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An Empirical Test Indicates Only Qualitatively Honest Aposematic Signaling Within a Population of Vertebrates

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Abstract.—Signaling is an important part of intraspecific and interspecific interactions. Theoretical work examining honest signaling in aposematic species (e.g., those with conspicuous colors and secondary defenses) has focused primarily on discerning the patterns between conspicuousness and defense within populations. Most empirical work, however, has investigated these patterns across populations or species. Here, we test for honest signaling across individuals within a population of the aposematic poison frog, *Ranitomeya imitator*. We find no evidence that increasing levels of the aposematic signal are correlated with increasing levels of defense in this species, indicating that our study population does not signal in a quantitatively honest manner, but rather that the signal is qualitatively honest. Additionally, we found no evidence that frogs with higher levels of defense behave more boldly as a result of the presumed increased ecological release from predation, an expected outcome in a qualitatively honest system. We discuss our findings in light of the ecology and evolution of *R. imitator*, and suggest mechanisms that may explain the absence of a relationship between toxicity and the aposematic signal.

Communication via signals is common in the animal kingdom, and signals are used to convey information to both conspecifics and heterospecifics. In some cases, interests align between the signaler and receiver, which can result in mutually beneficial communication (Weldon and Burghardt, 2015). Although signals are generally considered reliable, individuals may profit by "cheating" to gain a fitness reward (e.g., access to mates, food, etc.). Hence, a central question in animal behavior is whether the signals individuals produce are honest indicators of the information being conveyed to receivers (e.g., Zahavi, 1975, 1977; Dawkins and Guilford, 1991).

Honest signaling has often been investigated in the context of sexual selection (e.g., Velando et al., 2006; Vanpé et al., 2007; Emlen et al., 2012; Giery and Layman, 2015), but less frequently in the context of natural selection. Certain species signal directly to predators via traits that increase their probability of being detected. These aposematic species combine conspicuous signals with the presence of a secondary defense (e.g., venoms, poisons, spines, etc.), that is generally thought to be honest (barring cheaters, such as Batesian mimics) in the sense that signals advertise the presence of a defense (qualitative honesty: reviewed in Summers et al., 2015). Perhaps more intriguing is whether a species is characterized by *quantitative honesty,* or more specifically, is there a correlation between signal level and strength of defense (for example, increasing brightness or color saturation with increasing toxicity) that has evolved to communicate a level of defense to predators accurately? This question has been the increasing focus of both theoretical and empirical works over the last couple of decades (reviewed in Summers et al., 2015).

Importantly, whether we should predict quantitatively honest signaling remains unclear. Some theoretical analyses have suggested a tradeoff between defense and conspicuousness, wherein prey that are more toxic should invest less in the aposematic signal because they achieve higher fitness through investing in defense (e.g., Leimar et al., 1986; Speed and Ruxton, 2005). On the other hand, under alternative assumptions quantitative honesty is expected, particularly if there is competition for resources used in producing both the signal and defense within an organism (the resource-allocation framework, Blount et al., 2009) or if there is a tradeoff with future fecundity (Holen and Svennungsen, 2012). Few empirical tests have been conducted (particularly within populations), except in invertebrates. These empirical tests have found a positive correlation between brightness and poison gland size in Spanish paper wasps (*Polistes dominula*; Vidal-Cordero et al., 2012), elytra color and chemical defense in the Asian ladybird (*Harmonia axyridis*; Bezzerides et al., 2007), and color saturation and toxicity within ladybird species (Arenas et al., 2015). Those studies that have attempted to elucidate the mechanism underlying the production of quantitatively honest signaling provide support for the resource-allocation hypothesis (Bezzerides et al., 2007; Blount et al., 2012). Although these studies provide evidence that quantitative honesty exists within populations of insects, this relationship may depend on what aspect of the signal is considered (e.g., Winters et al., 2014). Additionally, whether quantitative honesty is generally applicable to other taxa is unclear. Studies investigating the relationship between signal level and toxicity across populations have found mixed results (e.g., Daly and Myers, 1967; Wang, 2011; Maan and Cummings, 2012; Arenas et al., 2015), but there seems to be a more consistent positive relationship between signal and toxicity across species (e.g., Summers and Clough, 2001; Cortesi and Cheney, 2010; Arenas et al., 2015). The only test of quantitative honesty within a vertebrate population found no evidence of quantitative honesty in aposematic newts (Mochida et al., 2013). So the issue of within-population relationships is particularly pertinent, because many insects (e.g., lepidopterans) acquire their toxicity as larvae before metamorphosing into adults (Duffey, 1980), whereas in many vertebrate aposemes, defense is acquired during either development and/or throughout later life (e.g., dendrobatid poison frogs: Daly et al., 1994; other poison frogs: Jeckel et al., 2015; newts: Hanifin and Brodie, 2002; snakes: McCue, 2006; mammals: Newman et al., 2005; Hunter, 2009). As a result, testing basic hypotheses in a variety of taxa that have different life histories is critical to determine if quantitative

