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Ontogenetic colour change in *Oreophryne ezra* (Anura: Microhylidae) reflects an unusual shift from conspicuousness to crypsis but not in toxicity

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Ontogenetic colour change (OCC) may signal a switch in defensive strategies across development. Commonly, juveniles use cryptic colours and transition to conspicuous coloration as adults. Extensive theoretical and empirical work suggest such a transition is more likely than the converse. *Oreophryne ezra*, a newly discovered frog from New Guinea, however, undergoes OCC that belies expectations. We investigated the notion juveniles are aposematic while the adults use crypsis. We quantified the extent of conspicuousness of *O. ezra* across life stages using spectrophotometry and models of avian vision; and analysed skin extracts for the presence of toxic alkaloids. As predicted, the juvenile colour patterns were estimated to be conspicuous to likely predators against the frogs natural viewing backgrounds, while adults consistently fell below the threshold of discriminability. However, we discovered no alkaloids in either juveniles or adults. This suggests the juveniles use alternative defensive chemicals, or the colour pattern has an alternative origin – to which we offer the hypothesis that juvenile frogs may be Batesian mimics of a synoptic species of weevil. This study highlights the potential for this system, and others like it, to inform our understanding of the adaptive significance of OCC and the selective drivers for different anti-predatory strategies.

ADDITIONAL KEYWORDS: adaptive coloration – mimicry – predation.

INTRODUCTION

Ontogenetic colour change (OCC) is a non-reversible change in body colour occurring as an animal transitions between different life stages (Booth, 1990; Grant, 2007; Wilson, Heinsohn & Endler, 2007). It occurs across a diversity of taxa from arthropods to primates, yet in most cases we have little understanding of why OCC exists. It has been suggested though that studies on animals that express OCC will be key to answering why and how animals have acquired conspicuous coloration (Stevens & Ruxton, 2012) – a question that has been vigorously debated for over a hundred years (Poulton, 1890; Cott, 1940; Mappes, Marples & Endler, 2005).

The variety of colour patterns seen in animals tends to reflect different defensive strategies (Ruxton, Sherratt & Speed, 2004). For example, aposematic

prey, when detected, deter predators by advertising unpalatability with conspicuous colours, while cryptic prey avoid detection altogether by matching the background colour patterns. Both strategies in their respective contexts are equally effective but just why one strategy is adopted over another is largely unknown. Theoretically, though aposematism is predicted to evolve when the opportunity costs associated with crypsis outweigh the costs of engaging in conspicuous behaviour (Ruxton *et al.*, 2004; Higginson & Ruxton, 2010). One such cost is constrained mobility, which may be imposed by the reduced effectiveness of cryptic coloration when in motion (Ioannou & Krause, 2009). This restriction in movement limits potential foraging range and/or mating opportunities, both of which are increasingly important as an animal develops from juvenile to adult (Merilaita & Tullberg, 2005).

Comparative analyses of taxa that use both aposematic and cryptic strategies overwhelmingly support the notion aposematism is more likely to occur in larger

species and at later developmental stages. In dart-poison frogs, for instance, larger species are more likely to be conspicuously coloured (Hagman & Forsman, 2003; Rudh, 2013). While in the polymorphic strawberry poison frogs the populations with conspicuous coloration have a greater mean body size than their cryptic counterparts (Rudh, 2013). Likewise, animals in general that switch between crypsis and aposematism adopt conspicuous colours later in their development, when they are much larger in size (Grant, 2007; Higginson & Ruxton, 2010; Valkonen *et al.*, 2014). A transition in the contrary direction, from aposematism to crypsis, is also theorized under certain circumstances, for instance, if advertising at a larger size increases the risk of predation by predators that are not toxin averse (Higginson & Ruxton, 2010). In such instances, then switching to another tactic may be of greater benefit. However, exemplar natural systems with which to examine these theoretical claims have yet to be empirically confirmed.

