

Experimental evidence for predator learning and Müllerian mimicry in Peruvian poison frogs  
(*Ranitomeya*, Dendrobatidae)

by

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**Abstract:** Poison frogs are characterized by bright coloration, striking patterns, and toxicity; they have thus become a classic example of aposematism. *Ranitomeya imitator* mimics three congeneric model species (*R. fantastica*, *R. summersi*, and two morphs of *R. variabilis*), creating geographically distinct populations of the species, including four allopatric mimetic morphs (a "mimetic radiation"). These complexes are thought to represent a case of Müllerian mimicry, but no empirical data exist on predator-learned avoidance to support this claim. In this study we used young chickens (*Gallus domesticus*) as naïve predators to determine if spotted mimetic morphs of *R. imitator* and *R. variabilis* contribute to learned avoidance by predators—a key component of Müllerian mimicry. Chickens exposed to either stimulus species demonstrated learned avoidance of both species; thus our results indicate that this complex functions as a Müllerian mimicry system. Furthermore, our study shows no difference between learned avoidance in stimuli frogs and a 'novel' morph of *R. imitator* which differed in both color and pattern—indicating that predator learned avoidance may be generalized in this system.



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(*Ranitomeya*, Dendrobatidae)

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## **Introduction:**

Poison frogs, family Dendrobatidae, are known for their unpalatability and toxicity as well as their bright aposematic coloration (Daly and Myers 1967; Saporito et al 2007). Their toxicity comes from skin alkaloids, which act as a deterrent to potential predators (Daly et al 2005; Darst and Cummings 2006). *Ranitomeya imitator* (formerly *Dendrobates imitator*—see Grant et al 2006 and Brown et al. 2011) is a poison frog endemic to the lowland and montane forests of the Peruvian Amazon (Schulte 1986). As its name implies, *R. imitator* mimics multiple sympatric species throughout its range (Symula et al. 2001; Yeager et al. 2012). Genetic analyses by Symula et al. (2001, 2003) have shown that *R. imitator* is a valid species that has undergone a rapid mimetic radiation to adverage on the aposematic signals of its sympatric congeners (Santos et al. 2009 and Yeager et al. 2012; but see Chouteau et al. 2011 for discussion). This indicates that *R. imitator* is the mimic in this system and evolved to resemble already established species (*R. fantastica*, *summersi*, and both lowland striped and highland spotted morphs of *variabilis*). Theory would suggest that the selection pressure driving this mimetic radiation involved experienced predators avoiding *R. imitator* that resembled the local model (Ihalainen et al. 2008); leading to frequency-dependent selection for Müllerian mimicry (Sherratt 2008).

While numerous authors (Symula et al. 2001; Sherratt 2008; Brown et al. 2011; Yeager et al. 2012) have argued that *R. imitator* is a Müllerian mimic, some key predictions of this hypothesis remain to be tested empirically. Müllerian mimicry is a phenomenon in which two or more evolutionarily distinct toxic species evolve to appear morphologically similar and thus share the burden of predator learning. Predators need to ‘sample’ individuals in order to learn

that they are toxic (Müller 1878, 1879) and, intuitively, this can have detrimental effect on those individuals ‘sampled.’ Commonly cited and well-studied examples of Müllerian mimicry are the “mimicry rings” that exist in *Heliconius* butterfly communities (Joron and Mallet 1998; Mallet and Joron 1999; Sherratt 2008). In these mimicry rings, novel or rare phenotypes are more likely to be attacked by predators if they are not recognized as toxic or unpalatable (Müller 1879; Mallet and Barton 1989; Kapan 2001; Sherratt 2008). Thus predators are thought to select against polymorphism in Müllerian mimics (Speed 1993; Speed 1999; Joron et al. 2001).

In contrast, Batesian mimicry describes systems in which the mimetic species is not toxic and does not contribute to learned predator avoidance of a shared morphological appearance (Bates 1862). These species take advantage of the protection provided by the model species, decrease the efficiency of learned avoidance by predators (Bates 1862; Speed 1993). This may impose a cost on the model species because Batesian mimics add palatable individuals and do not contribute to learned avoidance. The more abundant the mimic is, the less successful the toxic morph is, decreasing the effectiveness of the model’s signal (Speed 1993; Speed 1999). Batesian mimics are thus predicted to be rare relative to their model species.

Batesian and Müllerian mimicry are opposite extremes, but the middle ground is somewhat of a gray area. Verbal, analytical and computational models of the effects of variation in prey palatability, predator motivation, and predator communities support the concept of a “mimicry spectrum” (e.g. Turner 1984; Turner and Speed 1999). Species that fall in the middle of this spectrum are referred to as quasi-Batesian (or in some instances, Speedian) mimics (Speed 1999). Moderately defended, mimetic prey species can either help or hinder predator learning and therefore model survival (Speed 1993); in essence they can be either mutualistic or

parasitic. Under some circumstances (for example a common moderately defended species and a very rare species) this species may provide protection and thus act as a Müllerian mimic (Speed 1999). However, a moderately defended mimetic species can be parasitic when it increases the rate of attack on the model species, making it a quasi-Batesian mimic (Speed 1993, Speed 1999). One key component of quasi-Batesian mimicry is that although the model species may lose protection from predators, the mimetic species gains protection from the more defended model species (Speed 1999).

Mimicry tends to be directed at visual predators, and many authors have suggested that avian predators are the primary force driving the evolution of color and pattern in dendrobatid frogs (e.g. Symula et al 2001; Darst and Cummings 2006; Darst et al 2006; Saporito et al 2007; Noonan and Comeault 2009). There are a number of factors supporting this: 1) avian peak activity occurs at the same time as peak poison frog activity, early morning and late afternoon, and daylight is likely an important component of the aposematic signal (Schulte 1986; Duellman and Trueb 1994; Poulin et al 2001), 2) they are common predators of frogs in the Neotropics (Stiles and Skutch 1989), although Poulin et al. (2001) found that toxic dendrobatids are conspicuously absent in stomach contents, 3) birds are able to detect the conspicuous color signals of dendrobatid frogs (Siddiqi et al. 2004; Maan and Cummings 2012), 4) birds attack clay models of dendrobatid frogs (Saporito et al 2007; Noonan and Comeault 2009; Chouteau and Angers 2011) and 5) have been observed preying upon poison frogs (Master 1999). However, there is evidence that the combination of aposematic coloration and diurnal habits may be enough to deter many potential predators (Brodie 1993; Siddiqi et al 2004)—indicating an effective aposematic signal. Maan and Cummings (2012) demonstrated that *Oophaga (Dendrobates) pumilio* signals its toxicity honestly from the perspective of avian predators,

increasing in conspicuousness with increased toxicity. Further, Hegna et al. (2012) have shown that a green (less conspicuous) island morph exists in an area with a lower avian predation pressure than the mainland red (more conspicuous) morph. This indicates that these aposematic signals in dendrobatid frogs are directed towards potential avian predators.

