey's *post hoc* analysis was conducted for all ANOVA analyses to determine significantly heterogeneous subsets. Data transformations were performed in attempt to meet these assumptions when needed. All ANOVA analyses were performed in SPSS version 16.0.

Logistic regression analysis with a logit link function was used to analyse the variation in aggression using GenStat software. Analysis of the deviance of the data from the model was performed using a χ^2 test. Analysis of deviance is used to determine if there was a significant improvement from the null model with the inclusion of a predictor variable. The χ^2 value used was the difference in deviance (-2 log likelihood) of the two models under investigation and the χ^2 degrees of freedom was the difference in degrees of freedom of each model.

The statistical effect conspicuousness has as a predictor variable was determined with the same analyses as for between populations using conspicuousness as the predictor variable instead of population. Conspicuous groups were treated as a fixed factor for all general linear modelling.

The relationship of body size and exploration was investigated by performing Pearson's correlations. Since the aggression variable was a non-parametric variable, Spearman's ρ was used to calculate this relationship. All correlations were carried out in SPSS 16.0.

To account for differences in population or conspicuous group means, the exploration percentages were standardised for each population by the equation below:

$$\frac{X_{ij} - \bar{X}_j}{SD_j}$$

The difference between the population mean of trait X, \bar{X}_j , and the individual trait value, X_{ij} , is divided by the standard deviation of that population's values, SD_j . To estimate the covariation of the behavioural traits at an individual level Spearman's ρ correlation was conducted.

Animal health: After each field session all equipment was put in a heating/ drying cupboard above 45°C for at least an hour. This is sufficient to kill chytrid fungus and permanently inactivate ranavirus. Frogs were kept in small plastic cups for lengthy periods of time and to avoid the risks of heat exhaustion/stress water was sprayed on the cups and the frogs were placed in shade at all times. No tested frogs died.

Permission: Scientific permits were provided by A.N.A.M., the Panamanian authorities (Permit no.: SE/A-100-07). Permission to access land was sought from local community leaders and landholders at each locality where appropriate.

Results

We observed and recorded data from 78 male *D. pumilio* across eight populations (Table 1). Four males were not calling when caught, and they were determined to be males by their gular sac pigmentation. Sample sizes were slightly lower for the aggression trials due to some frogs escaping.

Table 1. Sample size, conspicuousness, mean snout-vent length with standard error, mean exploration with standard error and aggressive: non-aggressive of each of the 8 study populations.

Population	Sample size	Conspicuousness	Snout-vent length (mm)	Exploration (%)	Aggressive:non-aggressive
Cayo Nancy	14	High	17.58 ± 0.17	38.77 ± 3.16	6:4
Bastimentos, Red Frog Beach	9	High	18.71 ± 0.22	35.83 ± 2.06	6:2
Almirante	10	High	18.69 ± 0.30	35.58 ± 3.80	5:4
Isla San Cristobal	8	Intermediate	18.15 ± 0.23	42.28 ± 3.74	6:1
Isla Colon	11	Intermediate	18.04 ± 0.13	37.01 ± 4.12	6:3
Isla Pastores	8	Low	16.81 ± 0.17	24.88 ± 3.97	1:4
Cayo Agua	11	Low	17.50 ± 0.15	25.74 ± 2.18	1:8
Isla Popa	7	Low	15.60 ± 0.11	37.69 ± 5.33	4:1

Body size

A total of 78 male *D. pumilio* had their SVL measured. There was significant variation of SVL at the population level (ANOVA: $F_{\text{population}} = 23.574$, $df = 7, 70$, $p < 0.0001$; $F_{\text{Levene's}} = 2.145$, $df = 7, 70$, $p = 0.050$; Shapiro-Wilk = 0.991, $df = 78$, $p = 0.858$; Figure 4). The Isla Popa population clearly had the smallest SVL. High and intermediate conspicuous groups had significantly larger SVL than the low conspicuous group (ANOVA: $F_{\text{conspicuousness}} = 24.449$, $df = 2, 75$, $p < 0.0001$; $F_{\text{Levene's}} = 3.449$, $df = 2, 75$, $p = 0.037$; Shapiro-Wilk = 0.991, $df = 78$, $p = 0.889$; Figure 5).