Although body size is strongly correlated with aposematism, it is not the only trait that has been implicated in determining when aposematism is most appropriate. For instance, nocturnal animals are generally cryptic coloured to aid with hiding during the day, whereas animals that are diurnal may adopt a more conspicuous lifestyle (Merilaita & Tullberg, 2005). In frogs, species that are conspicuously coloured are strictly diurnal (Myers, Paolillo & Daly, 1991; Grant *et al.*, 2006). Animals that shift their diel activity across life stages potentially provide an opportunity to disentangle the relative importance of light conditions and body size as constraints on adaptive coloration strategies. In frogs, such shifts are unknown apart from our study animal.

The Papuan frog, *Oreophryne ezra* – only recently described from an island south-east of New Guinea (Kraus & Allison, 2009) – displays a dramatic age-related change in coloration and behaviour unlike other frogs. The original description described the juveniles as glossy black with yellow spots, whereas adults are uniform pink/peach with an iridescent blue iris (Kraus & Allison, 2009). Juveniles were also reported to sit conspicuously exposed on vegetation during the daytime and not easily disturbed, both of which are unique among Papuan frogs in the experience of F.K. (based on >6500 frogs of 220 species), whereas adults are only active at night and, like many other *Oreophryne* species, often call from hidden arboreal sites and are averse to human approach. On first appearances, the coloration in juvenile *O. ezra* has a striking general resemblance to patterns displayed by dart-poison frogs and, specifically, is similar dorsally to *Oophaga arborea*, *Ranitomeya vanzolinii* and some *Oophaga histrionica*. In addition, yellow contrasting with black is common among aposematic organisms (Summers *et al.*, 2015) more generally and has been

experimentally shown to ward off predator advances (Schuler & Hesse, 1985). In contrast, the lack of contrasting coloration in the adults, and their nocturnal behaviour, suggests they are not aposematic and are instead either cryptic or their body colour is not important for avoiding detection.

We provide the first investigation of the potential adaptive basis of coloration for *O. ezra*, which undergoes extreme OCC. Specifically, we tested the hypothesis that OCC, in *O. ezra*, reflects a shift in defensive strategy across life-history stages; from aposematism in juveniles to crypsis in adults. This makes two general predictions, which form the basis of our study. First, the colour patterns of juveniles should be conspicuous to potential predators and, conversely, adults should be relatively inconspicuous in their natural viewing conditions (Mappes *et al.*, 2005; Summers *et al.*, 2015). Second, the putatively aposematic juveniles should possess chemical defences (Summers *et al.*, 2015) while the adults dispel with such defence. Among conspicuously coloured diurnally active frogs, this often takes the form of alkaloids sequestered in the skin, which are a class of chemicals both distasteful and toxic to common anuran predators (Daly, 1995; Daly, Garraffo & Spande, 1999; Daly, Spande & Garraffo, 2005; Saporito *et al.*, 2011). We tested each of these expectations.

METHODS

HABITAT

Oreophryne ezra are restricted to a cloud forest, ~0.5 km², on the upper 170 m of Mt. Riu, Sudest Island (–11.5063S, 153.4311E). Sudest Island is 866 km² in extent and has a maximum elevation of 806 m a.s.l. lying 270 km ESE of the south-easternmost tip of New Guinea. The frogs were rather common during the survey period 5–18 August 2013. Forest was of short stature (3–5 m) with dense tangles of tree boles, shrubs and other vegetation.

ANIMAL COLLECTIONS AND BEHAVIOURAL OBSERVATIONS

Although juveniles had been collected on a previous trip, there were no notes on exactly where they were found or what the juveniles were doing when collected. We conducted an extensive search to locate them and to establish the range of backgrounds on which the animals perched if indeed they did. All juveniles were seen in a relatively open area just below the summit. Juveniles were then later collected opportunistically by hand, within this region, mostly during the morning from 0600 to 1200 h. We collected adults at night when they were active. Males engaged in advertisement calling, primarily from *Pandanus*, either on or

among the leaf litter or from the layered bark. On occasion, we observed them calling in the relative open on adjacent vegetation. Adults were never observed in the open during the day. Twenty-two focal juveniles were observed for 30 min each, to assess the activity budget and to describe the movement pattern to establish the range of backgrounds they may use.