*Ranitomeya imitator* has been hypothesized to be a Müllerian mimic of multiple sympatric congeners (Symula et al. 2001; Ruxton et al. 2004; Sherratt 2008). A recent field study by Chouteau and Angers (2011) studied *R. imitator in situ* using plasticine clay models which they reciprocally transplanted between two sites. Their highland site has spotted frogs of both *R. imitator* and *R. variabilis* while their lowland site has striped frogs of both species. This study demonstrated that local avian predators discriminate between the local morph and a novel morph from a nearby but geographically distinct location and that local morphs have a much lower rate of predation. Their study indicates that avian predators are a rapid, homogenizing selective force maintaining geographical organization in these two species (Chouteau and Angers 2011). However, it does not indicate whether both species (*R. imitator* and *R. variabilis*) are contributing to the protection of the local morph or whether one of these species (presumably *R. imitator*) is a Batesian parasite, gaining protection from the other similar species.

The aim of our study was to examine mimicry in a putative Müllerian system of *Ranitomeya* frogs and determine if both species (*R. imitator* and *R. variabilis*) contribute to learned avoidance by predators. We used naïve chicks (*Gallus domesticus*) as model predators to test this hypothesis, because birds are often hypothesized to be the drivers of color and pattern in poison frogs, previous studies have used chicks (e.g. Darst and Cummings 2006) and because Chouteau and Angers (2011) indicated that avian predators are able to differentiate between local

and novel morphs in *R. imitator/variabilis*. In addition to toxicity among all species involved, Müllerian mimicry predicts that mimetic species should confer benefits of learned predator avoidance to each other. Predators that have experience with Batesian mimics learn that at least some members of a mimetic population are palatable. Thus they do not contribute to learned predator avoidance and may even decrease the efficiency of a learned warning signal. We aimed to test one key component of the hypothesis of Müllerian mimicry: learned avoidance by predators of a shared appearance. We presented chicks with one of two stimuli, either the spotted morph of *R. variabilis* or the corresponding mimetic spotted morph of *R. imitator*. These naïve predators were given the opportunity to smell, taste, and prey upon wild-caught poison frogs in a series of learning trials. We compared their learned avoidance to baseline data we collected by recording the interaction time spent with these frogs in timed trials. Many studies have looked at differential predation rates between local morphs and novel or exotic morphs of poison frogs by experienced predators using plasticine clay models (Saporito et al. 2007; Noonan and Comeault 2009; Chouteau and Angers 2011). However, none of these studies have examined aposematism from the perspective of naïve predators as they are introduced to putatively toxic prey and their reaction. To our knowledge, this study represents the first test of learned avoidance of two anurans in a putative Müllerian system. In addition, we tested whether learned avoidance is generalized or exact through the addition of a geographically near morph of *R. imitator* in our study. If predators exhibit exact learning we would expect this to be at least a partial explanation of the evolution and maintenance of mimetic polymorphism in *R. imitator*. Generalized learned avoidance of frogs in our study may indicate a mechanism for the maintenance of polymorphism within populations as well as of the clines between morphs of *R. imitator* (as well as other species). These clines exhibit substantial phenotypic variation and

theory predicts that individuals in these areas should suffer higher predation rates due to a lack of phenotypic similarity.

### **Methodology:**

In order to test whether conferred protection from predators is reciprocal versus unidirectional (and therefore test whether *R. imitator* is a Müllerian mimic), predator-learning trials were carried out with chicks in one of two treatment groups, the model (i.e. the spotted morph of *R. variabilis*) or the corresponding mimetic spotted morph of *R. imitator*. These experiments were conducted in the department of San Martin, Peru in 2011 and 2012 and roughly followed the methodology presented in Darst and Cummings (2006). We used naïve chicks (*Gallus domesticus*) as naïve predators in this study since birds are known to differentiate colors well (Poulin et al. 2001) and have been widely implicated as a selective force in amphibian aposematic systems (Saporito et al. 2007; Noonan and Comeault 2009; Chouteau and Angers 2011, Maan and Cummings 2012). Chicks were given water *ad libitum*, were fed cracked corn twice daily, typically after trials were conducted, and were housed in a 1x2m chicken wire cage. This cage was similar to the arena that experiments were conducted in, a 1 m<sup>2</sup> wooden arena divided into four 50 cm<sup>2</sup> quadrants with an earthen floor. All *Ranitomeya* frogs were collected shortly before the initiation of trials as toxins are sequestered from prey items in wild frogs and we wanted to ensure that these frogs did not lose their toxicity through an extended period of captivity. Individuals of the spotted morph (both *R. imitator* and *R. variabilis*) were collected from a spotted population near Tarapoto, San Martin and striped *R. imitator* were collected from one of two populations near Pongo de Caynarachi or further east. Chicks were fed *Leptodactylus* control frogs prior to their use in the study to ensure that chicks recognized frogs as potential

prey items.