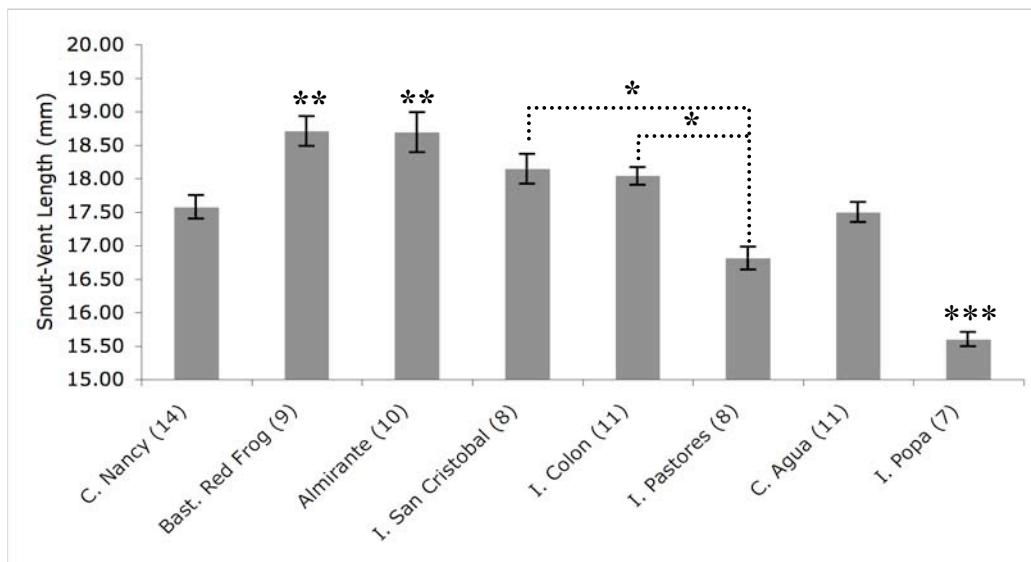


Figure 4. Mean of snout-vent length ± standard error of the mean of each population. Sample sizes are shown in parentheses. Populations connected by dotted lines with a ‘*’ indicate significant differences in mean SVL. ‘**’ indicates that the mean SVL of these populations was significantly larger than all other populations. ‘***’ indicates that the mean SVL of this population was significantly smaller than all other populations.

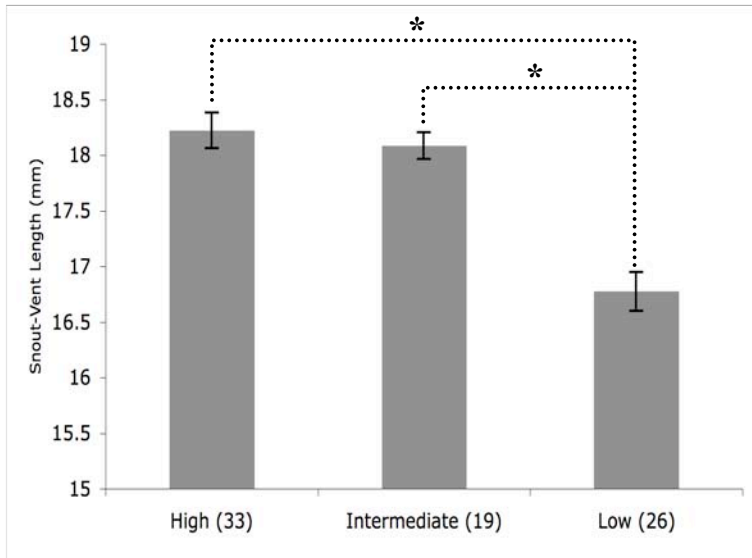


Figure 5. Mean of snout-vent length \pm standard error of the mean of each conspicuous group. Sample sizes are shown in parentheses. Groups connected by dotted lines with a ‘*’ indicate significant differences in mean SVL.