SPECTRAL REFLECTANCE

We measured frog colour ($n = 19$ juveniles, 30 adults) and background material in situ using a portable spectrophotometer fitted with a cosine corrector (Jaz EL-200, Ocean Optics, Dunedin, FL). We took readings with the probe held perpendicular to the measurement surface, maintained at a working distance of 5 mm by a polycarbonate probe-tip. All settings were kept consistent at an integration time of 180 ms, a boxcar width of three, and five spectra averaged per measurement. We recalibrated the spectrophotometer between measurements using a Spectralon 95% white standard. Owing to the minimum-achievable sampling area of *c.* 5 mm, readings of juvenile coloration came from either larger individuals that had yet to fully transform, or from larger spots found on the flank or thigh of smaller individuals. To quantify viewing backgrounds, we measured the reflectance of surfaces the juveniles were found on (e.g. fresh leaves and bark), and the accumulated leaf litter in the core of palm-like *Pandanus*, within which adults typically reside.

VISUAL MODELLING

To estimate the subjective conspicuousness of frogs across life stages, we used the log-linear receptor-noise-limited model with a tetrachromatic avian visual phenotype (Vorobyev & Osorio, 1998; Vorobyev *et al.*, 1998), as birds are almost certainly the predominant predator (Brass, 1959). This allows for the estimation of chromatic (hue/saturation; ΔS) and achromatic (luminance, or subjective 'brightness'; ΔL) distances between stimuli in units of Euclidean, noise-weighted 'just-noticeable distances' (JNDs), assuming the limits of colour discrimination are set by photoreceptor and neural noise (see Supporting Information for full model details). We used the package 'pavo' (v 1.0; Maia *et al.*, 2013) in R (v 3.2; R Core Team, 2014) for all spectral processing and visual modelling.

Following initial visual modelling, we used a two-step approach to estimate the chromatic and achromatic discriminability of colour patches against natural backgrounds, and pattern elements against one another (Maia & White, 2017). We first used a permutational multivariate analysis of variance

(PERMANOVA; Anderson, 2001) on chromatic (ΔS) and achromatic (ΔL) distances to statistically test the separability of groups in colour space. We used a PERMANOVA instead of parametric alternatives as it appropriately accounts for the multivariate nature of colour space distances, and does not rely on the assumptions of multivariate normality and homogeneity that are typically violated by visual model data (Maia & White, 2017). For our analysis of juvenile frogs, we included spots, dorsa and background material as distinct groups. When analysing adult frogs, we included dorsa and background material. If groups were identified as statistically distinct by PERMANOVA, we then used a bootstrap procedure to estimate whether patches were discriminable to avian viewers (i.e. perceptual 'effect sizes'). For a given run, we sampled points from each group – equal to the size of the original group – with replacement, and estimated the distance between the centre of each distribution. We repeated this process 1000 times, generating a distribution of subjective chromatic and achromatic distances between groups, from which we calculated a 95% confidence interval. We then inspected this interval to predict the discriminability of groups in colour space. If it contained, or was entirely less than, the threshold value of 1 JND, then the patches were predicted to be indiscriminable to an avian viewer under ideal (i.e. laboratory) conditions (Siddiqi, Cronin & Loew, 2004). Conversely, intervals that lie above this threshold suggested that such colour patches should be increasingly conspicuous to avian viewers (Fleishman *et al.*, 2016).

CHEMICAL COLLECTION AND ANALYSES

Alkaloid analysis

Whole-skin extracts from 20 juveniles and 20 adults were transferred to 100% methanol in 4 mL glass vials with Teflon-lined caps. We applied the procedure outlined in Saporito *et al.* (2010) for extracting alkaloids from the skins (see Supporting Information for full procedure).