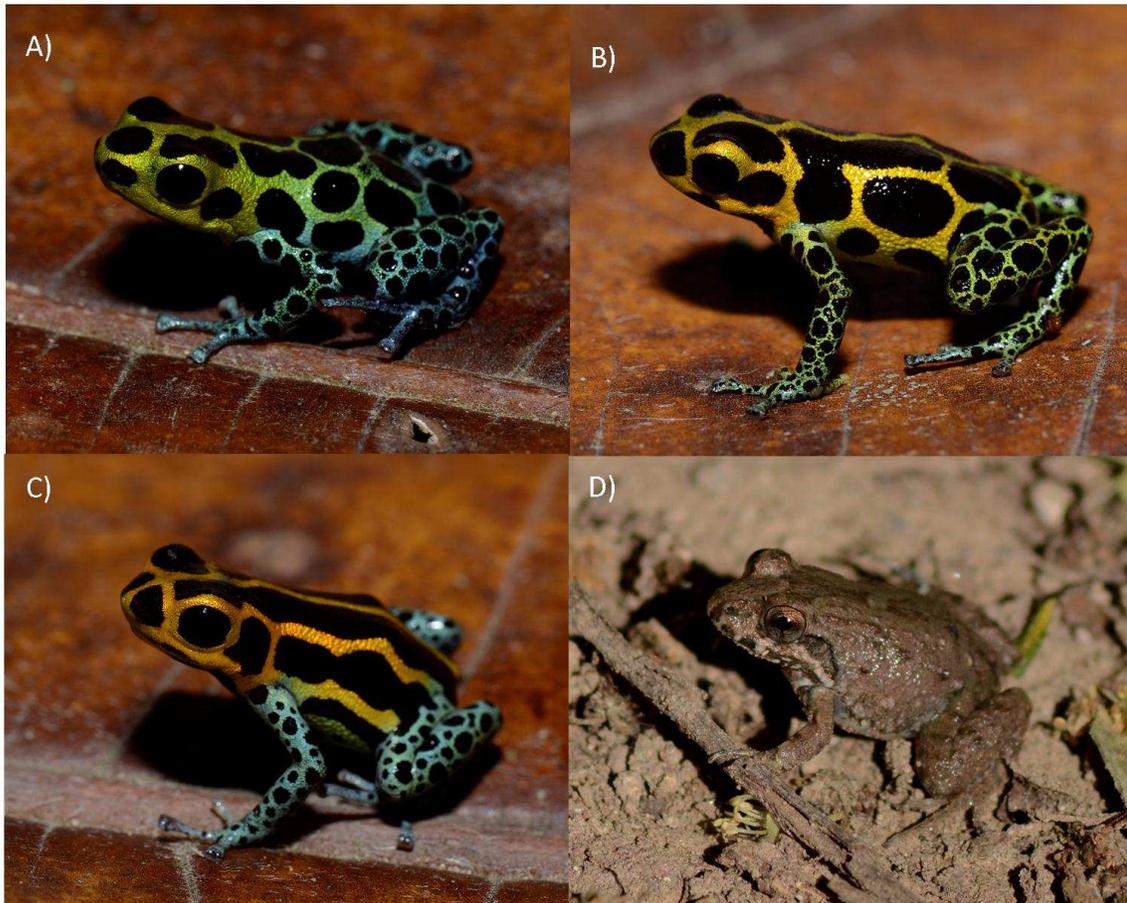


Figure 1. Frogs used in this study. A) *R. variabilis*, B) the spotted morph of *R. imitator*, C) the striped (novel) morph of *R. imitator*, D) *Leptodactylus* control frog

We then conducted pre-learning trials which were used as baseline data to test naïve chicks' behavior when presented with a poison frog. During pre-learning trials we paired cryptic *Leptodactylus spp* control frogs with one of 3 aposematic and presumably toxic *Ranitomeya* frogs, the spotted morph of *R. variabilis* (model), the spotted morph of *R. imitator* (mimic), or the striped morph of *R. imitator* (representing a novel morph). We chose to use a novel morph in our experiments because theory indicates that novel or rare phenotypes are more likely to be

attacked by predators if they are not recognized as toxic or unpalatable (Muller 1879; Guilford and Dawkins 1993; Sherratt 2008) and we were interested in determining whether learning is exact or generalized in this system. Every chick (n=35) was tested for each of these 3 experimental pairings once per day for 3 consecutive days. The order of these trials was randomized. Frogs were randomly assigned to quadrants and placed under glass domes (8x8x3.5cm) with white bottoms to enhance visibility to chickens and make both the cryptic and aposematic frogs of approximately equal visibility. Trials lasted for 2 minutes and were video recorded with a Sony DSC-W20 (2011) and a Nikon D3100 (2012). We recorded the number of pecks directed at each frog, total number of separate attack events, and interaction time, which we defined as time spent in the same quadrant and directly oriented towards a frog.

Following this we conducted a series of learning trials in which chicks were randomly assigned to two groups and presented with a putatively toxic dendrobatid frog (either spotted *R. variabilis*, n=18, or the corresponding spotted morph of *R. imitator*, n=17) in a glass dome with the top removed. Chicks were observed for 2 minutes or until an attempted predation event and subsequent consumption of or loss of interest in the presented frog. Each chick was offered the same individual learning stimulus (except in the event of death of the learning stimulus) for 8 learning trials; trials were conducted twice daily.

Post-learning trials followed the completion of learning trials and were conducted in the same manner as pre-learning trials. In addition to these trials, chicks were offered palatable *Leptodactylus* control frogs prior to pre-learning trials, following learning trial number 4, and prior to post-learning trials.

We compared pre-learning baseline data to post-learning data. We analyzed data for interaction time, number of pecks, and the number of separate attack events for each stimulus in a series of paired t-tests, corrected for multiple analyses using a false discovery rate. We used false discovery rates because they correct for multiple analyses but are less conservative than other corrections, eg Bonferroni (García 2004). In an effort to keep the number of type I errors at a minimum, corrections such as Bonferroni penalize harshly. In situations where multiple tests are run this can decrease statistical power until it is too low to detect differences, resulting in increases the number of type II errors (Verhoeven et al. 2005). Correcting using a false discovery rate controls for the proportion of type I errors, as opposed to trying to control the chance of making a type I error (Verhoeven et al. 2005). Use of this test has been advocated in ecological situations (García 2003, 2004).

We further analyzed the difference between pre- and post-learning using a one-way ANOVA and a repeated measures ANOVA between groups to analyze whether learning is exact (specific to the spotted morph) or generalized (all things bright and colorful—including the ‘novel’ striped morph).

### **Results:**

On average our chicks weighed 305.2g (304.5g for *R. variabilis* stimuli chicks and 305.9g for *R. imitator* stimuli chicks). On average, *R. variabilis* frogs weighed 0.46g and *R. imitator* frogs weighed 0.48g; frogs thus were 0.15% and 0.16% of the average chicken weight respectively. Although *R. imitator* had a larger mass on average than *R. variabilis*, this was not statistically significant (2-tailed t-test  $t_{66}=0.992$ ,  $p=0.325$ ). *Ranitomeya variabilis* was slightly larger in SVL

(17.3mm) than *R. imitator* (17.2), but this was not a significant difference (2 tailed t-test  $t_{66}=0.546$ ,  $p=0.587$ ).