honesty is a general trend or if it occurs only because of specific life histories.

Aposematism comes with a putative release from predation pressure, that may allow aposematic species to use novel habitats or gain unique foraging opportunities (Santos and Cannatella, 2011; Cummings and Crothers, 2013). Because defended individuals are not relying on stationary crypsis to avoid the attention of predators, aposematic individuals are free to move throughout the landscape and actively forage and attract mates. Under quantitative honesty, we would expect aposematic individuals to be bolder, and further we hypothesize that the most toxic (i.e., most chemically defended) individuals will be the boldest within a population. Given the relationship between toxicity and the aposematic signal, predators would then be expected to avoid the brightest individuals, because they are also likely to be the most toxic. This potential predation release for brighter and/or more toxic individuals would likely have a positive impact on their foraging success, mate acquisition, or overall fitness. In systems with purely qualitative honesty, however, we may not expect the same degree of ecological release from predation pressure for more toxic and/or brighter individuals if predators are merely concerned with the presence of toxins, and not the level of toxicity per se. Therefore, under the alternative hypothesis of qualitative honesty we would not expect a positive relationship between toxicity and behavioral boldness. Thus, by testing for increased boldness we can investigate specific potential benefits conferred via aposematism within a population.

In this article, we test the hypothesis of quantitative honesty and examine the relationship between conspicuousness and toxicity within an aposematic vertebrate, *Ranitomeya imitator*, a Peruvian poison frog (Dendrobatidae) that possesses alkaloid defenses (Stuckert et al. 2014a,b). We measure the conspicuousness of the visual signal with the use of two different methods. First, we use receiver-independent measures of total spectral brightness and second, we use receiver-dependent visual models of both chromatic and achromatic contrast. Both of these measurements are important, as receiver-independent honesty may indicate a resource-allocation tradeoff, whereas predator visual models may indicate that predators enforce quantitative honesty. We then compare both measures of conspicuousness to total alkaloid content (a measure of toxicity) from 10 individual males that held contiguous territories within a single population. Lastly, we test the hypothesis that brighter or more toxic individuals may benefit more from predation release and look at individual boldness by examining male calling behavior within our focal population of *R. imitator* to determine if highly toxic individuals are released from predation pressure.

Materials and Methods

Field Work.—Territories of 10 male *R. imitator* were identified near Tarapoto, San Martin, Peru, over a 2-wk period (Fig. 1). Although both males and females in this population have a yellow-green spotted aposematic phenotype, males are more engaged in territorial behavior, and therefore are likely the most visible to predators and researchers (Brown et al., 2008a), a trait common amongst dendrobatids (Pröhl, 2005). Many male behaviors, such as territory maintenance via calling, also reveal a male's location to potential predators.

