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Does aggression and explorative behaviour decrease with lost warning coloration?

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For prey, many behavioural traits are constrained by the risk of predation. Therefore, shifts between warning and cryptic coloration have been suggested to result in parallel changes in several behaviours. In the present study, we tested whether changes in chromatic contrast among eight populations of the strawberry poison-dart frog, *Dendrobates pumilio*, co-vary with behaviour, as expected if selection is imposed by predators relying on visual detection of prey. These eight populations are geographically isolated on different island in the Bocas del Toro region of Panama and have recently diverged morphologically and genetically. We found that aggression and explorative behaviour were strongly correlated and also that males tended to be more aggressive and explorative if they belonged to populations with conspicuously coloured individuals. We discuss how evolutionary switches between predator avoidance strategies and associated behavioural divergence between populations may affect reproductive isolation. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **108**, 116–126.

ADDITIONAL KEYWORDS: Anura – Amphibia – aposematism – co-evolution – evolutionary innovation – *Oophaga pumilio* – population divergence.

INTRODUCTION

When prey are toxic, warning coloration has been found to decrease the probability of attack by predators by increasing the learning efficiency of avoidance (Gittleman & Harvey, 1980; Gittleman, Harvey & Greenwood, 1980) reviewed by (Mappes, Marples & Endler, 2005; Ruxton, Sherratt & Speed, 2006). Avoiding detection by predators is beneficial to prey, although this avoidance constrains the expression of both morphological and behaviour traits. Speed, Brockhurst & Ruxton (2010) argued that aposematism could function as an evolutionary innovation, widening habitat use by prey as a result of their enhanced protection from predators. The enhanced protection gained by aposematism might not only allow widened habitat use, but also, in a more general

sense, alter the selection pressure on all behaviours that affect detection (e.g. sexual display and aggression). We recently suggested that population divergence in predator avoidance strategy (aposematism and crypsis) may favour the rapid build-up of reproductive isolation by causing large shifts in selection on traits influencing pairing patterns (Rudh *et al.*, 2011). In the present study, we focus on two other important behavioural traits: aggression and exploration.

More aggressive and explorative individuals facilitate range expansion (Duckworth & Badyaev, 2007), have better abilities to secure territories (Huntingford & Turner, 1987; Duckworth, 2006), and experience higher reproductive success (Huntingford & Turner, 1987). However, there are also fitness costs associated with being aggressive and explorative, such as injury (Huntingford & Turner, 1987), higher energy consumption (Chellappa & Huntingford, 1989), and an increased risk of predation (Jakobsson, Brick &

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Kullberg, 1995; Dunn, Copelston & Workman, 2004). Aposematic individuals that are explorative and aggressive should experience a lower risk of predation than cryptic individuals that are aggressive and explorative. Therefore, we expect a nonrandom association between these two behaviours and coloration.

Territoriality and aggressive behaviours are widespread in Dendrobatid frogs (Pröhl, 2005) and have been repeatedly described for the strawberry poison-dart frog, *Dendrobates pumilio* Schmidt, 1857; Anura: Dendrobatidae (Pröhl, 2005). This species is indigenous to the eastern lowlands of Nicaragua and Costa Rica (Savage, 2002), where it is relatively monomorphic, with bright red bodies and red, blue or black limbs (Hagemann & Pröhl, 2007; Wang & Shaffer, 2008). Its range also includes north-western Panama in the Bocas del Toro region (Savage, 2002), where there is prominent population differentiation, both genetically (Hagemann & Pröhl, 2007; Rudh, Rogell & Höglund, 2007; Wang & Shaffer, 2008) and in morphological characters, such as coloration and size (Summers, Cronin & Kennedy, 2003; Siddiqi *et al.*, 2004; Pröhl *et al.*, 2007; Rudh *et al.*, 2007). In Bocas del Toro, populations of strawberry poison-dart frogs are

found on several islands and on the mainland close to the Caribbean coast (Fig. 1). In these locations, the frogs occupy shaded habitats (Rudh *et al.*, 2011), covered with canopy or other high vegetation (e.g. primary or secondary forest, dense banana or cacao plantations). The frogs appear to avoid open areas, such as clearings and fields, as well as very wet and moist habitats (Daly & Myers, 1967; A. Rudh, pers. observ.).