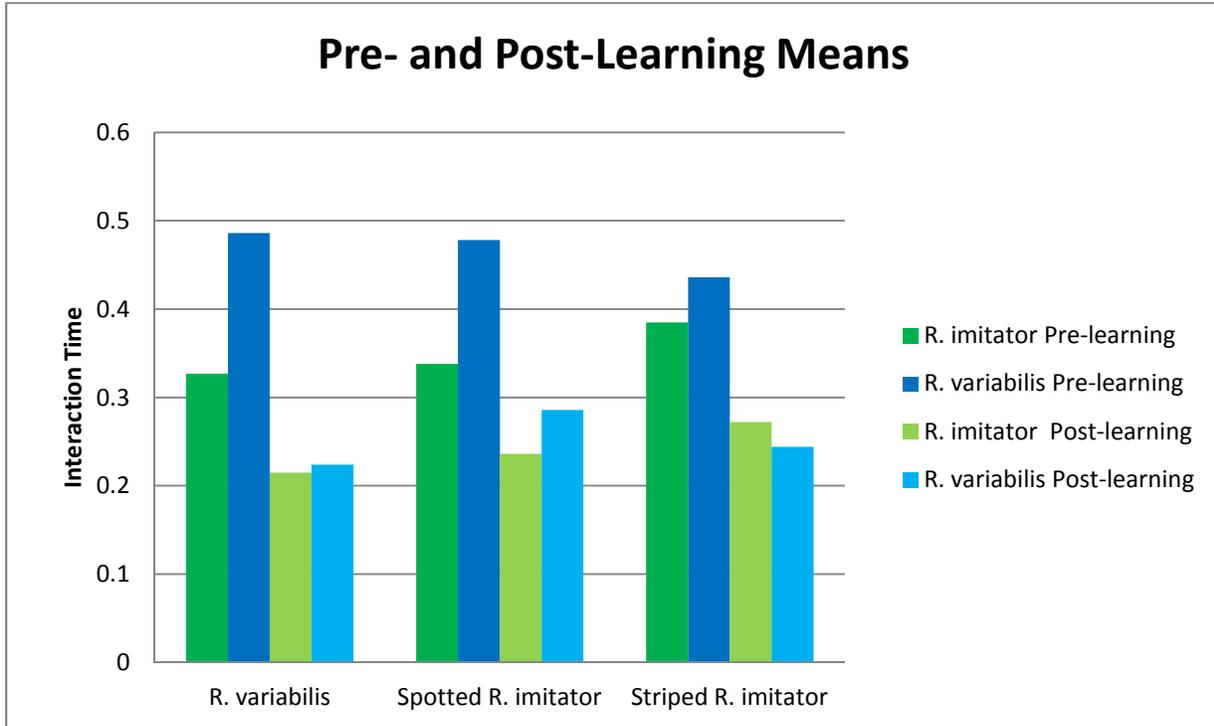


Figure 2. Pre- and post-learning means for both *R. imitator* (n=17) and *R. variabilis* (n=18) stimuli chicks across all three trial types.

When chicks were given the opportunity to smell, taste, and prey upon poison frogs some chicks expressed innate neophobia and did not taste either species. However, all chicks expressed interest in the poison frogs and actively investigated them. The majority of chicks sampled the stimulus frogs by taking them in their bills and then immediately dropping them and moving on; some expressed signs of distress and distaste such as bill wiping and eating dirt. However, it should be noted that some chicks did consume poison frogs during learning trials (n=3 for *R. imitator* and n=1 for *R. variabilis*). Those chickens which consumed *R. imitator*

(n=3) weighed 340g on average while the one chick that ate *R. variabilis* weighed 385g. Chicks used in our study weighed 305.2g on average.

	<i>R. imitator</i> stimuli (n=17)	<i>R. imitator</i> stimuli, eaters removed (n=14)	<i>R. variabilis</i> stimuli (n=18)	<i>R. variabilis</i> stimuli, eaters removed (n=17)
<i>R. variabilis</i>	0.058	0.0206	<0.001	<0.001
Spotted <i>R. imitator</i>	0.058	0.0117	0.0094	0.0031
Striped <i>R. imitator</i>	0.058	0.0395	0.0105	0.0105
All spotted frogs (imitator and variabilis)	0.058	0.0075	<0.001	<0.001
All <i>Ranitomeya</i> frogs	0.058	0.0100	<0.001	<0.001

Table 1. P- values from 1-tailed, paired sample t-tests controlled for multiple comparisons using a false discovery rate. In all treatments a *Ranitomeya* frog was paired with a non-toxic, cryptically patterned *Leptodactylus* frog. The top row is the stimulus chicks were trained on and the first column represents the poison frog used in each trial to test for learned avoidance.

Comparisons of pre-learning and post-learning behavior among chicks for both *R. imitator* and *R. variabilis* stimulus chicks were done using 1-tailed, paired sample t-tests for the average ratio of time spent with each frog per trial. These analyses were 1-tailed because we were only interested in if chicks exhibited learned avoidance. In analyses of interaction time between pre- and post-learning (Figure 1 shows interaction times, Table 1 comparisons between them), chicks trained on *R. variabilis* learned to avoid both their own species ( $t_{17}=4.663$ ,  $p<0.01$ )

and the spotted morph of *R. imitator* ( $t_{17}=2.704$ ,  $p=0.0094$ ) and the novel striped morph of *R. imitator* as well ( $t_{17}=2.544$ ,  $p=0.0105$ ). Chickens trained on the *R. imitator* stimuli learned to avoid both the spotted and striped morph of *R. imitator* as well as the model *R. variabilis* ( $t_{16}=1.730$ ,  $p=0.058$ ,  $t_{16}=1.705$ ,  $p=0.058$  and  $t_{16}=1.660$ ,  $p=0.058$  respectively). Additionally, we analyzed the data excluding the few chicks that ate frogs. We did this due to the ratio of the size of predators (on average >300g) and the prey (typically <0.5g), with the poison frogs roughly 0.15% of the chickens' weight on average, potential differences in predator motivation or hunger, as well as these chickens being much larger than the vast majority of potential avian predators in the wild. Of particular interest were chickens trained on the *R. imitator* stimuli, which showed a marked increase in learned avoidance ( $t_{13}=2.822$ ,  $p=0.0117$  and  $t_{13}=2.391$ ,  $p=0.0206$  for spotted *R. imitator* and *R. variabilis* respectively and  $t_{13}=1.905$ ,  $p=0.0395$  for the novel striped morph of *R. imitator*).

Chicks did not learn to avoid all frogs. They were fed *Leptodactylus* control frogs after pre-learning, the 4<sup>th</sup> learning trial, and the last learning trial (before the post-learning trials); almost all chickens ate these frogs immediately and with a gusto. Further, total interaction time (in seconds) nearly doubled between pre- and post-learning trials. For the *R. imitator* stimulus chicks, total time spent with frogs trended on increasing in trials with *R. variabilis* and control frogs ( $t_{16}=-1.957$ ,  $p=0.068$ ) and increased in trials with both spotted and striped *R. imitator* ( $t_{16}=-2.613$ ,  $p=0.019$  and  $t_{16}=-2.583$ ,  $p=0.020$  respectively). Total interaction time in *R. variabilis* stimulus chicks also increased, but not statistically ( $t_{17}=-1.268$ ,  $p=0.222$  for *R. variabilis*,  $t_{17}=-2.015$ ,  $p=0.060$  for spotted *R. imitator* and  $t_{17}=-1.790$ ,  $p=0.091$  for striped *R. imitator*).