We repeatedly and opportunistically recorded male calling activity in the morning (0630–1100 h) when males were calling over a period of 2 mo. The total number of calls over 2-min period was recorded after the initiation of a calling bout (mean ± SD number of calling-bout observations per frog: 16.3 ± 9.7), after which we located the perch from which the male was calling (mean ± SD number of perch observations per frog: 6.3 ± 3.5). After frogs moved, we placed an imitator-sized frog clay model where the frog was located and recorded visibility (as a percentage of the male visible) from a distance of 1 m in the four cardinal directions and from directly above. We used a compass to indicate the cardinal directions, and measured 1 m distances by tape measure. Visibility of the clay model was determined from the height of the frog's perch. These were then averaged to give us a measurement of perch visibility, which we used as a proxy for visibility to predators. This is similar to work done by Willink et al. (2013) and functionally tests the hypothesis that better-defended males use more open territories and sites to advertise. An early pilot study indicated that observing male activity directly was not feasible. Because of the structure of the forest, observing males from >5 m is impossible because of physical barriers blocking views of the male. Further, observations from distances <5 m yielded noticeable behavioral differences (such as a hunkering down), presumably caused by the proximity of the observer.

Spectral Measurements.—We measure spectral reflectance with an USB4000 spectrometer (Ocean Optics, Inc., Largo, Florida) with an LS-1 tungsten–halogen light source and SpectraSuite software (Ocean Optics, Inc.). A 45° angled tip was used on the probe, standardizing distance and angle to frog skin. Ocean Optics WS-1-SL white standards were used between every frog measured to account for lamp drift. Spectral data were recorded from each frog on a total of eight spots on the dorsum and were processed from 450 to 700 nm in R v3.2 (R Core Team, 2015) in the package 'pavo' (Maia et al., 2013). Data were initially imported from 400 to 700 nm, but data below 450 nm proved to be too noisy for use. A subsample of the individual spectra were smoothed using a loess smoothing function at various levels and visualized; we then used the lowest smoothing span that produced a smooth curve (span = 0.2) for all spectra. Spectra were then aggregated into a single mean spectrum for each frog, after which we recorded mean brightness of each individual's spectrum. We chose a priori to use mean brightness (receiver independent) as opposed to intensity (maximum reflectance value) because itensity is sensitive to noise and slight changes in lamp alignment (Montgomerie, 2006; Maia et al., 2013); however, we subsequently compared median brightness, which did not produce qualitatively different results. Additionally, results using total brightness and intensity yielded qualitatively similar results during visual data exploration. We ignored measures of coloration for this particular receiver-independent analysis, as interpretation of color largely depends on psychophysical parameters; therefore, we consider coloration per se only in the context of predator vision.

The primary predators of poison frogs remain unclear. Although there is growing evidence of predation by many taxa (see Discussion), evidence from anecdotal studies (Master, 1999; Alvarado et al., 2013) and clay model studies (e.g., Noonan and Comeault, 2009; Chouteau and Angers, 2011; Hegna et al., 2011; Paluh et al., 2014) indicate that birds are a primary selective force, and often a source of purifying selection towards a single local aposematic phenotype. Following this, we analyzed receiver-dependent measures of brightness from the average violet-sensitive avian visual perception from multiple species of birds with known visual acuities (Hart, 2001) and using the

FIG. 1. Map of our study site near Tarapoto, in the Department of San Martin, in Peru. Tarapoto is indicated with a triangle.

visual model function provided in the 'pavo' package (Vorobyev et al., 1998) against the average reflectance of three *Dieffenbachia* leaves taken in the field. We chose to use *Dieffenbachia* reflectance because *R. imitator* frequently breeds in *Dieffenbachia* (Brown et al., 2008b) and all males were seen on these plants during this study. The visual model function is based on stimulation of different cone types, and assumes that color discrimination is in large part limited by receptor noise (Vorobyev et al., 1998). This calculation allows us to examine both chromatic (dS, color-based) and achromatic (dL, luminance or brightness) contrast to the background in units of just-noticeable differences (JNDs), a unit of differentiation in which JND = 1 indicates a difference that is at the threshold of discrimination for a viewer (derived from Vorobyev et al., 1998). We used the average avian visual system and ideal, white illumination in our visual model (data provided within 'pavo').