Exploration trials

A total of 78 male *D. pumilio* underwent trials to examine their explorative behaviour. Exploration differed significantly between populations (ANOVA $F_{\text{population}} = 2.917$, $df = 7, 70$, $p < 0.05$; $F_{\text{Levene's}} = 1.106$, $df = 7, 70$, $p = 0.369$; Shapiro-Wilk = 0.993, $df = 78$, $p = 0.964$) (Figure 6). The Isla Pastores and Cayo Agua populations were clearly less explorative. High and intermediate conspicuous groups were significantly more explorative than the low conspicuous group (ANOVA $F_{\text{conspicuousness}} = 5.631$, $df = 2, 75$, $p < 0.01$; $F_{\text{Levene's}} = 0.911$, $df = 2, 75$, $p = 0.407$; Shapiro-Wilk = 0.993, $df = 78$, $p = 0.963$; Figure 7). Isla Popa stood out as an unusually explorative population for its low conspicuous group (compared with the Isla Pastores and Cayo Agua populations). Its mean of $38\% \pm 5\%$ was more similar to the intermediate and high conspicuous populations’ means. Overall, there was a non-significant correlation between SVL and exploration (Pearson’s correlation: $r^2 = 0.022$; $p = 0.195$), even though the low conspicuous group tended to show lower levels of exploration.

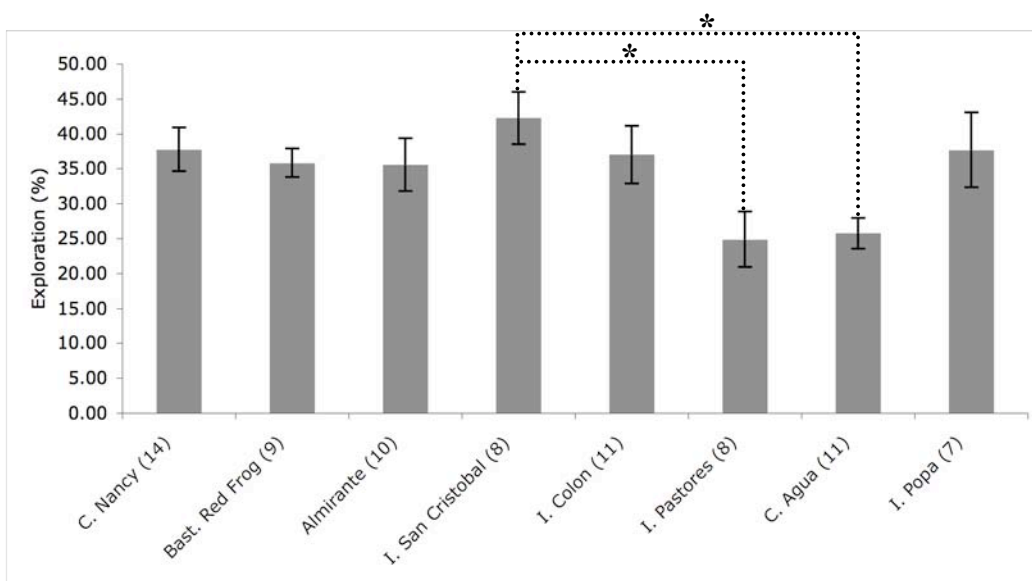


Figure 6. Mean of exploration \pm standard error of the mean of each population. Sample sizes are shown in parentheses. Populations connected by dotted lines with a ‘*’ indicate significant differences in mean exploration.