Additionally, we compared the interaction time of chickens during the pre-learning baseline time using 2-tailed t-tests to the expected interaction time of 50% with each frog given that these chickens had no experience with poison frogs (Table 2). What we found is that chickens that were to-be-trained on *R. variabilis* showed no difference in interaction time from what we expected. However, chickens that were to-be-trained on *R. imitator* showed a highly significant difference from what we would expect. These chicks spent significantly less time with all three types of poison frogs paired with *Leptodactylus* control frogs, *R. variabilis* ( $t_{16}=-2.990$ ,  $p=0.0113$ ), the spotted morph of *R. imitator* ( $t_{16}=-3.714$ ,  $p=0.0033$ ), and the striped morph of *R. imitator* ( $t_{16}=-1.725$ ,  $p=0.1040$ ). Although interaction time in our baseline (pre-learning) data for chicks to-be-trained on the *R. variabilis* stimulus did not differ from this expected value, chicks to-be-trained on *R. imitator* spent significantly less time with poison frogs than expected. In essence, these two groups (which were randomly assigned) differed slightly in their pre-learning interaction time for unknown reasons.

	<i>R. imitator</i> stimuli (n=17)	<i>R. variabilis</i> stimuli (n=18)
<i>R. variabilis</i>	0.0113	0.7440
Spotted <i>R. imitator</i>	0.0033	0.7440
Striped <i>R. imitator</i>	0.1040	0.7440

Table 2. P values from 2-tailed t-tests with the expected value of 0.5 (50% of interaction time directed towards poison frogs), corrected with a false discovery rate for multiple comparisons.

We also compared pecks and independent attack events to an expected 50% in 2-tailed t-tests. In these analyses, chickens to-be-trained on both stimuli (*R. variabilis* or *R. imitator*)

directed many more pecks and independent attack events towards *Leptodactylus* control frogs than expected ( $t_{16} < -7$ ,  $p < 0.001$  and  $t_{17} < -4$ ,  $p < 0.001$  respectively), for all three treatment types (*Leptodactylus* frog with *R. variabilis*, the spotted morph of *R. imitator*, and the striped morph of *R. imitator*). This indicates that chicks trained on both stimuli directed significantly more pecks and independent attack events towards control frogs than would be expected given even odds.

We also ran one-way ANOVAs and repeated measures ANOVAs on the differences between pre- and post-learning interaction times. This data was not significant ( $p > 0.05$  in all cases), indicating that there was no difference between treatments in the learned avoidance. Additionally, we analyzed the number of pecks and number of independent attack events between pre- and post-learning using paired t-tests. None of these data were statistically significant for either *R. imitator* or *R. variabilis*. Although these data do not support our prediction that predator learned avoidance would decrease the number of pecks and attacks directed at poison frogs, this is likely an effect of our baseline data being so heavily skewed away from the poison frogs ( $p < 0.001$  for both *R. imitator* and *R. variabilis* for all three treatment types).

## **Discussion:**

Müllerian mimicry has been proposed for a number of anuran systems, including *Ranitomeya imitator* (Symula et al. 2001; Sherratt 2008; Brown et al. 2011), other *Ranitomeya* complexes (Brown et al. 2011), mantellid frogs (Schaefer et al. 2002), and across families in the putative Müllerian mimics *Amereega picta* and *Leptodactylus lineatus* (Prates et al. 2012). However, to date no study has demonstrated reciprocal learned avoidance by predators of a

shared morph—a key component of Müllerian mimicry. Predator learned avoidance is reciprocal between model (spotted *R. variabilis*) and mimic (spotted *R. imitator*) in this system; thus providing the first experimental evidence of learned avoidance by predators consistent with Müllerian mimicry in anurans.

Some chickens showed innate neophobia or conservative behavior when presented with either of the spotted stimuli frogs (*R. variabilis* and *R. imitator*). This was especially true for chicks to-be-trained on *R. imitator*, which interacted with poison frogs much less than expected in our pre-learning baseline data. Further, both *R. imitator* and *R. variabilis* stimuli chicks directed many more pecks and independent attack events toward the *Leptodactylus* control frog than expected ( $p < 0.001$  in all cases). This experimental evidence of an innate neophobia towards an anuran is the first documentation of this phenomenon to our knowledge. However, this phenomenon may be common, and further testing is warranted. Other studies have demonstrated innate neophobia by potential avian predators in response to potential prey from other taxa. Smith (1975) has shown that motmots have an innate neophobia and avoidance of coral snake color and patterns. Further, Marples et al. (1998) demonstrated dietary conservatism of blackbirds (*Turdus merula*) and robins (*Erithacus rubecula*) when presented with novel-colored pastry baits in the presence of familiar-colored baits.

This conservative behavior is probably important in the maintenance of aposematic signals. This likely works in the favor of aposematic species in two ways: 1) by a decrease in overall attack rates and 2) through a slight hesitation to attack (often seen in our study) which can give an aposematic individual time to escape a potential predator. These are especially important because predator communities are continually changing due to recruitment,

immigration, emigration, etc. Recruited, naïve individuals that display conservative behavior or innate neophobia are less likely to attack aposematic prey items, and juveniles are more wary than adults with aposematic prey (Marples et al. 1998; Lindström et al. 1999). Attacks, if not a direct cause of mortality, may have implications later on. A small proportion of individuals in the wild have loss of digits/limbs or scarring (AMMS pers. obs.) and attacks/injuries may lead to infection. Further, attacks may lead to a decrease in fitness either through physical injury (eg. loss of digits or limbs) or reduced sexual fitness due to the effects of scarring on mate choice or changes in behavior to decrease the risk of further attack.

Once chicks sampled poison frogs, the majority immediately dropped them and lost interest. Some chicks exhibited distress signals such as bill wiping after sampling frogs. This indicates that these frogs have a noxious taste and elicit an aversive reaction from potential predators due to their chemical defenses. Although a few chickens did consume poison frogs (three *R. imitator* stimulus chicks and one *R. variabilis* stimulus chick), it is worth noting that chickens used in this study likely weigh much more than the vast majority of potential avian predators that are likely to encounter these frogs in the wild. Due to the minute stature of these frogs (typically under 0.5g), toxin dilution could reduce the effectiveness of chemical defenses with heavier predators. Indeed, Exnerová et al. (2008) noted that larger bird sizes, as well as food storing behaviors, increase a predator's ability to handle the chemical defenses of heteropteran insects (see also Veselý et al. 2006). In this case then the prey-to-predator weight ratio could be an important consideration in studies exploring the mimicry spectrum. As a result, we hypothesize that larger species may play an important predatory role in this system (tinamous, chachalacas, guans, etc). Chicks that consumed poison frogs showed no ill signs of poisoning afterwards. However, there could be a delayed effect on fitness or mortality of wild

birds who consume these frogs, as is seen in native *Sceloporus undulatus* fence lizards that consume *Solenopsis invicta*, an introduced, toxic red fire ant (Langkilde and Freidenfelds 2010).