Because the populations in north-western Panama have become physically isolated as a result of a rise in sea level during the last 10 000 years (Summers *et al.*, 1997; Anderson & Handley, 2002; Wang & Shaffer, 2008), this system provides us with a unique opportunity to study processes occurring at early stages of population differentiation. The recent isolation and continuous distribution of red coloured populations on the mainland (Hagemann & Pröhl, 2007; Wang & Shaffer, 2008) supports the view that populations in north-western Panama have lost their red coloration. Furthermore, there is genetic support for several independent shifts from conspicuous to dull (Wang & Shaffer, 2008). Several mechanisms have been proposed to be involved in shaping the striking

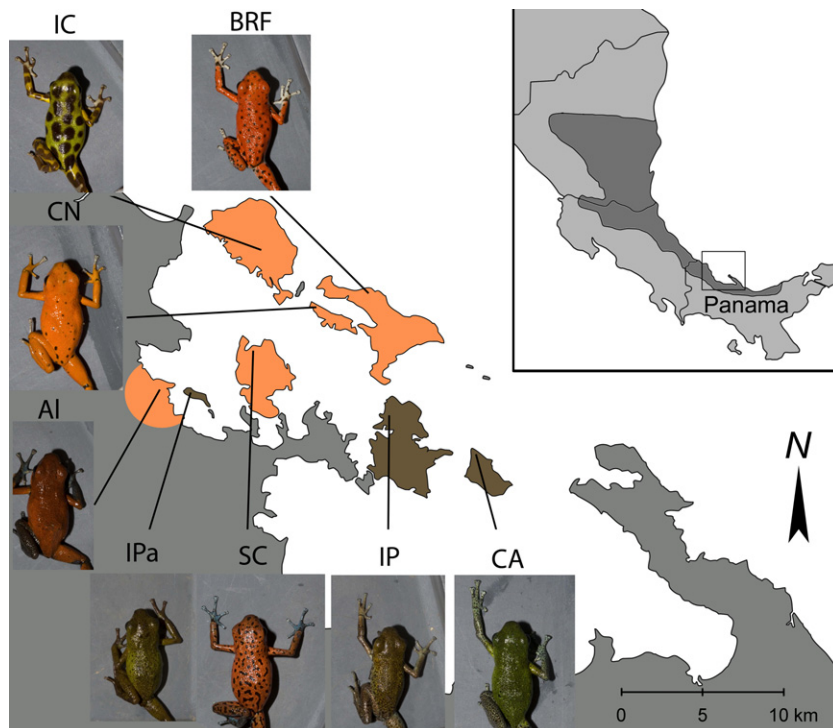


Figure 1. Study populations of *Dendrobates pumilio*, showing population abbreviations and representative individuals. Species distribution is shown by the shaded area in the inset. Distribution in north-western Panama (large image) is fragmented into small islands and mainland populations, separated by wetlands and rivers. Colour on the large map indicates whether the population is assigned to be cryptic (dark green) or conspicuously coloured (light red) (size of coloured area of population AI was arbitrarily selected). AI, Almirante; BRF, Bastimentos Red Frog Beach; CA, Cayo Agua; CN, Cayo Nancy; IC, Isla Colon; IP, Isla Popa; IPa, Isla Pastores; SC, Isla San Cristobal.

variation in colour of these populations. For example, differences in natural selection that are imposed by different ecological conditions (Maan & Cummings, 2009; Brown *et al.*, 2010), such as predator communities (Reynolds & Fitzpatrick, 2007), have been proposed and differences in toxicity have been found (Daly & Myers, 1967; Saporito *et al.*, 2004, 2006, 2007a; Maan & Cummings, 2012). Additionally, sexual selection is known to act on the coloration of *D. pumilio* because females have been observed to associate more with individuals of their own colour (Summers *et al.*, 1999; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008) and, in some populations, prefer brighter males (Maan & Cummings, 2009). Drift in combination with selection (Summers *et al.*, 2003; Reynolds & Fitzpatrick, 2007; Rudh *et al.*, 2007; Maan & Cummings, 2009), as well as the effects of differences in population size (Rudh *et al.*, 2011), have also been suggested to affect population differentiation.