ETHICS STATEMENT

All handling and processing of frogs followed approved protocols from PNG National Research Institute. We also followed the ABS (Animal Behavior Society)/ASAB (Association for the Study of Animal Behaviour) 'Guidelines for the treatment of animals in behavioural research and teaching'. The PNG Department of Environment and Conservation, PNG National Research Institute, and Milne Bay Provincial Government approved the work and provided permission for all fieldwork activities.

RESULTS

BEHAVIOUR AND OBSERVATIONS

All juveniles we observed were perching diurnally in the open (Fig. 1A) some of which were sitting in full sunlight. Of the juveniles collected/observed, 39% were seen on trunks of palms, young saplings or small trees; 56.5% were perched on leaves of vines, *Pandanus*, or fronds of ferns and palms; and a single individual was on the ground. Apart from one individual observed scaling a tall palm (~3 m), individuals on woody material were often found on pieces hollowed out by ants. The twenty-two focal juveniles did not move for most of the 30-min observation period ($n = 22$; $99.24 \pm 0.88\%$ time/individual). Only three individuals, however, did not move at all. Movements consisting of walking ($n = 19$; $0.70 \pm 0.85\%$ time/individual). The walking was discontinuous, with individuals moving in repeated short spurts ($n = 19$; 1.73 ± 0.97 cm/individual, max = 6.5 cm) punctuated by pauses (often in mid-stride; Fig. 1C). Paused animals would often hold that posture for minutes before progressing further. Changes in direction of movement first involved rotating the head ($n = 14$; $0.03 \pm 0.03\%$ time/individual) towards the new direction and pausing before setting a course in that direction. Other movements we observed included jumping

($n = 9$; $0.02 \pm 0.03\%$ time/individual; 2.75 ± 1.37 cm/individual), striking with tongue ($n = 5$; $0.009 \pm 0.02\%$ time/individual) and body push-up ($n = 1$; 0.001% time/individual). In contrast, during the day adults were not active and were only found by actively searching in the leaf litter that accumulates in the central leaf axils of *Pandanus* (Fig. 1D).

COLOUR

Smaller juveniles were primarily glossy black with strongly contrasting yellow spots (Fig. 1E), although to the naked eye the spots in the groin or adjacent region were more green, but these are typically hidden when at rest. Larger juveniles (Fig. 1F; SVL: 13–15 mm, $n = 5$) and ‘subadults’ (Fig. 1G) were found in various stages of colour transition (Fig. 1E/1F; G; see also Kraus & Allison, 2009: fig. 1). Melanin appeared to disappear from the snout first, and the spots on the abdomen appeared to persist the longest. In contrast, the dorsal colour of adults was typically uniform in colour (i.e. without markings), but the prevailing colour varied between individuals (Fig. 1F–H). From a qualitative, human perspective, adults ranged from apricot to bright red/red-orange to variations of brown.