Alkaloid Identification.—Alkaloids from individual frogs were extracted with the use of the methodology presented in Stuckert et al. (2014b). Frogs were euthanized and skins were placed into 4-mL, Teflon-lined glass vials containing 100% methanol to extract alkaloids. An internal 10-µg nicotine standard ([-]-nicotine ≥99%, Sigma-Aldrich, Milwaukee, Wisconsin) was added to samples, which were then fractionated to isolate alkaloids. We performed gas chromatography–mass spectrometry (GC-MS) analysis in electron impact (EI MS) and chemical ionization (CI MS) mode on a Saturn 2100T ion trap MS instrument (Varian, Inc., Ringoes, USA) coupled to a 3900 GC (Varian, Inc.) with a 30 m × 0.25–mm i.d. Factor Four VF-5 ms fused silica column (Varian, Inc.). We identified alkaloids with MS peaks and GC retention times in combination with previously published anuran alkaloids (Daly et al., 2005). We determined alkaloid quantities by comparing individual alkaloid peaks to that of the internal nicotine standard; alkaloids <0.5 µg were not included because

of the unreliability of identification and quantification of these trace alkaloids.

Statistical Analyses.—Following alkaloid identification and quantification, we visually inspected data for deviations from normality. Finding none, we ran linear regressions comparing the receiver-independent brightness of each individual to the total quantity of alkaloids each frog possessed (adjusted for frog mass). Similarly, we ran a linear regression with the results from the average avian visual system and alkaloid content. We ran linear mixed effects models with the package 'lmer4' to compare calling behavior to brightness and alkaloid content with individual frogs as a random effect because we repeatedly recorded calling behavior from males (Bates et al., 2014). Degrees of freedom for this test were calculated based on the Satterthwaite approximation of the denominator degrees of freedom in the R package 'lmerTest' (Kuznetsova et al. 2017). We ran two, independent models fitted with restricted maximum likelihood, one with number of calls over a 2-min period and another using perch visibility. The linear mixed effects model for receiver-independent brightness had a singularity in the estimate of the random effect, so we collapsed the model to a single measure of median perch visibility and ran a simple linear model. We also ran both of these models with receiver-dependent measures of chromatic and achromatic contrast relative to a *Dieffenbachia* leaf background. Summary statistics are reported as means $\pm$ SE and $\alpha = 0.05$ for all tests.

RESULTS

All males in our study possessed alkaloids, indicating that aposematism in *R. imitator* is at least qualitatively honest. The most common alkaloid groups by quantity were indolizidines, histrionicotoxins, and decahydroquinolines, followed by small quantities of allopumiliotoxins (Fig. 2). These are primarily ant-derived alkaloids, although allopumiliotoxins are derived from mites (Saporito et al. 2012, 2015). These alkaloid data are similar to those we collected (Stuckert et al. 2014a) in a previous study examining alkaloids across mimicry complexes of *Ranitomeya* sp., indicating that our data set is comparable in both the quantities of alkaloids and variance to other populations and studies.

We found that frogs were viewed as substantially different from *Dieffenbachia* leaves, and that birds should be able to distinguish frogs from the background. Additionally, there is variation between frogs in coloration, indicating that birds should be able to distinguish individual frogs from each other (mean: 39.7 JNDs, median: 42.9 JNDs). We did not calculate formal statistics because this method compares each individual frog to every other frog in the data set in terms of color discrimination, so any analyses would be inherently pseudo-replicated. When we compared individual receiver-independent brightness to the quantity of alkaloids adjusted for mass, we found no relationship ($F_{1,8} = 0.042$, $P = 0.843$, adjusted $R^2 = -0.119$). Similarly, when we compared brightness from the avian perspective to the adjusted quantity of alkaloids we found no relationship in achromatic contrast (dL) to a *Dieffenbachia* leaf ($F_{1,8} = 1.413$, $P = 0.269$, adjusted $R^2 = 0.044$). Further, we compared chromatic contrast (dS) to a *Dieffenbachia* leaf from the avian perspective to the adjusted quantity of alkaloids and found no difference in this either ($F_{1,8} = 0.6721$, $P = 0.436$, adjusted $R^2 = -0.039$).