D. pumilio males and, to a lesser extent females, are intrasexually competitive (Pröhl & Berke, 2001; Haase & Pröhl, 2002). Pröhl & Berke (2001) reported that females choose their breeding territories based on the presence of tadpole rearing sites, whereas males choose their territories based on the presence of females. Males display on elevated perch sites (Limerick, 1980) and are very aggressive towards conspecific males when guarding their territory (Pröhl, 2005). A strong 'prior residence effect' has been observed that commonly determines the winner of contests, regardless of the size of the intruder (Baugh & Forester, 1994; Pröhl & Hödl, 1999). After territorial battles, shifts have been observed where winning males gain access to the most female dense territory, resulting in the successor obtaining more mates (Pröhl & Berke, 2001). Thus, aggression appears to be beneficial because it may lead to the acquisition of higher quality territories. Exploratory behaviour has not been investigated in *D. pumilio*, although it is likely that it affects the intensity by which males without a territory explore and occupy vacant territories. Furthermore, because males rarely visit the borders of their territories (Pröhl & Berke, 2001), an increase in exploratory behaviour at the fringes of a held territory could affect territory safeguarding. This behaviour could then increase the probability of occupying more female dense territories, as well as help to increase a male's territory size. However, as mentioned above, aggression and exploration should also be costly. The risk of predation should vary depending on whether individuals are using an aposematic or cryptic predation avoidance strategy, and, thus, recently diverged populations of *D. pumilio* provide an excellent study species for investigating co-variation between coloration and behaviour.

The present study aimed to test whether male aggression towards population-specific coloured intruders and a willingness to explore novel habitats differed among populations displaying considerable differences in dorsal coloration. To our knowledge, this is the first study that has compared either aggressive or explorative behaviour between several aposematic and conspicuous populations. We predict a positive relationship between visual conspicuousness (measured by chromatic contrasts) and the level of expressed behaviour. We expect to observe this pattern because, in populations of aposematic frogs, males are expected to be relieved from the cost of predation and should therefore be freer to be more explorative and more aggressive. Thus, we expect to find a positive relationship between these two behaviours and conspicuousness across the recently diverged populations.

MATERIAL AND METHODS

In November 2007, we visited eight populations of *D. pumilio* in Bocas del Toro, Panama: Cayo Nancy (CN), Bastimentos Red Frog Beach (BRF), Almirante (Al), Isla San Cristobal (SC), Isla Colon (IC), Isla Pastores (IPa), Cayo Agua (CA), and Isla Popa (IP) (Fig. 1, Table 1). We measured spectral reflectance, exploration, and aggression of males from the different populations. Biases potentially introduced by differences in temporal activity were minimized by conducting our behavioural trials after the morning activity peak (09.15 h) and before any increased activity in afternoons (15.30 h) (Haase & Pröhl, 2002; Graves, Stanley & Gardner, 2005). Males were captured, kept in transparent plastic cups with netting, and placed in a shaded spot to avoid heat stress. We identified target males either by their calls, because only males call, or by the presence of a dark vocal sac, which was visually inspected after capture ($N = 4$) (Bunell, 1973).

BEHAVIOURAL TRIALS

All behavioural trials were performed in cylindrical arenas (diameter 36 cm, height 25 cm), made of opaque plastic (thickness 0.5 mm), which was approximately 20 times the length of the mean size male in the present study. The trials were recorded with a vertically positioned digital video recorder attached to a tripod and covered with a black umbrella.

The mean male territory core area of *D. pumilio* has been estimated to be approximately 3 m², with home ranges of approximately 20 m² (Pröhl & Berke, 2001). By placing the arena for the aggression trials as close to the male's calling site as possible, we maximized our chances of being within his core area to invoke a natural aggressive response. Aggression

Table 1. Mean levels of exploration (%) and aggression (0–1) with SEs across eight isolated populations of *Dendrobates pumilio*

Locality	Conspicuousness (N)	Conspicuousness group	Aggression (N)	Aggressiveness (0–1)	Exploration (N)	Exploration (%)
Cayo Nancy (CN)	39	High	13	0.69 ± 0.13	14	38.77 ± 3.16
Bastimentos, Red Frog Beach (BRF)	25	High	9	0.78 ± 0.15	9	35.83 ± 2.06
Almirante (Al)	20	High	10	0.60 ± 0.16	10	35.58 ± 3.80
Isla San Cristobal (SC)	18	High	8	0.88 ± 0.12	8	42.28 ± 3.74
Isla Colon (IC)	32	High	9	0.67 ± 0.17	11	37.01 ± 4.12
Isla Pastores (IPa)	20	Low	6	0.33 ± 0.21	8	24.88 ± 3.97
Cayo Agua (CA)	24	Low	9	0.11 ± 0.11	11	25.74 ± 2.18
Isla Popa (IP)	20	Low	6	0.83 ± 0.17	7	37.69 ± 5.33

Sampling locality name abbreviations are given in parentheses.

was tested with mirror-image stimulation using a mirror (height 10 cm, length 22.5 cm) that was placed inside each arena, opposite an observer. A frog was released into the arena either voluntarily after removing the netting off the plastic cup or by gentle taps/shakes. The observer sat within 1.5 m of the arenas to prevent climbing frogs reaching the top of the arena where they could escape.