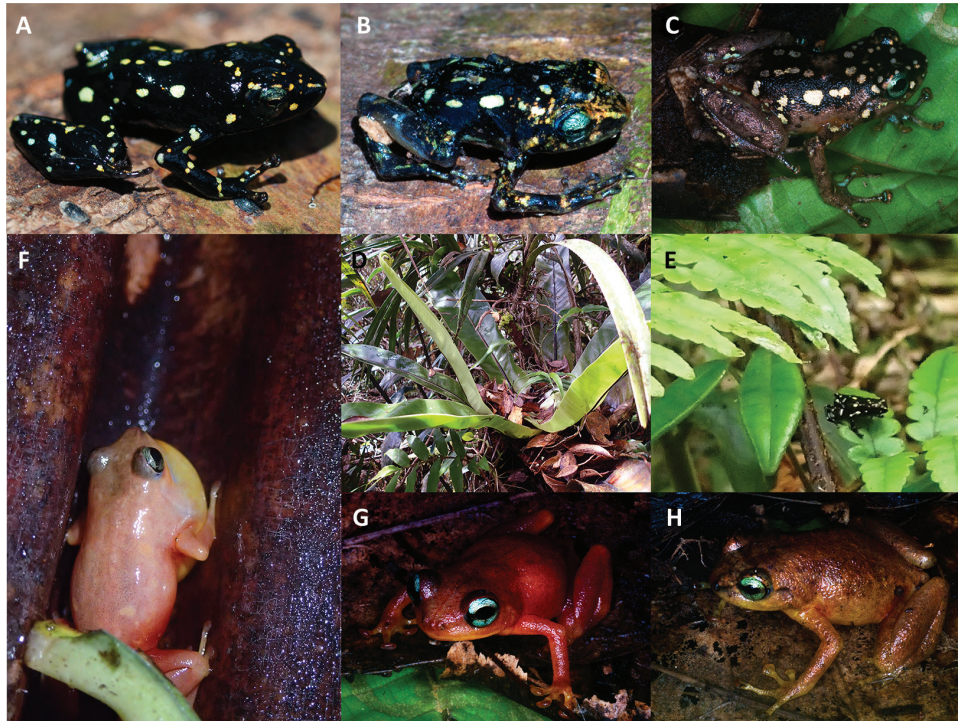


Figure 1. Juveniles ‘basking’ in the open (A) and on ant-hollowed wood (B); juvenile paused in mid-stride held for ~3 min (C); (D) leaf litter filled *Pandanus* – adult hiding place during the day; (E, F) transition of coloration from juvenile to sub-adult; (G, H) adult colour variation and example of a typical calling location (H).

VISUAL MODELLING

We found a distinct shift in the subjective conspicuousness of frogs across life stages: juveniles were predicted to be relatively conspicuous to potential visual predators, whereas adults were chromatically and achromatically cryptic in their environments. Specifically, we found that the colour-pattern elements and backgrounds of juvenile frogs were chromatically (PERMANOVA, $F_{2,140} = 4.94$, $P < 0.01$) and achromatically (PERMANOVA, $F_{2,140} = 90.69$, $P < 0.01$) separable in avian colour space (Fig. 2A, B). Our subsequent bootstrap-based analysis predicted that the contrast of juvenile spots against background material, as well as within-pattern contrasts (spots vs. surrounding dorsal base colour), is chromatically conspicuous (Fig. 2A, B; Table 1). The chromatic contrast of dark dorsal ground colour with the background material falls below the perceptual threshold of avian viewers. Achromatically, juvenile dorsa were predicted to generate strong contrast against background materials and exhibited high within-pattern contrast (Table 1), but the spots themselves were for the most part as bright as the background.

We found the dorsal coloration of adult frogs to be chromatically distinct from background material in avian colour space (PERMANOVA, $F_{1,111} = 8.36$, $P < 0.01$), though this difference was subsequently estimated to fall below the perceptual threshold of avian viewers (Fig. 2C, D; Table 1). Achromatically, adult dorsa and backgrounds were statistically inseparable (PERMANOVA, $F_{1,111} = 0.17$, $P = 0.68$).

CHEMICAL ANALYSES

GC-MS analysis of skin extracts revealed no detectable lipophilic alkaloids (Fig. 3).

DISCUSSION

We investigated the hypothesis that OCC in *O. ezra* reflects a shift in defensive strategies, more specifically, that juveniles use aposematism while adults rely on crypsis. Juveniles were perched during the day on high-contrast backgrounds, with some even found in full sunlight; behaviour consistent with being

