The impact of sexual and natural selection on signal divergence in a polytypic poison dart frog (*Oophaga pumilio*, Dendrobatidae)

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Summary

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In the presented project I aim to identify the evolutionary forces driving and maintaining phenotypic divergence among geographically separated populations of a color-diverse neotropical frog species. To evaluate which factors facilitated the evolution of phenotypic variation in the strawberry poison frog (*Oophaga pumilio*), I examined the roles of sexual selection and natural selection favoring divergence on color signals between genetically distinct populations. To address this question I selected six study populations across the distribution range of *O. pumilio* including populations of both genetic groups in which this species has diverged. I consider the selection of populations of both genetic groups to be of particular interest, as the Northern genetic lineage consists of monomorphic red populations, while the Southern genetic lineage embraces more than 15 distinct color morphs of the Bocas del Toro province in Panama.

In the first part of the project I investigated predation pressure as a mean of **natural selection**. I evaluated, how predation pressure varies among populations and among different color phenotypes. In particular I tested whether local colors are less prone to be attacked by predators compared to nonlocal aposematic or cryptic colors. Furthermore, I investigated whether the composition of predator communities differs among populations. Additionally, applying visual models I expanded former studies of predation pressure on different colors of clay model frogs with an evaluation of the conspicuousness of certain clay colors to different predators. Compared to previous investigations, I extended my experiments to a broader geographic scale. The results proved that predation pressure varies strongly among populations and birds are the major predators of *O. pumilio*. Even though frog coloration is an honest indicator of toxicity in this species, clay frogs of local and nonlocal coloration were equally attacked. Interestingly, elevated predation pressure by birds was linked to high conspicuousness values of the local frogs to the avian visual system. Hence, it is likely that avian predation pressure as a component of natural selection facilitates the evolution of conspicuous aposematic coloration.

In my second study on **sexual selection**, I integrated two geographically diverged sexual signals: advertisement calls and coloration. Unlike previous studies, I did not only focus on unimodal signals. Instead, I included the two signals which, in isolation, have been linked to pre-mating isolation and divergence between populations of *O. pumilio*. Also in contrast with previous studies, I evaluated the preference of definite receptive females to ensure that detected preferences are related to mate selection as opposed to other social interactions (e.g. rivalry), and thus can serve as an indicator of sexual selection. Furthermore, all experiments were conducted under the least possible disturbance of the females in their natural habitat under field conditions. The results demonstrated a strong preference for local over nonlocal advertisement calls in Southern populations, whereas the females from Northern populations were less selective. On the other hand, presence of model frogs of local and nonlocal coloration during bimodal experiments did barely affect female choice. I conclude, that female mate choice in *O. pumilio* is primarily guided by advertisement calls, which are perceptible over distances of several meters, while coloration might be employed as a secondary sexual signal during close-range intersexual interactions. These mate choice experiments not only highlight geographical differences in the strength of female preferences for traits involved in pre-mating behavioral isolation, but also demonstrate that multiple signal modalities differ in their importance for sexual selection, and that the outcome of experiments under field conditions might lead to different conclusions as expected from laboratory experiments.

For the third part of my project, I measured reflectance data of courting couples in three populations to test whether variability of coloration within a population predicts reproductive success of individual frogs. Although sexual dimorphism has been described for one Panamanian population of *O. pumilio,* and laboratory experiments demonstrated female preferences for brighter males, no study has investigated whether pronounced brightness entails reproductive success under natural choice situations. Visual modeling revealed that in one of three populations, male and female frogs which were engaged in courtship had significantly higher brightness and lower color contrast indices compared to unmated animals of the same population, which provides first evidence that brightness predicts reproductive success in natural populations of *O. pumilio.*

I here present a project investigating the importance of sexual selection on coloration and calls – operating via assortative mating preferences exerted by females of *O. pumilio* - and natural selection on coloration of frogs – acting predominantly through predation. To understand the evolution of phenotypic signals used in intraspecific communication, it is critical to address both of these classic forms of selection, given that signals involved in mate selection, such as (conspicuous) coloration, advertisement calls or chemical cues, are often perceptible for predators and parasites as well as for conspecifics. The interactions between these forces deserve further investigation, as they increase the dimensionality of the selective landscape of any given trait. Investigating six populations, I aim to clarify, if the importance of natural and sexual selection for signal divergence is consistent across the distribution range of *O. pumilio*. All experiments demonstrated that selection pressures strongly vary among populations, which highlights the prevalence of geographic variation on the strength of selection and emphasizes the importance of studying multiple populations in research on evolutionary drivers of divergence. Together with other previous and recent studies, I provide evidence showing that geographic color variation in the study species is the result of simultaneous sexual and natural selection.