In the aggression trials, 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° of the mirror. This arrangement is assumed to elicit a natural reaction from the frog (i.e. matching the reaction when an intruder of the same colour and size was found in its territory). Our aim was not to separate whether aggression relates to conspicuousness through a genetic or plastic change but, rather, to measure the behaviour of males in the setting of their population. The male's behaviour during 5 s after he detected his reflection was used to categorize the frog as either 'aggressive' (1) (called or tracked statically, called or tracked when advancing, charged and veered, or charged and contacted the mirror) or 'not aggressive' (0) (did not move, attempted to escape or hid) (Baugh & Forester, 1994).

Exploration trials were conducted in standardized patches of habitat (flat ground with mixed leaf litter and no large vegetation) away from male capture sites. Frogs were introduced to the arena when the trial started, ensuring they were naïve to the environment. Movements during the first 5 min were recorded on video and later traced using IMAGEJ (Abramoff, Magelhaes & Ram, 2004) to produce an image of the movement path. To calculate exploration, we divided the circular arena to smaller units by overlaying an image of a square divided in 10 × 10 equally sized units. Each unit hence represents a proportion of the total arena size with the units that were cut off as a result of the outer circle of the arena being smaller. By overlaying the traced path of movements on the grid image, we could count each visited unit and calculate the exploration percentage of the total area. Each unit was only counted once.

CONSPICUOUSNESS ESTIMATION

Birds prey on many tropical anurans (Poulin *et al.*, 2001) and attack clay models of both *D. pumilio* (Saporito *et al.*, 2007b) and other species of poison dart frogs (Noonan & Comeault, 2009). The diurnal nature and colour signalling by *D. pumilio* further suggest that birds are important predators. Therefore, spectral sensitivity data of *Pavo cristatus* (peafowl) with SWS1 : SWS2 : MWS : LWS cone proportions of 1 : 1.9 : 2.2 : 2.1 were used in the visual model to represent a bird with a typical violet sensitive vision

system (Hart, 2002). Variation in the incident light spectra is similar within and among the localities (Rudh *et al.*, 2011). We therefore chose a typical natural forest light spectrum (similar to a D65 standardized forest light) from locality Cayo Nancy and a quantum flux of 10 000, which represents moderately shaded day light conditions (Osorio *et al.*, 2004).

Dorsal reflectance of trial frogs and additional individuals (Table 1) were measured using a telera-diometer. The telera-diometer was calibrated *sensu* Sumner, Arrese & Partridge (2005). It consisted of an Avantes spectrophotometer (AvaSpec-2048FT-SPU), with a 400-nm optic cable, a modified Nikon FM2 camera with a Nikon Rayfact PF10545MF-UV Quartz lens, a quartz filter and a Nikon HS-8 s lens shade. We used a lens aperture of 4.5 and a focus distance of 48 cm. Before measuring each individual, the telera-diometer was calibrated to a white diffuse reflection standard. We recorded spectra with the AVASOFT, version 7.0 (Avantes). We visually selected the median from three measurements of the dorsal coloration of each individual. For some individuals, this was not possible because the dark dorsal pattern prevented measurements of three different regions of the dorsal surface.

The median of five measurements of each of nine typical substrates (local brown soil, common bark, live green leaves, dead yellow leaves, reddish leaves, greyish leaves, and three brown coloured leaves) was used to represent each substrate. Frog dorsal reflectance spectra were contrasted to these nine substrate spectra using chromatic contrast calculations (ΔS) (Osorio *et al.*, 2004). Bespoke software (Håstad & Ödeen, 2008) was used to calculate colour distance (ΔS) (Vorobyev & Osorio, 1998; Osorio *et al.*, 2004) between frog dorsal spectra and the natural substrate spectra with the setting defined above, from 320 to 700 nm and interpolated to a step width of 1 nm. This resulted in a measure of perceived difference between two colours in units of 'just noticeable differences'. Achromatic contrast (ΔL) can also influence visual detection (v. Campenhausen & Kirschfeld, 1998), and was calculated *sensu* Siddiqi *et al.* (2004) using the double cone spectral sensitivity of the peafowl (Hart, 2002).