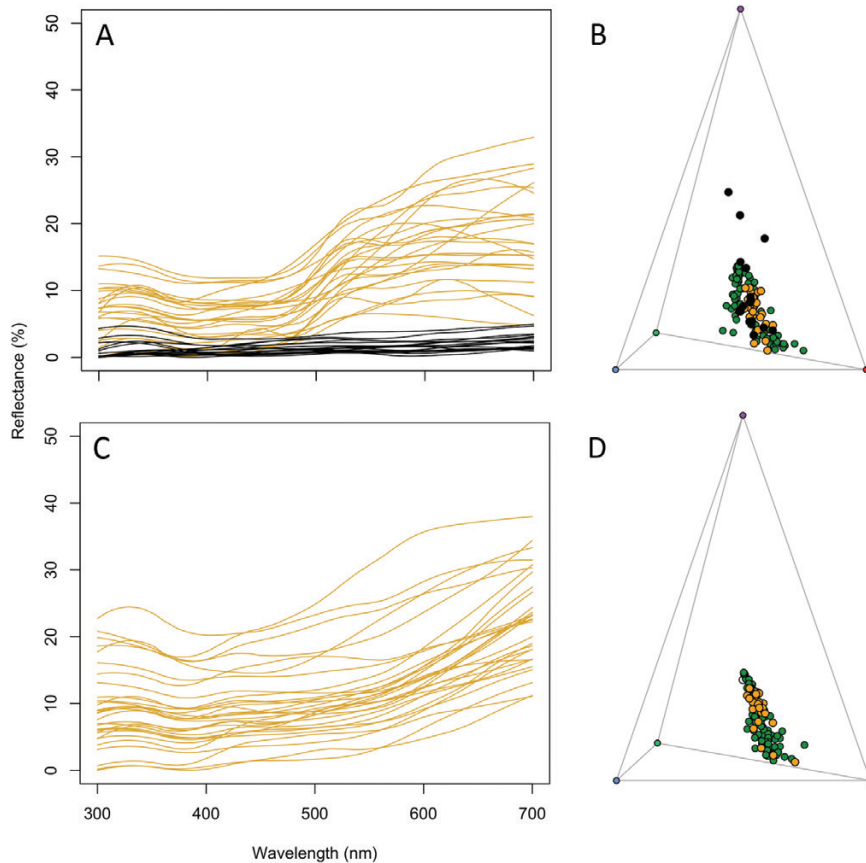


Figure 2. Reflectance spectra of (A) juvenile (black lines, dorsum; yellow lines, spots) and (C) adult (dorsum) body regions; (B, D) the spectra as modelled in a tetrahedral (average-UV avian) colour space, along with background foliage (green points).

aposematic. They also moved erratically with long pauses between short-movement bouts of walking, similar to behaviours seen in aposematic frogs with spotted appearances (Rojas & Devillechabrolle, 2014). As previously recorded (Kraus & Allison, 2009), adults were not active during the day. Visual modelling supported the hypothesis that juvenile colour patterns are relatively conspicuous to potential avian predators and that adults are cryptic in their respective microhabitats (Table 1). However, the change in discriminability

Table 1. Estimated chromatic (ΔS) and achromatic (ΔL) contrasts (JNDs) of juvenile and adult body regions and backgrounds

Comparison	ΔS	ΔL
Juvenile		
Dorsum/background	0.56–1.82	18.54–23.49
Spot/background	2.34–4.24	0.07–2.76
Dorsum/spot	2.27–4.84	19.44–24.79
Adult		
Dorsum/background	0.96–2.87	Not significant

Ranges represent 95% CIs for centroid distances between groups in a tetrahedral (average-UV avian) colour space, estimated using a bootstrap procedure. Groups whose intervals exceed the threshold value of 1 are predicted to be discriminable on average, and are indicated in bold. An estimate of achromatic adult-vs.-background contrast was not calculated since these groups were statistically indistinguishable (see main text). CI, confidence interval; JND, just-noticeable distance.

was not mirrored by the chemical analyses, which failed to detect any alkaloids in either life stage.