Keywords: natural selection, sexual selection, phenotypic divergence.

Zusammenfassung

Der Einfluss von ökologischer und sexueller Selektion auf die phänotypische Divergenz beim Erdbeerfröschchen (*Oophaga pumilio*, Dendrobatidae)

Das vorgestellte Projekt untersucht, welchen Einfluss natürliche und sexuelle Selektion auf die phänotypische Divergenz zwischen verschiedenen Populationen einer polytypischen neotropischen Froschart (*Oophaga pumilio)* haben. Hierzu wählte ich sechs Populationen entlang des Verbreitungsgebiets des Erdbeerfröschchens aus. Zwei der untersuchten Populationen stammen hierbei aus der nördlichen genetischen Gruppe, in welcher alle Populationen rot gefärbt sind, während die restlichen Populationen aus den über 15 unterschiedlichen Farbmorphen der südlichen genetischen Gruppe ausgewählt wurden.

Im ersten Teil des Projektes untersuchte ich den Prädationsdruck als Maß für die Wichtigkeit von **natürlicher Selektion** auf die stammesgeschichtliche Veränderung von Merkmalen. Hierbei ergänzte ich die Studie durch visuelle Modelle, mit denen ich ermittelte, ob die Wahrscheinlichkeit eines Angriffs von der Auffälligkeit der Froschmodelle für die jeweiligen Prädatoren abhängt. Die Ergebnisse beweisen, dass sich der Prädationsdruck zwischen den Populationen stark unterscheidet und Vögel für den größten Teil der registrierten Angriffe verantwortlich sind, wobei Angriffe anderer potentieller Fressfeinde selten waren. Interessanterweise zeigte die Analyse, dass die lokale Färbung der Frösche für das visuelle System von Vögeln in Populationen mit hohem Prädationsdruck besonders auffällig erscheint. Die Angriffswahrscheinlichkeit war zwar direkt abhängig von der Färbung der Froschmodelle, jedoch nicht davon, ob die Froschmodelle der lokalen Färbung der Frösche entsprachen. Dieses Ergebnis überrascht, da die Färbung der Erdbeerfröschchen mit ihrer Giftigkeit korreliert, d.h. auffällig gefärbte Populationen giftiger sind als kryptische. Insgesamt deuten die Ergebnisse darauf hin, dass der Prädationsdruck durch Vögel – und somit natürliche Selektion - die Evolution der verschieden gefärbten Populationen beim Erdbeerfröschchen beeinflusst hat.

Für die Studie zu **sexueller Selektion** machte ich mir zunutze, dass sich die einzelnen Populationen des Erdbeerfröschchens im Laufe der Jahrtausende bezüglich mehrerer phänotypischer Merkmale auseinanderentwickelt haben. Sowohl die Färbung als auch die Anzeigerufe spielen bei der Partnerwahl von *O. pumilio* eine Rolle und unterscheiden sich zwischen den beiden genetischen Gruppen und deren Populationen. Weisen die Weibchen einer Froschgruppe starke Präferenzen für die Merkmale lokaler Männchen auf, kann dies über präzygotische Isolation die fortschreitende Divergenz zwischen Populationen fördern, und somit als Indikator für sexuelle Selektion herangezogen werden. Um zu ermitteln, wie ausgeprägt die Präferenzen der Weibchen für lokale über fremde Merkmale (Rufe bzw. Rufe und Färbung) sind, testete ich ihr Wahlverhalten in uni- und bimodalen Playback-Experimenten. Im Gegensatz zu bestehenden Studien wurden hierbei ausschließlich eindeutig rezeptive Weibchen getestet um sicherzustellen, dass die gefundenen Präferenzen auf die