Despite this, chickens trained on both stimuli learned to avoid these frogs, although *R. imitator* chicks were trending on significance ( $p=0.058$  for all treatment types) prior to treating chickens that ate frogs as outliers. We think this is justified because our chicks have much more mass than most possible avian predators, larger predators have been shown to function differently as predators (Veselý et al. 2006; Exernová et al. 2008), and predator communities are extremely varied and chemical defenses may not deter all predators (Endler and Mappes 2004). Further, the difference in our baseline data of chicks to-be-trained on *R. imitator* and *R. variabilis* should be taken into account in our final results. *Ranitomeya imitator* chicks all interacted with poison frogs significantly less than the expected ratio of 50% of the time ( $p<0.05$  in all treatments) in pre-learning trials whereas *R. variabilis* chicks did not ( $p>>0.05$  in all treatments). This likely explains at least some of the discrepancy between learned avoidance results between *R. imitator* and *R. variabilis* chicks.

We used learned avoidance by predators as a proxy for toxicity. As there is a slight difference in interaction times, there may be a difference in toxicity from the predators' perspective. Although this difference is not statistically significant between learning stimulus species, more chickens consumed *R. imitator* than *R. variabilis* (three chicks ate *R. imitator* and one ate *R. variabilis*). This may derive from differences in either the quantities of toxins and/or the actual toxins present in these species. As a result, *R. variabilis* may have more types of toxins or a suite of toxins that is more aversive from a predator's viewpoint and reduce consumptive attacks compared to *R. imitator*. Future work should investigate the chemical suites

that these frogs possess, as well as from other known mimetic populations. This knowledge may further elucidate the mimetic relationship between *R. imitator* and its congeneric models, help explain *R. imitator*'s polymorphism, and why *R. imitator* switches model species throughout its geographical range.

Polymorphism in a Müllerian mimic is theoretically detrimental. This is because predator learned aversion is thought to be exact and predators primarily learn to avoid individuals of the same morphological appearance (Sherratt 2008). *In situ* studies of wild predator populations uphold this prediction (Saporito et al. 1007; Noonan and Comeault 2009; Chouteau and Angers 2011). However, we found no difference between the learned avoidance between 'local' spotted *R. imitator* and *R. variabilis* and 'novel' striped *R. imitator* (one-way and repeated-measures ANOVAs). These results indicate that our predators did not discriminate between their learning stimuli (the spotted morph) and the 'novel' striped morph (i.e. they displayed generalized learning).

These data are contrary to how Müllerian systems are assumed to work: with rapid and intense negative selection acting against novel or rare phenotypes (Benson 1972; Mallet and Barton 1989; Kapan 2001; Pinheiro 2003, Ihalainen et al. 2008; Sherrat 2008; but see Ihalainen et al. 2006 in support of our findings). Furthermore, *in situ* studies of predation using clay models of poison frogs (including the spotted morph of *R. imitator/variabilis*) have demonstrated that novel phenotypes are attacked more frequently by avian predators and experience negative selection, thus maintaining the common shared aposematic signal (Saporito et al. 2007, Noonan and Comeault 2009, Chouteau and Angers 2011). This is important not only for the evolution of mimicry in these systems but additionally in the maintenance of mimicry complexes as even

experienced predators will continually be a source of purifying selection against rare or novel morphs and push populations towards phenotypic homogeneity (Saporito et al. 2007, Noonan and Comeault 2009, Chouteau and Angers 2011).

The difference in our findings (generalized learning in ours versus exact in these other studies) may result from exposure to both the learning stimuli morph (spotted) and the novel phenotype (striped) immediately prior to learning trials. These results may indicate how populations with great phenotypic variation persist (for example, clines where *R. imitator* transitions from one morph to another and exhibits great phenotypic variation) when theory holds that both intrapopulation phenotypic variation and mimetic polymorphism should be rare in Müllerian systems (Speed 1993). If predators are exposed to individuals that vary significantly in appearance but also share similar traits (eg. color, pattern elements, or perhaps just an appearance of aposematism) they may attribute unpalatability to the entire spectrum of individuals they are exposed to. Predator learning is often rapid (Kapan 2001; Rowland et al. 2007, Chouteau and Angers 2011), and this may contribute to generalized learning in instances where predators are exposed to varying aposematic signals.

Further, research has shown that avian predators focus primarily on colors and not patterns (Aronsson and Gamberale-Stille 2008; Exernová et al. 2006; Exernová et al. 2008). As a result, predators may cue in on specific colors, or the combination of colors, in the learning phase. Predator generalization may arise from a finite capacity to remember the myriad of palatable and unpalatable prey items that predators are exposed to (MacDougall and Dawkins 1998; Ruxton et al. 2004). Thus mimicry may not need to be perfect in Müllerian systems if prey have evolved similar characters that predators hone in on (Ruxton et al. 2004). Individuals

may become protected by learned avoidance if their coloration is perceived as ‘close enough’ by predators. This may partially explain the continued existence of some species which are polymorphic within a population or the clines between distinct morphological populations.

Both learning and reinforcement of aposematic signals occurs better in populations with higher densities (Lindström et al. 2001; Ruxton et al. 2004). In many sites in the province of San Martín, Peru, *R. imitator* is more commonly observed than its model species *R. variabilis*, although there may be sites where this trend is reversed, and there are sites above *R. imitator*’s elevational range where *R. variabilis* is very common as well (AMMS, pers. obs.).

Microgeographical variation in predator and prey communities and interactions can lead to spatial and temporal variation in the functioning of and selective pressures on aposematic systems (Mappes et al. 2004). The abundance of each species, and the rates at which predators encounter them, is an important factor in understanding mimicry in this system. Further, the effects of mixed-species learning stimuli and density-dependence (Rowland et al. 2007) are unknown in this system and we therefore cannot explain exactly how this mimicry complex functions. Further work should explore this avenue of research and will help to fully explain how mimicry in Peruvian *Ranitomeya* frogs functions.

*Ranitomeya imitator* evolved its color and pattern to mimic congeneric species throughout its geographical range in a ‘mimetic radiation’ (Symula et al. 2001, 2003). However, *R. imitator* has become much more abundant, or at least more frequently encountered by terrestrial biologists, than these congeneric model species (AMMS pers. obs.). As a result, the majority of predator learned avoidance may now be driven by *R. imitator*. Furthermore, the abundance of mimetic individuals in these populations may lead to a decrease in overall toxicity

(perhaps especially evident in *R. imitator*) as a result of resource competition (Blount et al. 2009). Frogs of the genus *Ranitomeya* appear to eat primarily ants (AMMS, unpublished data) which are extremely chitinous and likely energetically expensive to digest. A decrease in consumption of costly prey items, prey specialization, or toxin sequestration could allow more energy to be directed towards the energetically expensive process of monogamous biparental care (Brown et al. 2010; Tumulty et al. in press). Future work should test the efficacy of this hypothesis.