We also compared alkaloid quantity and brightness to the number of territorial calls males produced, and found no

significant influence of male defense (estimate: 0.002 ± 0.006, $t_{5.85} = 0.384$, $P = 0.712$) or brightness (estimate: -1.05 ± 1.52, $t_{6.99} = -0.693$, $P = 0.515$) on boldness via calls. Running the same comparison with the use of chromatic and achromatic contrast from the avian visual perspective produced similar results. We found that brighter males called from perches that are less visible from 1 m away (Fig. 3; estimate: -6.25 ± 2.39, $t_7 = -2.626$, $P = 0.034$), but there was no effect of alkaloid quantity (estimate: $-0.012 \pm 0.0.0092$, $t_7 = -1.354$, $P = 0.218$). When we analyzed these data from the perspective of avian viewers, however, we found no effect of alkaloid quantity (estimate: -0.015 ± 0.015, $t_6 = -1.03$, $P = 0.343$), chromatic contrast (dS, estimate: 0.043 ± 0.18, $t_6 = 0.234$, $P = 0.823$), or achromatic contrast (dL, estimate: 0.208 ± 0.65, $t_6 = 0.32$, $P = 0.758$).

DISCUSSION

In this study, we investigated whether the aposematic signal is quantitatively honest within a population of the poison frog *R. imitator*, a key prediction of aposematic theory. Furthermore, a key benefit posited for aposematism is ecological release from predation pressure; more toxic or brighter individuals should have more freedom to conduct daily activities because of a decreased likelihood of predation. Hence, we tested for increased behavioral boldness in more toxic or brighter individuals by examining territorial calling activity. All individuals sampled in this study possessed defensive alkaloids, but we found no relationship between the level of the defense and the level of the aposematic signal. Further, we did not find any evidence that individuals with higher levels of chemical defense behaved more boldly, as more toxic males did not call more or from more obvious perches. We did, however, find that that brighter males called from perches that were less open than more dull males. The findings of our study indicate that males in this population of *R. imitator* have a qualitatively honest aposematic signal, but do not signal in a quantitatively honest manner. Although our sample size is small, we view this is an ecologically relevant sample size, as it is unlikely that predators sample many poison frogs before they learn avoidance (e.g., in lab experiments, model predators learn to avoid poison frogs rapidly; Darst and Cummings, 2006; Stuckert et al., 2014a). Therefore, predators apparently do not use frog brightness as an indicator of toxicity to adjust their attack probability. This is similar to newts (*Cynops pyrrhogaster*), which do not signal honestly within populations (Mochida et al., 2013). Therefore, although evidence suggests there is general quantitative honesty across vertebrate species (e.g., Summers and Clough, 2001), quantitative honesty likely does not occur within populations, and likely varies extensively across populations (Daly and Myers, 1967; Wang, 2011; Maan and Cummings, 2012).