Modelling predicted that bird predators should detect juvenile *O. ezra* more readily than adults. The yellow colour patches of the juveniles are predicted to be chromatically distinguishable from both the background substrate as well as the juveniles' own dark dorsal ground colour. The brightness of the markings contrasted strongly with the dark dorsa but not the perching substrate, suggesting the saliency of the yellow markings may rely more on the internal-pattern contrast rather than overall contrast with the background. Of interest is the apparent dominance of the dark coloration relative to the yellow markings. It has been shown, using model caterpillars exposed to wild birds, that models with higher proportions of black to yellow markings had greater survivability than if the model caterpillars were entirely yellow (Barnett, Scott-Samuel & Cuthill, 2016). An animal uniformly brightly coloured can be perceived from a farther distance than one that has smaller patches, and it potentially runs the risk of attracting a predator not deterred by the coloration. In contrast, adult *O. ezra* were indistinguishable from their background and, unlike juveniles, varied somewhat in their predominant coloration. This variation in coloration broadly matches the mixed coloration of the leaf litter under which the frogs hide (Fig. 1D; Table 1), and the cryptic behaviour of adults during the day likely further inhibits detection by avian predators.

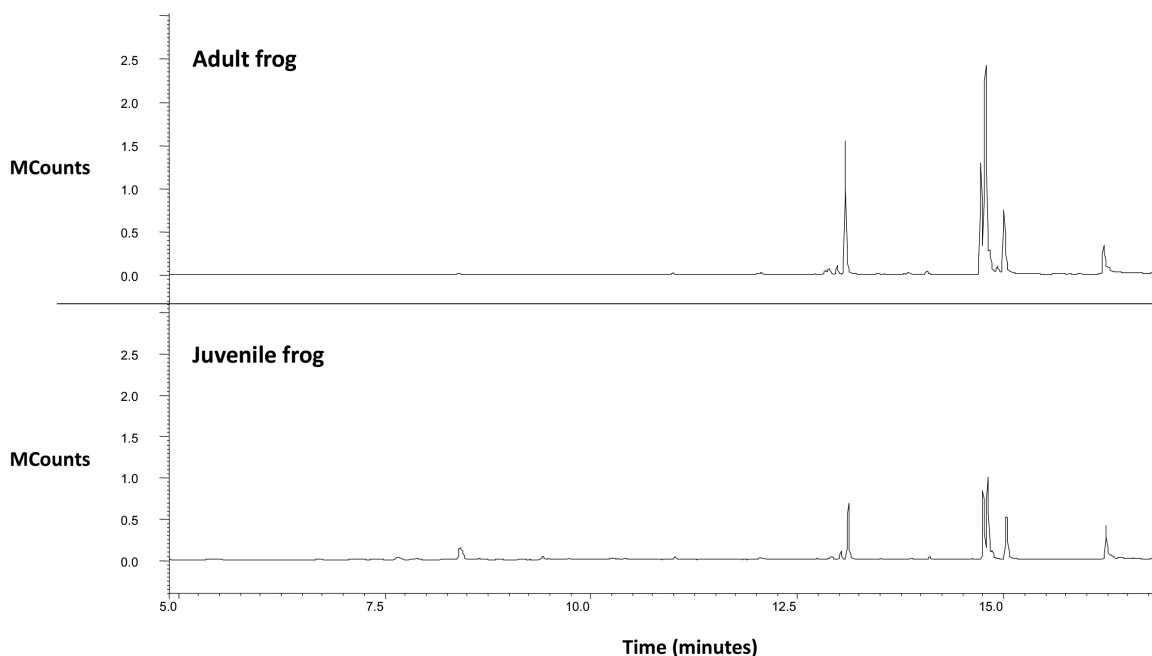


Figure 3. Gas chromatogram of skin extracts from an adult and a juvenile *Oreophryne ezra*. Chemical profiles were very similar among samples, and consisted largely of fatty acids. No alkaloids were detected.