To our knowledge, these data represent the first experimental evidence for learned avoidance by predators in the context of Müllerian mimicry in any anuran system. Further research should focus on the relative toxicity between model and mimic species as well as the suite of chemical toxins that they possess. Additionally, studies using both wild populations of poison frogs and potential predators should be done to understand how native predators interact with these species. Given the inherent variation among populations of these frogs, this system promises to provide interesting insights into mimicry for years to come.

## References:

- Aronsson, M and G Gamberale-Stille. 2008. Domestic chicks primarily attend to colour, not pattern, when learning aposematic coloration. *Anim. Behav.* **75**:417-423.
- Bates, HW. 1862. Contributions to an insect fauna of the Amazon valley. Lepidoptera: Heliconidae. *Trans. Linn. Soc. Lond.* **23**:495-566.
- Benson WW. 1972. Natural selection for Müllerian mimicry in *Heliconius erato* in Costa Rica. *Science* **176**:936–938.
- Blount, JD, MP Speed, GD Ruxton, and PA Stephens. 2009. Warning displays may function as honest signals of toxicity. *Proc. R. Soc. B.* **276**:871-877.
- Brodie, EDI. 1993. Differential avoidance of coral snake banded patterns by free-ranging avian predators in Costa Rica. *Evolution* **47**(1):227-235.
- Brown J.L., Morales V., Summers K. 2010 A key ecological trait drove the evolution of biparental care and monogamy in an amphibian. *Am. Nat.* **175**(4), 436-446.
- Brown JL, Twomey E, Amézquita A, Barbosa de Souza M, Caldwell JP, Lötters S, von May R, Melo-Sampaio PR, Mejía-Vargas D, Perez-Peña P, Pepper M, Poelman EH, Sanchez-Rodriguez M, Summers K (2011). A Taxonomic Revision of the Neotropical Poison Frog Genus *Ranitomeya* (Amphibia: Dendrobatidae). *Zootaxa* **3083**:1–120
- Chouteau, M and B Angers. 2011. The role of predators in maintaining the geographic organization of aposematic signals. *Am. Nat.* **178**(6):810-817.

- Chouteau, M, K Summers, V Morales, and B Angers. 2011. Advergence in Müllerian mimicry: the case of the poison dart frogs of Northern Peru revisited. *Biol. Lett.* **7**:796-800.
- Daly, JW and CW Myers. 1967. Toxicity of Panamanian poison frogs (Dendrobates): Some biological and chemical aspects. *Science* **156**(3777):970-973.
- Daly, JW, TF Spande, and HM Garraffo 2005. Alkaloids from amphibian skin: A tabulation of over eight-hundred compounds. *J. Nat. Prod.* **68**(10 ):1556-1575.
- Darst, CR and ME Cummings. 2006. Predator learning favours mimicry of a less-toxic model in poison frogs. *Nature* **440**(9):208-211.
- Darst, CR, ME Cummings, and DC Cannatella. 2006. A mechanism for diversity in warning signals: Conspicuousness versus toxicity in poison frogs. *Proc. Natl. Acad. Sci. USA* **103**(15):5852-5857.
- Duellman, WE and L Trueb. 1994. *Biology of amphibians*. Baltimore, MD: John Hopkins University Press. 670 pp.
- Endler, JA, and J Mappes. 2004. Predator mixes and the conspicuousness of aposematic signals. *Am. Nat.* **163**(4):532-547.
- Exnerová, A, K Svádová, P Fousová, E Fučíková, D Ježová, A Niederlová, M Kopečková, and P Štys. 2008. European birds and aposematic Heteroptera: review of comparative experiments. *B. Insectol.* **61**(1):163-165.
- Exnerová, A, K Svádová, P Štys, S Barcalová, E Landová, M Prokopová, and R Socha. 2006. Importance of colour in the reaction of passerine predators to aposematic prey:

- experiments with mutants of *Pyrrhocoris apterus* (Heteroptera). *Biol. J. Linn. Soc.* **88**:143-153.
- García LV. 2003. Controlling the false discovery rate in ecological research. *Trends in Ecol. Evol.* **18**:553-554.
- García, LV. 2004. Escaping the Bonferroni iron claw in ecological studies. *Oikos* **105**(3):657-663.
- Gotmark, F. 1996. Simulating a Colour Mutation: Conspicuous Red Wings in the European Blackbird Reduce the Risk of Attacks by Sparrowhawks. *Funct. Ecol.* **10**(3):355-359.
- Grant, T, DR Frost, JP Caldwell, R Gagliardo, CFB Haddad, PJR Kok, DB Means, BP Noonan, WE Schargel, WC Wheeler. 2006. Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). *Bull. Am. Mus. Nat. Hist.* **299**:1-262.
- Greenwood, JJD, PA Cotton, and DM Wilson. 1989. Frequency-dependent selection on aposematic prey—some experiments. *Biol. J. Linn. Soc.* **36**:213-226.
- Guilford, T, and MS Dawkins. 1993. Receiver psychology and the design of animal signals. *Trends Neurosci.* **16**(11):430-436.
- Hegna, RH, RA Saporito, MA Donnelly. 2012. Not all colors are equal: predation and color polytypism in the aposematic poison frog *Oophaga pumilio*. *Evol. Ecol.*, published online, Sept. 15.

- Ihalainen, E, L Lindström, J Mappes, and S Puolakkainen. 2008. Can experienced birds select for Müllerian mimicry? *Behav. Ecol.* **19**:362-368.
- Ihalainen, E, L Lindström, and J Mappes. 2006. Investigating Müllerian mimicry: predator learning and variation in prey defences. *J. Evolution. Biol.* **20**(2):780-791.
- Langkilde T, Freidenfelds NA (2010) Consequences of envenomation: red imported fire ants have delayed effects on survival but not growth of native fence lizards. *Wildlife Res.* **37**:566–573.
- Lindström, L, RV Alatalo, A Lyytinen, and J Mappes. 2001. Strong antiapostatic selection against novel rare aposematic prey. *Proc. Natl. Acad. Sci. USA* **98**:9181-9184.
- Joron, M, and JLB Mallet. 1998. Diversity in mimicry: paradox or paradigm? *Trends Ecol. Evol.* **13**(11):461-466.
- Joron, M, IR Wynne, G Lamas, and JLB Mallet. 2001. Variable selection and the coexistence of multiple mimetic forms of the butterfly *Heliconius numata*. *Evol. Ecol.* **13**:721-754.
- Kapan, DD. 2001. Three-butterfly system provides a field test of Müllerian mimicry. *Letters to Nature* **409**:338-340.
- Maan, ME and ME Cummings. 2012. Poison frog colors are honest signals of toxicity, particularly for bird predators. *Am. Nat.* **179**(1):E1-E14.
- MacDougall, A and MS Dawkins. 1998. Predator discrimination error and the benefits of Müllerian mimicry. *Anim. Behav.* **55**:1281-1288.