The type of substrate that frogs were seen sitting on when first observed was examined for 384 frogs during the sampling of individuals in 2007 and 2008. These proportions of background use were used to weight the nine chromatic contrasts (ΔS) values for each individual proportionally to create a final measurement of conspicuousness per individual that includes the behaviour of the species. As a reference for these chromatic contrasts, we calculated a habitat substrate chromatic noise, which was defined as the mean of all pairwise substrate chromatic contrasts.

STATISTICAL ANALYSIS

To categorize populations into conspicuousness groups, we tested whether frog-to-background chromatic contrast for each population was higher than the mean substrate chromatic contrast using one-way *t*-tests. We used a Bayesian approach implemented in MCMCglmm, version 2.15 (Hadfield, 2010) in R (R-Development-Core-Team, 2012) where the effect of chromatic contrast group on aggression and exploration was analyzed using linear mixed-effects models. Population was implemented as a random effect nested within the chromatic contrast group. The binary variable 'aggression' was analyzed using a categorical residual distribution and the exploration percentage was arcsine-transformed and analyzed using a normal distribution. All prior distributions were chosen to be rather flat, thus representing little prior knowledge. For each analysis, two chains were run and after a burn-in of 8×10^5 , the 100th of the subsequent 9.2×10^6 iterations were saved, giving a total of 2×92000 draws from the posterior distribution. The posterior mode and highest posterior density (HPD) intervals of each statistic were calculated from the posterior distribution. The posterior mode and HPD interval of the high conspicuousness group represent the difference compared to the low conspicuousness group. If the HPD does not include 0, then there is a significant difference between conspicuousness groups. We also tested for a correlation between the two behaviours among populations using Pearson's product moment correlation test.

RESULTS

The substrate used by 384 frogs was observed and classified as bark ($N = 130$), live green leaves ($N = 23$), dead leaf litter ($N = 203$), and soil ($N = 14$). Leaf litter (54.9% of total observations) was photographed at the natural habitats and the proportions of leaf types were later manually scored as yellow leaves (0.04), reddish leaves (0.15), greyish leaves (0.15), and brown leaves (0.66). Brown-type background (i.e. bark, soil and leaf litter) was the predominant background type in all populations in the present study (75% to 100% of the observations, total number of observations: BRF, $N = 23$; CN, $N = 18$; IP, $N = 5$; SC, $N = 19$; IC, $N = 26$; Ipa, $N = 8$; Al, $N = 20$; CA, $N = 23$). Therefore, because substrates that could significantly alter the outcome of the subsequent combined estimation of chromatic contrast (red, yellow, and green leaves) were so rare, the results were robust to errors in the estimation of leaf litter composition. No clear preference for a specific type of substrate was observed. The mean of all pairwise substrate chromatic contrasts was used as a 'background noise' reference because it reflects the colour variation in the natural background.

Population means of ΔS ranged from 3.85 in Isla Popa to 14.00 in Cayo Nancy. Populations with a higher chromatic contrast between frogs and their native substrates than the mean substrate chromatic contrast were defined as having high conspicuousness (Almirante: $t_{19} = 8.0$, $P < 0.001$; Bastimentos Red Frog Beach: $t_{24} = 26.3$, $P < 0.001$; Cayo Nancy: $t_{38} = 23.1$, $P < 0.001$; Isla San Cristobal: $t_{17} = 4.3$, $P < 0.001$; Isla Colon: $t_{31} = 4.9$, $P < 0.001$). Populations with equal or lower chromatic contrast (i.e. cryptic appearance) were defined as having low conspicuousness (Cayo Agua: $t_{23} = -5.0$, $P = 1$; Isla Popa: $t_{19} = -4.6$, $P = 1$; Isla Pastores: $t_{19} = -0.5$, $P = 0.69$) (Fig. 2). This ordinal variable was subsequently used as a predictor in subsequent statistical analyses. Achromatic contrasts (ΔL) were similar to chromatic contrast (ΔS) (Fig. 2), except for Almirante, where individuals had dark red dorsal coloration, resulting in a high chromatic contrast but low achromatic contrast.