A hypothesis of aposematism predicts the subject animal to be defended (Summers *et al.*, 2015), often chemically, but neither adult nor juvenile *O. ezra* tested positive for alkaloids in our study. Juvenile *O. ezra* appear to share traits with dart-poison frogs in that they are diurnal, perch in obvious locations, make no attempt to hide when approached, more haltingly, contrast with their background, and eat ants, which serve as a source of many dietary alkaloids (Daly *et al.*, 2000; Clark *et al.*, 2005). Given these parallels, we hypothesized that juvenile *O. ezra* may contain alkaloid-based chemical defences. Our finding of no alkaloids in their skins falsifies the second of these hypotheses; however, it does not falsify the hypothesis of toxicity more generally because it remains possible that they use some other toxic chemical instead. A diversity of peptides and steroid-based defences have also been implicated in anuran defence and, unlike alkaloids, are synthesized directly by the frogs themselves (Daly, 1995). In contrast, alkaloids are generally sequestered from the frogs' diet and potentially may take time to accumulate so other biosynthesized chemicals may be more readily adopted (Jeckel, Saporito & Grant, 2015). Testing for other classes of chemicals is thus required before we can fully discount that *O. ezra* uses a chemical defence.

Should *O. ezra* not rely on chemical defence, then an alternative explanation for its conspicuous colour pattern is required. Bright coloration used in advertising is positively correlated with body size in frogs, such that adults are more likely to be brightly coloured than juveniles (Hagman & Forsman, 2003). Juvenile *O. ezra* contradict this trend, suggesting some factor restricts the colour form to these smaller juveniles. A potential alternative explanation to being toxic themselves is that they may be Batesian mimics of a syntopic species that is toxic (Smith, 1981). Intriguingly, we encountered a species of yellow-on-black weevil in the genus *Pantorhytes* (Stibick, 1978). Like *O. ezra*, it was day active observed moving slowly across the same vegetation as *O. ezra* and unlike other weevils it did not death feign when touched but instead would reach out with its front legs. The likely species is *P. stanleyanus* (Gressitt, 1966). The colour of the spots varies between collection locations, but on Sudest the spots are described as pale greenish yellow/white which corresponds with our own observations (Fig. 4). *Pantorhytes* species tend to range from 8 to 13 mm in length (Gressitt, 1966), which overlaps the size range of juvenile *O. ezra* (SVL: 10.37 ± 2.08 ; $n = 31$) but not the adults (SVL: 25.72 ± 2.08) measured in this study. *Pantorhytes* species have been suggested to be either chemically protected or distasteful because predation on adults has not been observed (Smith, 1981). Assuming the weevils on Mt. Riu do not attain



Figure 4. *Pantorhytes* sp. – likely *P. stanleyanus* – a potential model for Batesian mimicry by the juveniles of *Oreophryne ezra*.

a larger size, adoption of the colour pattern by the frogs when bigger would reduce the effectiveness of any mimicry by reducing their match to the model and by making them conspicuous at greater distances. The markings displayed by *O. ezra* resemble those of the lizard *Heliobolus lugubris* (Huey & Pianka, 1977). Juveniles of this South African lizard are black with yellow spots and are said to mimic carabid beetles that use a chemical defence against predators. So, the suggestion of a vertebrate mimicking an invertebrate is not unprecedented.

In conclusion, we have shown that the colour pattern of juvenile *O. ezra* is likely conspicuous to potential visual predators, which differs from the cryptic coloration of adults. However, both life stages lack alkaloid defences in their skins. Two additional areas of research are immediately suggested for obtaining a better understanding of the evolutionary forces producing the remarkable ontogenetic shift in color pattern in this species. First, the presence of additional potential toxins beyond alkaloids needs to be investigated in this species. It may be that *O. ezra* uses peptides or other compounds are used for defence in this species, as is known for other frogs (Daly, 1995). Should such compounds prove absent, conclusively falsifying the hypothesis of aposematism, then the hypothesis that *O. ezra* juveniles mimic toxic models should be investigated. We have proposed *Pantorhytes* weevils as one possible syntopic model but there may be others that we did not observe during our observations.

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