This seems to be a departure from similar invertebrate systems, which generally indicate quantitative honesty across and within populations (Bezzerides et al., 2007; Blount et al., 2012; Vidal-Cordero et al., 2012; Arenas et al., 2015). Therefore, insect systems appear to have proximate mechanisms that maintain quantitative honesty, whereas we found no evidence in our data for quantitative honesty in this population of poison frogs. Whether this is generally true in vertebrates is unclear, however, and should be viewed with some skepticism in light of our small sample size. In insects, some evidence indicates a tradeoff between production of the aposematic signal and toxins
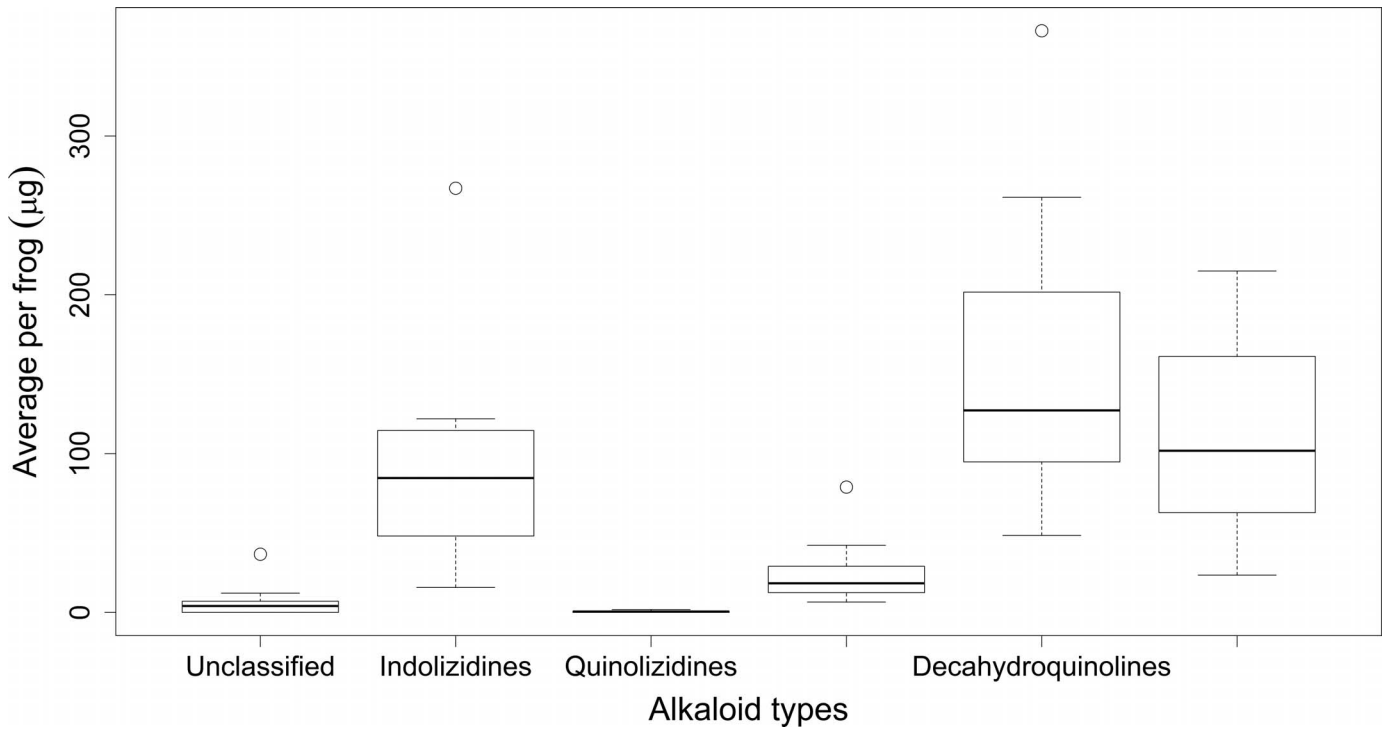
FIG. 2. Box and whisker plot of quantities of alkaloids based on group classification. The box represents the first and third quartile, the horizontal line is the median, and open circles represent outliers.