The mean of male aggression ($N = 70$) varied substantially among populations and ranged from 0.11 (Cayo Agua) to 0.88 (Isla San Cristobal) (group mean: high = 0.72, low = 0.43) (Fig. 3, Table 1). Population conspicuousness significantly predicted male

aggressiveness at 90% HPD (0.194, 2.673, posterior mode 1.522) but not at 95% HPD (Table 2).

The mean of male exploration ($N = 78$) varied between 24.88% (Isla Pastores) to 42.28% (Isla San Cristobal) (conspicuousness group mean: high = 37.69%, low = 29.44%) (Fig. 3, Table 1). Frogs from more conspicuous populations were significantly more explorative only at a 90% HPD (0.011, 0.168, posterior mode 0.090) but not at 95% HPD (Fig. 3, Table 2).

Aggression and exploration was strongly correlated among populations ($r = 0.93$, $t = 6.184$, d.f. = 6, $P < 0.001$). Two of the low chromatic contrast populations, IPa and CA, had lower aggression and explorative behaviour than all other populations, including the third low chromatic contrast population IP.

DISCUSSION

Aggression towards population-specific males and exploration of a novel habitat tended to be higher in populations with more conspicuous individuals among eight populations of *D. pumilio*. We suggest that the results obtained in the present study reflect different levels of constraint on behaviours that were caused by different predator avoidance strategies (i.e. aposematism versus crypsis). A difference in the level of aggression may have two underlying causes. First, males that are more conspicuous may be intrinsically more aggressive. Second, there could be a higher level of aggression directed towards conspicuous competitors. In either case, the level of aggression would be

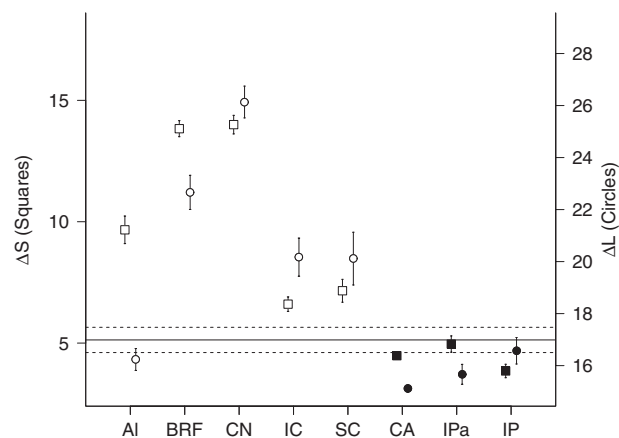


Figure 2. Mean *Dendrobates pumilio* population values of conspicuousness. Conspicuousness is shown by chromatic contrast (ΔS) and achromatic contrast (ΔL), both obtained from a visual model for an avian observer, weighted by natural substrate composition and frog behaviour. Chromatic contrast is indicated by squares (\pm SE) and scaled to the left axis. Horizontal lines indicate the mean of all pairwise substrate chromatic contrasts (solid) with SEs (dashed). Achromatic contrast is indicated by circles (\pm SE) and scaled to the right axis. The conspicuousness group is indicated by white and black symbols, representing high and low conspicuousness, respectively. AI, Almirante; BRF, Bastimentos Red Frog Beach; CA, Cayo Agua; CN, Cayo Nancy; IC, Isla Colon; IP, Isla Popa; IPa, Isla Pastores; SC, Isla San Cristobal.

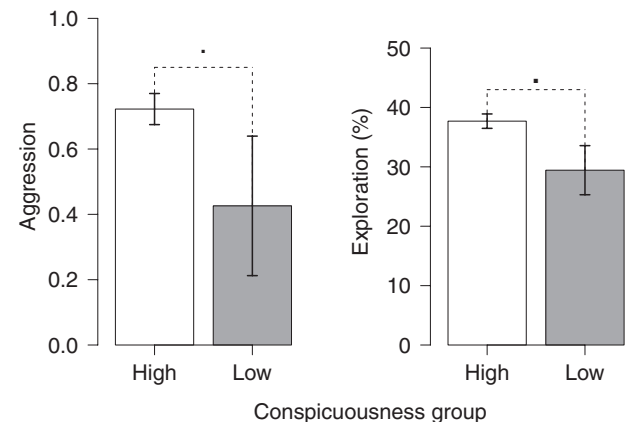


Figure 3. *Dendrobates pumilio* frogs from conspicuously coloured populations tended to behave more aggressively and exploratively compared to individuals belonging to cryptic populations. Conspicuousness group mean aggression is shown on the left. Mean exploration is shown on the right. Bars indicate the SE. Significant differences between groups at 90% highest posterior density intervals are indicated by a point.