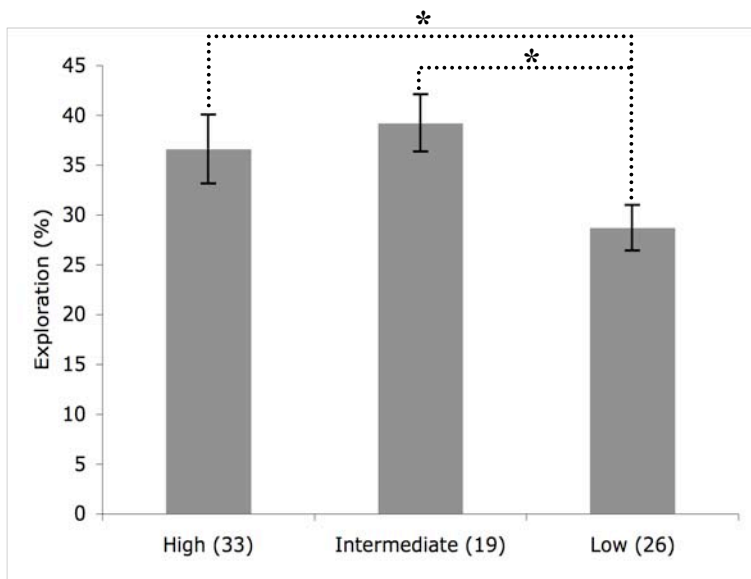


Figure 7. Mean of exploration \pm standard error of the mean of each conspicuous group. Sample sizes are shown in parentheses. Groups connected by dotted lines with a ‘*’ indicate significant differences in exploration

Aggression trials

A total of 62 male *D. pumilio* underwent trials to determine their aggressive status. Population information significantly improved the fit of the logistic regression model when compared with the null model (*i.e.* including y-intercept only) (Table 2). Discriminating between the aggressive statuses of the different populations with these data was difficult due to low sample sizes, but it can be seen that Cayo Agua and Isla Pastores populations were generally less aggressive than the others (Table 3). Conspicuous group information significantly improved the fit of the logistic regression (Table 2). High and intermediate conspicuous populations were generally more aggressive than the low conspicuous group (Table 3). Isla Popa was, again, a clear exception to the general rule for low conspicuous populations as its pattern of aggressive to not aggressive individuals was similar to the conspicuous populations and most unlike the other cryptic populations. There was a significant positive correlation between SVL and aggression (Spearman’s ρ : $n = 62$, $\rho = 0.278$, $p = 0.029$).

Table 2. Analysis of deviance of logistic regression models, showing the difference in -2 log likelihood χ^2 value (the deviances), the difference in degrees of freedom and the p-value of the χ^2 test.

Models compared	Chi ²	df	p-value
Null vs Population	16.606	7	0.020*
Null vs Conspicuous group	7.628	2	0.022*

Table 3. Logistic regression models of aggression: coefficients, standard error and t-test p-value for each parameter.

Model	Parameter	Coefficient estimate	S.E.	P-value
$z = \beta_{\text{intercept}} + \beta_{\text{population}}$	Constant	0.267	0.327	0.414
	C. Nancy	0.139	0.647	0.830
	Bast. Red Frog	0.832	0.779	0.285
	Almirante	-0.043	0.666	0.948
	I. San Cristobal	Reference	-	-
	I. Colon	0.427	0.694	0.539
	I. Pastores	-1.653	1.022	0.106
	C. Agua	-2.346	0.975	0.016
	I. Popa	1.12	1.022	0.273
$z = \beta_{\text{intercept}} + \beta_{\text{conspicuous}}$	Constant	0.285	0.286	0.318
	High	0.245	0.367	0.504
	Intermediate	0.813	0.439	0.064
	Low	Reference	-	-

Covariation of individual responses

No exploration-aggression correlations were significant after exploration was standardised for differences within each population and conspicuous group. (Spearman's ρ correlation: Population, $\rho = -0.045$, $p = 0.731$; Conspicuous group, $\rho = 0.093$, $p = 0.474$).