(the resource-allocation framework; Blount et al., 2009, 2012). Additionally, predators can discern differences in the aposematic signal, and they pay attention to the level of the signal produced by insects and use that information to determine whether to attack (Arenas et al., 2015). This unifying selective force is surprising, because evidence indicates that a predator's decision on whether to attack is highly nuanced and that predators continually reassess based on their own toxin loads,
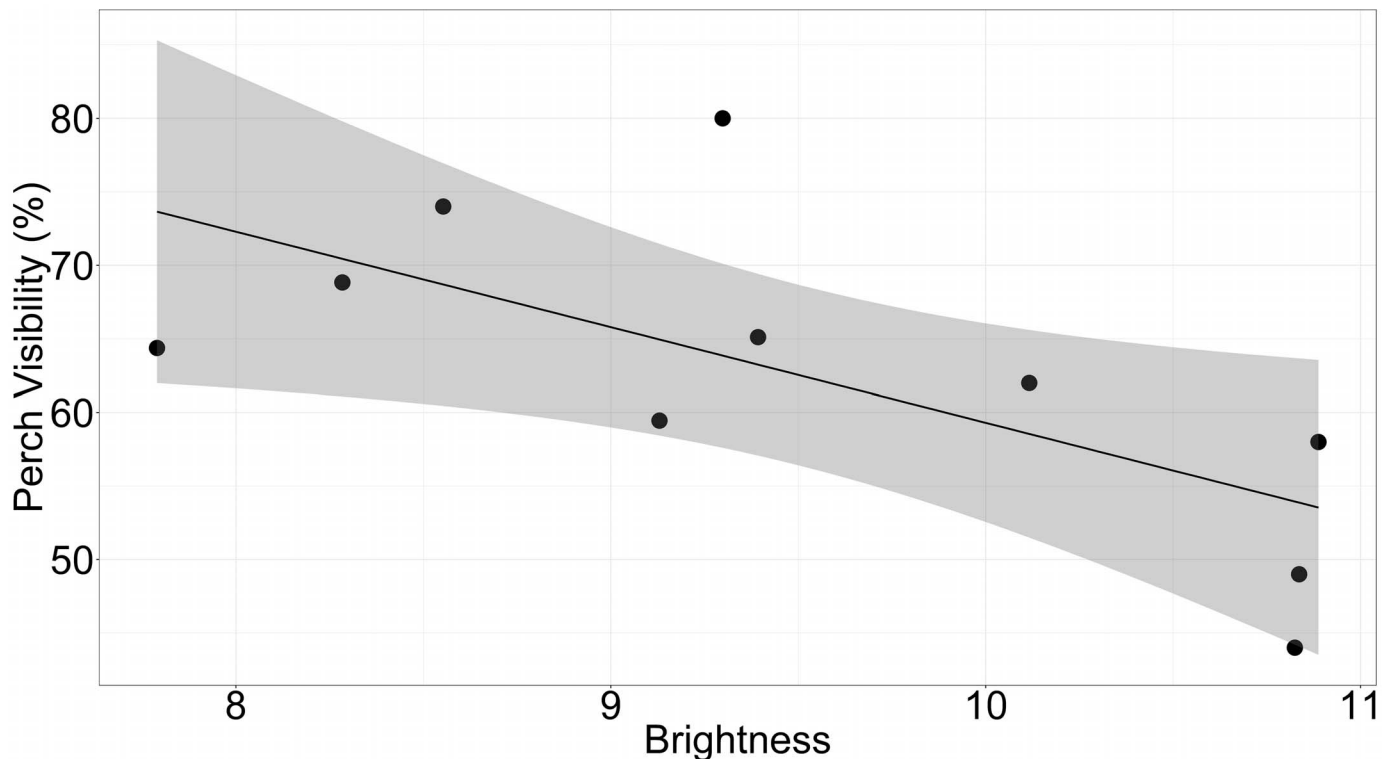

FIG. 3. Results from a comparison of individual boldness to brightness, indicating brighter males choose less conspicuous perches. Linear model comparing receiver-independent brightness to median perch visibility from 1 m distance in all directions (% of total) in individuals. Points are the mean for each individual; the gray bar represents the 95% confidence interval.

hunger, availability of other prey, etc. (Skelhorn et al., 2016). In fact, Flores et al. (2015), found that the attack rate on clay models that resemble the aposematic poison frog *Dendrobates auratus* are not dependent on model brightness (note, however, that this study used clay models of juvenile size).