Table 2. *Dendrobates pumilio* frogs from conspicuously coloured populations tend to behave more aggressively and be more explorative compared to individuals belonging to cryptic populations

	Aggression		Exploration	
	Posterior mode	95% HPD	90% HPD	90% HPD
Conspicuousness group				
Low	-0.462	-1.626, 0.905	-1.405, 0.648	0.235, 0.360
High	1.522	-0.061, 2.989	0.194, 2.673*	-0.011, 0.168*

The effects of conspicuousness on aggression and exploration are represented by posterior mode and two levels of highest posterior density (HPD) intervals (generalized linear mixed-model fitted by Laplace approximation). Values given for the high conspicuousness groups represent differences compared to the low conspicuousness groups. Significant differences between groups are defined as when the HPD intervals for the high conspicuousness group do not include 0 (indicated by an asterisk).

higher in populations with predominantly conspicuous males.

The long-lasting notion of an association between aposematism and conspicuous behaviour (Wallace, 1889; Poulton, 1890) has recently regained scientific attention. By using simulated prey evolution and human predators, Sherratt, Rashed & Beatty (2004) showed that unprofitable prey that survived ‘attacks’ evolved slower movements. Merilaita & Tullberg (2005) combined simulated evolution and phylogenetic comparative analysis of Lepidoptera species and found that there could also be an influence of behaviour on the evolution of antipredator coloration (more precisely, the evolution of crypsis can be constrained in diurnal species). The evolutionary relationships between gregarious behaviour and aposematism have been repeatedly addressed (Ruxton & Sherratt, 2006) and are supported by phylogenetic comparative studies (Sillén-Tullberg, 1988). Male aggression was recently found to positively correlate with colour brightness in both focal and intruder males in one aposematic island population of *D. pumilio* (Crothers, Gering & Cummings, 2011). Furthermore, Pröhl & Ostrowski (2011) found a difference in behaviour that affected exposure (e.g. activity and time spent hidden) in two island populations. It was suggested that this behaviour was connected to frog coloration (bright red versus green). Escape behaviour has also been found to be lower in *D. pumilio* than in an edible frog species (Cooper, Caldwell & Vitt, 2009). In the present study, we report a positive trend between the strength of antipredator coloration and the level of aggressive and exploratory behaviour across eight divergent populations of the same species. Thus, the findings of the present study fill the gap between studies comparing individuals belonging to one or two populations of the same species and studies comparing across species that diverged a long time ago. Our findings also support the view that divergence in antipredator strategies causes a general constraint of behaviours.

It is worth noting that one of the eight populations observed in the present study strongly deviated from the expected pattern in both aggression and exploration. Males from Isla Popa did not behave as predicted by their low chromatic contrast. Instead, they showed similar levels of aggression and exploration as conspicuously coloured populations (Table 1). Because of the difficulty to locate, observe, and capture the cryptic and small individuals from Isla Popa, we only managed to sample relatively few individuals ($N = 7$), which means that there may have been a bias in our sample towards more bold and aggressive males. Alternatively, there may be other factors affecting aggression and exploration that overrule the effects of coloration in this particular population, such as

microclimate effects or population density, although this would be impossible to test statistically based on only one deviating population.

How could a relationship between antipredator coloration and behaviour influence the future evolutionary trajectories of these populations? Speciation is generally assumed to be hampered by gene flow. This is because classical population genetic models indicate that low levels of gene flow are needed to counteract population differentiation (Mayr, 1963; Coyne & Orr, 2004). Divergence under gene flow is possible; however, because it generally requires divergent selection on two or more genes, strong linkage disequilibrium by chromosomal rearrangements, for example, is required to prevent the association of these genes to break down with recombination (Pinho & Hey, 2010). Therefore, any mechanism that reduces the probability of hybridization events between diverging populations plays an important role on the likelihood of population differentiation, and, ultimately, speciation. Explorative behaviour and aggression are interesting traits from this point of view because they directly affect interactions between individuals and could reduce the probability of hybridization events, even at early stages of population divergence. Most of the morphologically diverged populations of *D. pumilio* are physically isolated from each other at present, with clear genetic differentiation and very low gene flow between populations (Rudh *et al.*, 2007; Wang & Shaffer, 2008; Wang & Summers, 2010).