Discussion

In the present study significant variation in male-male aggression, male boldness when exploring novel habitats and in male body size between different populations of *D. pumilio* frogs was found to occur. As predicted, males belonging to more conspicuous populations were larger, thus likely producing a stronger aposematic signal, warning potential predators of their unprofitability as prey. Also as predicted, males of these more conspicuous populations were significantly more aggressive and more explorative, likely explained by their relatively low risk of predation and selective advantages of these behaviours. These combined results present, for the first time in any taxa, data on the coevolution of risk-taking behaviour with aposematic colouration and body size.

Our results support the hypothesis that more aposematic populations were less risk averse, as they were more aggressive and explorative. Therefore, it is inferred that these behavioural traits are either (1) heritable and genetically linked to conspicuousness, which is heritable and under selection, or (2) an adaptive plastic response to the perceived reduced predation pressure caused by conspicuousness, which is heritable and under selection.

Overall, the populations can be classified into two different categories based on the main strategies used by the males. These strategies include (1) populations with conspicuous, larger, and more risk keen individuals, and (2) populations with cryptic, smaller and more risk averse individuals. These strategies may represent two adaptive peaks on an adaptive landscape. Investigations into the fitness levels experienced by the trait combinations are required, but it would be assumed that since there were consistent significant differences in phenotype across conspicuous groups these differences are likely to be adaptive and represent fitness peaks, rather than convergent maladaptive strategies.

Due to our data giving evidence for conspicuousness being a strong predictor of variation in exploration, aggressiveness and size, we hypothesise that size would be less tightly linked with exploration and aggression than conspicuousness. It is likely that *D. pumilio* size is linked with conspicuousness, and this would be because more conspicuous populations were significantly larger than less conspicuous populations, and that these bigger frogs would provide a more effective warning signal to predators (Gamberale & Tullberg, 1998; Gamberale & Tullberg, 1996; Forsman & Merilaita, 1999; Lindström *et al.*, 1999; Hagman & Forsman, 2003). In accordance with this prediction, we see slight size-behaviour inconsistencies, including the non-significant correlation between size and exploration. Looking closely at this non-significant correlation, it can be seen that the largest frogs (Size: #1 = Bast. Red Frog; #2 = Almirante) were not the most explorative (Explorative: #1 = I. San Cristobal; #2 = C. Nancy, I. Colon or I. Popa), and Isla Popa, the smallest frogs, were as explorative as the largest frogs. We cannot rule out the possibility that size is an indirect predictor of exploration and aggression, but instead a more likely scenario of size being linked with conspicuousness is proposed, and that conspicuousness has driven the coevolution of the risky behaviours, exploration and aggression.

Isla Popa was an exception to the general rule of more conspicuous populations being more aggressive, more explorative and bigger. This population had similar levels of exploration and similar ratios of aggressive to not-aggressive individuals as the conspicuous populations, but had one of the lowest levels of conspicuousness (Rudh, *pers. comm.*) and had the smallest frogs. It is possible that the size of Isla Popa frogs falls below a certain “size threshold” (Mean body size of populations in mm: I. Popa = 15.6; I. Pastores = 16.8; C. Agua = 17.5; C. Nancy = 17.6; I. Colon = 18.0; I. San Cristobal = 18.2; Almirante = 18.7; B. Red Frog = 18.7), thus presenting a third fitness peak. Perhaps below this threshold, individuals are so small that they are just not worth eating by predators, or they are just too