- Master, TL. 1999. Predation by rufous motmot on black-and-green poison dart frog. *Wilson Bull.* **111**(3):439-40.
- Mallet, JLB and NH Barton. 1989. Strong natural selection in a warning-color hybrid zone. *Evolution* **43**(2):421-431.
- Mallet JLB and M Joron. 1999. Evolution of diversity in warning color and mimicry: Polymorphisms, shifting, balance, and speciation. *Annu. Rev. Ecol. Evol. Syst.* **30**:201-233.
- Mappes, J, N Marples, JA Endler. 2005. The complex business of survival by aposematism. *Trends Ecol. Evol.* **20**(11):598-603.
- Marples, NM, TJ Roper, and DGC Harper. 1998. Responses of wild birds to novel prey: evidence of dietary conservatism. *Oikos* **83**(1):161-165.
- Master, TL. 1999. Predation by rufous motmot on black-and-green poison dart frog. *Wilson Bull.* **111**(3):439-40.
- Müller, F. 1879. Ituna and Thyridia: A remarkable case of mimicry in butterflies. *Trans. Entomol. Soc. Lond.* 20-29.
- Müller, F. 1878. Über die Vortheile der Mimicry bei Schmetterlingen. *Zool. Anz.* **1**:54–55.
- Noonan, B.P. and A.A. Comeault. 2009. The role of predator selection on polymorphic aposematic poison-frogs. *Biol. Lett.* **5**(1):51-54.

- Patrick, LD, and M Sasa. 2009. Phenotypic and molecular variation in the green and black poison-dart frog *Dendrobates auratus* (Anura: Dendrobatidae) from Costa Rica. *Rev. Biol. Trop.* **57**:313-321.
- Pinheiro, CEG. 2003. Does Müllerian mimicry work in nature? Experiments with butterflies and birds (Tyrannidae). *Biotropica* **35**(3):356-364.
- Pough, FH. 1988. Mimicry of vertebrates: Are the rules different? *Am. Nat.* **131**:S67-S102.
- Poulin, B, G Lefebvre, R Ibanez, C Jaramillo, C Hernandez, AS Rand. 2001. Avian predation upon lizards and frogs in a neotropical forest understorey. *J. Trop. Ecol.* **17**:21-40.
- Prates, I, MM Antoniazzi, JM Sciani, DC Pimenta, LF Toledo, CFB Haddad, and C Jared. 2012. Skin glands, poison and mimicry in dendrobatid and leptodactylid amphibians. *J. Morphol.* **273**(3):279-290.
- Rowland, HM, E Ihalainen, L Lindström, J Mappes, and MP Speed. 2007. Co-mimics have a mutualistic relationship despite unequal defences. *Nature* **448**:62-67.
- Ruxton, GD, TN Sherratt, MP Speed. 2004. *Avoiding attack: The evolutionary ecology of crypsis, warning signals, and mimicry*. New York: Oxford University Press. 260 pp.
- Santos, JC, LA Coloma, K Summers, JP Caldwell, R Ree, and DC Cannatella. 2009. Amazonian amphibian diversity is primarily derived from late Miocene Andean lineages. *PLoS Biology* **7**(3):448-461.
- Saporito, RA, M Isola, VC Maccachero, K Condon, MA Donnelly. 2010. Ontogenetic scaling of poison glands in a dendrobatid poison frog. *J. Zool.* **282**:238-245.

- Saporito, RA, R Zuercher, M Roberts, KG Gerrow, MA Donnelly. 2007. Experimental evidence for aposematism in the dendrobatid poison frog *Oophaga pumilio*. *Copeia* **4**:1006-1011.
- Schaefer, H-C, M Vences, and M Veith. 2002. Molecular phylogeny of Malagasy poison frogs, genus *Mantella* (Anura: Mantellidae): homoplastic evolution of colour pattern in aposematic amphibians. *Org. Divers. Evol.* **2**:97-105.
- Schulte, R. 1986. Eine neue *Dendrobates*—art aus ostperu (Amphibia: Salienta: Dendrobatidae). *Sauria* **8**:11-20.
- Sherratt, TN. 2008. The evolution of Müllerian mimicry. *Naturwissenschaften* **95**:681-695.
- Siddiqi, A, TW Cronin, ER Loew, M Vorobyev, K Summers. 2004. Interspecific and intraspecific views of color signals in the strawberry poison frog *Dendrobates pumilio*. *J. Exp. Biol.* **207**:2471-2485.
- Smith, SM. 1975. Innate recognition of coral snake pattern by a possible avian predator. *Science* **187**(4178):759-760.
- Speed, MP. 1999. Batesian, quasi-batesian or Müllerian mimicry? Theory and data in mimicry research. *Evol. Ecol.* **13**:755-776.
- Speed, MP. 1993. Muellierian mimicry and the psychology of predation. *Anim. Behav.* **45**:571-580.
- Turner, JRG and MP Speed. 1999. How weird can mimicry get? *Evol. Ecol.* **13**:807-827.
- Stiles, FG and AF Skutch. 1989. *A guide to the birds of Costa Rica*. Ithaca, NY: Cornell University Press. 656 pp.

- Symula, R, R Schulte, and K Summers. 2001. Molecular phylogenetic evidence for a mimetic radiation in Peruvian poison frogs supports a Müllerian mimicry hypothesis. *Proc. R. Soc. B.* **268**:2415-2421.
- Symula, R, R Schulte, and K Summers. 2003. Molecular systematics and phylogeography of Amazonian poison frogs of the genus *Dendrobates*. *Molecular Phylogenetics and Evolution* **26**:452-475.
- Turner, JRG. 1984. The palatability spectrum and its consequences. In: *The biology of butterflies*. Vane-Wright RI and Ackery P, editors. Princeton: Princeton University Press. 429 pp.
- Verhoeven, KJF, KL Simonsen, and L McIntyre. 2005. Implementing false discovery rate control: increasing your power. *Oikos* **108**:643-647.
- Veselý, P, S Veselá, R Fuchs, and J Zrzavý. 2006. Are gregarious red-black shieldbugs, *Graphosoma lineatum* (Hemiptera: Pentatomidae), really aposematic? An experimental approach. *Evol. Ecol. Res.* **8**:881-890.
- Yeager, J, JL Brown, V Morales, M Cummings, K Summers. 2012. Testing for selection on color and pattern in a mimetic radiation. *Curr. Zool.* **58**(4):667-675.

## APPENDIX: Funding and institutional approval

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