The crucial issue then becomes whether the observed differences in behaviour of individuals decrease the probability of migration events between populations that results in gene flow. For example, if a cryptic *D. pumilio* male were to migrate into a conspicuous population, he would most likely be at a disadvantage when competing for territories with the local aggressive males. Furthermore, because access to a territory is a prerequisite for acquiring mates in this species (Pröhl & Berke, 2001), a cryptic male's chances of attracting an aposematic female in an aposematic population should be low. However, if he adjusted his behaviour to match the aggression of local conspicuous males, he would increase his exposure to predators. This is potentially very costly because cryptic individuals suffer higher predation rates compared to those with an aposematic strategy (Saporito *et al.*, 2007b). Moreover, because cryptic males display at less exposed sites, this will make them less attractive to females in the presence of aposematic males (Rudh *et al.*, 2011). Thus, successful migration and reproduction of cryptic males into conspicuous populations is not very likely.

By contrast, a conspicuous male that migrates into a cryptic population is likely to experience a competitive advantage for females over local cryptic males.

This advantage rests on three lines of inference: first, the conspicuous males likely have an advantage when competing with cryptic males over territories (as shown in the present study); second, conspicuous males display at more exposed sites (Rudh *et al.*, 2011), which increases their exposure to females; and, third, conspicuous males may be preferred more by females (Maan & Cummings, 2009). Thus, an immigrant conspicuous male could potentially exclude less aggressive cryptic males from better breeding sites. However, bold and aposematically coloured males in a cryptic population would not only experience more attention from females, but also have a higher risk of attracting the attention from naïve predators. Therefore, we argue that successful immigration (with reproduction) between the different populations of *D. pumilio* is likely to be counteracted by a combination of natural and sexual selection.

Most studies on the effect of male aggression on the speciation process have focused on negative frequency-dependent selection. A repeated observation is that males express more antagonistic behaviour towards males with similar colour morphs (Kral, Jarvi & Bicik, 1988; Saetre, Kral & Bicik, 1993; Seehausen & Schluter, 2004; Grether *et al.*, 2009). This type of negative frequency-dependent selection facilitates the invasion of new colour morphs and the maintenance of colour polymorphism. Thus, gene flow between differently coloured populations should then always be promoted. However, there is growing evidence for colour-related aggression biases, as well as for asymmetric dominance relationships between colour morphs, indicating that the role of male contest competition in speciation may be more complex than previously assumed (Dijkstra & Groothuis, 2011). For example, cichlid males belonging to the sympatric species pair *Pundamilia pundamilia* (with blue males) and *Pundamilia nyererei* (with red males) from two wild populations were found to direct more aggression towards red male rivals than towards blue rivals (Dijkstra *et al.*, 2007). In *D. pumilio*, Crothers *et al.* (2011) presented evidence for increased male aggression towards more brightly coloured males within a single aposematic population, and we show in the present study that there is a trend for higher aggression and exploration in populations with conspicuous individuals. Studies on the heritability of behaviour (e.g. aggression and exploration) in *D. pumilio* are needed to better understand the suggested co-evolution between morphology and behaviour.

When populations adapt to their environment, complexes of traits may change non-independently of each other, either as a result of genetic correlations between them or because of synergistic effects on fitness. The latter, where a number of genetically independent traits form a joint target of divergent selection, has

been suggested as a favourable condition for speciation (Nosil, Harmon & Seehausen, 2009). We argue that population divergence in predator avoidance strategy by shifts between aposematism and crypsis represents a compelling example of this condition. An evolutionary gain of warning coloration may function as a key innovation that relieves a whole suite of behavioural traits from the constraint caused by predation risk because the efficiency in avoiding predation no longer relies on the ability to hide (Speed *et al.*, 2010; Rudh *et al.*, 2011). The subsequent combined divergence in both morphology and behaviour may then affect the level of reproductive isolation between populations that have different predation avoidance strategies. Reproductive isolation may be promoted by both mating behaviour differences (e.g. territoriality, sexual display or sharing of mating space) and by natural selection against migrants and hybrids with suboptimal trait combinations.

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