cryptic for a predator to detect even if they move. The other cryptic populations in this study might be out of this size threshold, therefore moving has a great cost as it increases their chances of being detected by predators, and they are big enough to be worth eating. Below this size threshold it may just not be beneficial enough to produce aposematic colouration, since the benefits are likely to be very small and the signal would be weak, and therefore the relative cost of production would be high. Alternatively, perhaps this population has experienced a different evolutionary history from the other sampled populations, leaving it on this island without colour variation (thus explaining its cryptic morph), being small in size and on an island without predators. This would provide a similar scenario to that which the conspicuous morphs experience, where no predation is likely experienced, and this might allow the frogs to evolve into being generally bolder and more aggressive. Alternatively, if these behavioural traits are heritable and 'visible' to females, then the females could prefer bolder, more aggressive males. This would impose sexual selection pressures on these traits, resulting in the patterns seen. Another possible explanation might be that the sample population's phenotype was differentiated from other Isla Popa populations due to adaptation to the local environment, since on Isla Popa there is a large amount of between population morphological variation (Andreas Rudh *pers. comm.*). Therefore, perhaps the degree of variation on Isla Popa was so great that it encompassed this unique combination of phenotypic traits. But why Isla Popa would uniquely experience these pressures and variation remains unknown. Other information that might help to explain this paradoxical result could include the population's small sample size, as it was the least sampled population ($n = 7$), and that individuals of this population were noticeably harder to locate, observe and then capture than all other populations sampled. To overcome this anomaly, greater behavioural sampling effort of the Isla Popa population(s) is required as well as of more *D. pumilio* populations.

The present study provides no evidence that aggression and exploration are linked as a behavioural syndrome (*i.e.* at the level of the individual), even if this link has been found previously in other taxa (Sih *et al.*, 2004). This raises three issues: (1) that the link between these two behavioural traits is being confounded by a stronger link between the behaviours and another factor, for example with conspicuousness, (2) that the mechanism of the previously found link of these traits may not be found within *D. pumilio*, or (3) that our sample size did not provide sufficient power to detect such trends, as it is well known that behavioural traits generally have high degrees of variation and require substantial sampling effort (Réale *et al.*, 2007).

To be decisive about the findings of the present study, one would need to conduct a suite of experiments to determine such information as (1) whether the patterns of variation of behavioural traits measurements in the present study are repeatable and consistent through time, populations and contexts; (2) if/how heritable these behavioural traits are and if selection experiments can elucidate whether it is possible to manipulate the traits; (3) the role environmental factors play in variation of these behavioural traits; (4) if these behavioural traits are genetically linked with other traits, specifically conspicuousness and/or body size, and if so in which way are they linked; (5) to what extent and in what manner conspicuousness and body size are genetically linked; (6) what the fitness implications of these behavioural traits are; (7) what role does individual variation in behaviour traits play in female choice in *D. pumilio*; (8) what are the predators of *D. pumilio*.

The rapid evolution of differences in degree of aposematic colouration across populations belonging to the same species may provide the means for speciation, at least in its early stages. It is often proposed that behavioural shift precedes genetic change (Mayr, 1963), but how this would be the case in *D. pumilio* is hard to fathom. It is comprehensible to picture an aposematic population becoming more risk-averse if these risk-taking behaviours were no longer adaptive. But the alternative scenario would be very hard to imagine, where a cryptic morph increases its risk-taking behaviour, leading to an

increase in aposematic colouration. It would appear that any increase in risk-taking behaviour of a cryptic frog would rapidly lead to death and hence be strongly selected against. Although, this might be precisely what has occurred on Isla Popa where these frogs do not behave as they “should” according to the pattern described. Perhaps, as alluded to by Price *et al.* (2003), the way in which phenotypic plasticity affects evolutionary change depends on the adaptive landscape that the population finds themselves on. So, perhaps on Isla Popa there really are no predators and so there are no selective pressures to maintain or return to aposematic colouration, but it may be beneficial to be aggressive and explorative to secure the best territory available. And so for this population, they have the phenotypic plasticity to shift from one adaptive peak (cryptic, small, risk-averse) to another peak (cryptic, very small, risk-taking), which is potentially better adapted to their given environment. Exploring the fitness returns of various trait combinations, the degrees to which they are genetically determined or plastic, and the fitness landscapes that the combinations are exposed to would seem appropriate in this study species and would help untangle the evolutionary questions presented.

In conclusion, the data provides further evidence for the breadth of divergence *D. pumilio* populations present at the phenotypic level, and supports the aposematic theory that predicts more aposematic organisms express less predator avoidance and that they are bigger. We suggest that adaptive behavioural adjustments reinforces rapid divergence in aposematic colouration across populations of the same species, which in turn may reflect speciation, at least in its early stages.

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