Several alternative explanations may potentially explain why we see qualitative, but not quantitative, honesty in *R. imitator*. First, unlike invertebrates, which generally sequester all their toxins at the larval stage, there is likely an ontogenetic disconnect between color production and toxicity in many vertebrate species (dendrobatids: Daly et al., 1994; other poison frogs: Jeckel et al., 2015; newts: Hanifin and Brodie, 2002; aposematic snakes: McCue, 2006). Together, these examples likely indicate a substantial difference from examined insect cases in which the resource-allocation framework is more plausible. Therefore, although the resource-allocation hypothesis has some support in invertebrate systems, this proximate mechanism does not appear to be ecologically relevant in many vertebrate systems. Second, predator avoidance may be independent of the quantity of alkaloids as long as they are present in amounts sufficient to make them unpalatable and so typically avoided by potential predators (e.g., Speed et al., 2012). Therefore, a threshold level of defense may very well be predator dependent (e.g., birds, arthropods, snakes), above which quantitative honesty is uninformative and therefore not selected by predators. Further, we might predict different selective pressures from nonavian predators. Anecdotal evidence of predation on dendrobatids corroborates this, as only one bird species has been observed preying on poison frogs (Master, 1999; Alvarado et al., 2013), whereas multiple other predator guilds have been observed preying on dendrobatids (e.g., Myers et al., 1978; Summers, 1999; Lenger et al., 2014). In fact, there is evidence that certain arthropod predators (bullet ants and banana spiders) impose different selective pressures on the dendrobatid frog *Oophaga pumilio* in Costa Rica based on different thresholds of defense (Murray et al., 2016).

Predation Release.—In addition to testing quantitative honesty within a population, we also tested the prediction that increased toxicity and brightness is correlated with an increase in behavioral boldness, using the number of calls males gave in a 2-min period as well as the visibility of the perch that males called from as a proxy for boldness. We found no evidence for increased boldness with increasing chemical defense. We did find evidence that brighter males are more likely to call from less visible perches; however, and importantly, we did not see the same relationship when examining chromatic and achromatic contrast from the avian visual perspective against a host plant leaf, and therefore, the ecological significance is unclear. This may be an example of bet-hedging (Slatkin, 1974), in which duller males of potentially lower quality attempt to stand out by using conspicuous perches, simultaneously entailing an increased risk of predation. Brighter males on the other hand may be of higher quality, so gain little by choosing a more conspicuous perch relative to the increased risk of predation. This is largely speculative, however, and some work in a related species *O. pumilio* has shown either the opposite relationship, that more conspicuous morphs are bolder (*O. pumilio*, Pröhl and Ostrowski, 2011; *Oophaga granulifera:* Willink et al., 2013), or no relationship at all (Dugas et al., 2015).

Concluding Remarks.—In this study, we tested the hypothesis that quantitative honest signaling exists within a population of *R. imitator,* a key prediction of a substantial body of theoretical work on signaling. We found that adult males within a population of *R. imitator* all possess alkaloids, and therefore their aposematic signal is qualitatively honest; however, we found no evidence for quantitative honesty, a corresponding increase in the level of the signal with the level of the defense. Additionally, we tested the hypothesis that an increase in toxicity yields an increase in boldness because of ecological niche release. We found no evidence that more toxic males behaved more boldly under our metrics. We did, however, find that brighter males call from less-visible perches, suggesting that males may be pursuing a bet-hedging strategy with respect to calling behavior. We suggest that alternative mechanisms are acting on the variation in the intensity of the aposematic signal. We view the ontogenetic disconnect between toxin sequestration and the setting of coloration to be a plausible hypothesis in many vertebrate taxa, and a crucial difference with respect to invertebrate systems (and with respect to the assumptions of many theoretical models).

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