Partnerwahl bezogen sind. Des Weiteren wurden alle Experimente unter möglichst geringer Störung der Tiere in ihrem natürlichen Habitat durchgeführt. Die Weibchen der südlichen genetischen Gruppe zeigten eine starke Präferenz für lokale über fremde Rufe, während die Weibchen nördlicher Populationen weit weniger wählerisch waren. In allen Populationen wurde die Wahl der Weibchen kaum durch die Kombination der Rufe mit lokal bzw. fremd gefärbten Froschmodellen beeinflusst. Die Ergebnisse belegen, dass die Partnerwahl der weiblichen Erdbeerfröschchen in erster Linie auf den Anzeigerufen der Männchen, die über mehrere Meter hörbar sind, basiert. Die Färbung hingegen könnte später während sexueller Interaktionen im Nahbereich von Bedeutung sein. Visuelle und akustische Signale sind also von unterschiedlicher Wichtigkeit für sexuelle Selektion und die Präferenzen für diese Merkmale unterscheiden sich stark zwischen geographisch getrennten Populationen.

In einer südlichen Population von *O. pumilio* wurde Sexualdimorphismus beschrieben, und in Laborexperimenten zeigten Weibchen eine Präferenz für hellere Männchen, was auf sexuelle Selektion bezüglich hellerer Färbung hinweisen kann. Jedoch fehlten bislang Studien, die belegen, dass eine hellere Färbung den Fortpflanzungserfolg unter natürlichen Bedingungen begünstigt. Im dritten Teil des vorgestellten Projekts untersuchte ich, ob farbliche Unterschiede zwischen Individuen innerhalb einer Population einen Einfluss auf den Fortpflanzungserfolg haben. Hierzu wurden die Reflektionsspektren von Männchen und Weibchen balzender Froschpaare in drei Populationen gemessen und mit den Daten nicht balzender Tiere verglichen. Visuelle Modelle belegten, dass sich die Männchen und Weibchen der balzenden Froschpaare in einer der drei Populationen signifikant von nicht balzenden Tieren unterschieden: sie wiesen höhere Helligkeitskontraste und niedrigere Farbkontraste auf. Die vorliegenden Daten liefern erste Beweise, dass hellere Individuen zumindest in manchen natürlichen Populationen des Erdbeerfröschchens eine höhere Fortpflanzungswahrscheinlichkeit besitzen.

Studien zur Evolution phänotypischer Signale sollten den Einfluss von natürlicher und sexueller Selektion betrachten, da meist mehrere Evolutionsfaktoren gleichzeitig agieren. Bei der Partnerwahl genutzte Signale, wie z.B. auffällige Färbung, Laute oder Duftstoffe, sind häufig auch für Prädatoren oder Parasiten wahrnehmbar. Das vorgestellte Projekt untersucht den Einfluss sexueller Selektion auf die Färbung und die Anzeigerufe der Männchen (ausgedrückt durch Präferenzen für lokale Merkmale) sowie den Einfluss natürlicher Selektion mittels Prädationsdruck auf die Färbung der Frösche. Durch die Untersuchung von sechs Populationen entlang des Verbreitungsgebietes von *O. pumilio* konnte ich belegen, dass der Einfluss beider Evolutionsfaktoren zwischen den Populationen stark variiert. Somit unterstreichen die Ergebnisse die Wichtigkeit der Untersuchung mehrerer Populationen sowie verschiedener, simultan wirkender Evolutionsfaktoren bei der Erforschung evolutionärer Auslöser phänotypischer Divergenz. Die präsentierte Studie liefert weitere Belege dafür, dass die Farbvielfalt geografisch getrennter Populationen des Erdbeerfröschchens das Ergebnis gleichzeitig wirkender sexueller und ökologischer Selektion ist.

Schlagworte: natürliche Selektion, sexuelle Selektion, phänotypische Divergenz.

1. Introduction

1.1 Consequences of signal divergence

Sexual signals like mating calls, color patterns and ornaments are important for intersexual communication and often vary among populations of the same species or closely related species. Variation in sexual traits may affect mate selection, and hence, be involved in behavioral prezygotic isolation. Prezygotic isolation includes the preferences for the local signals over signals from nonlocal populations in one or both sexes. Strong preferences for local traits will prevent hybridisation among groups that differ in sexual signals. Prezygotic behavioral isolation is a crucial mechanism of divergence between populations which are undergoing speciation, and is often more important than postzygotic isolation (Coyne and Orr, 1997). Prezygotic isolation through assortative mating based on acoustic signals has been reported in insects, birds and anuran species (Wilkins et al., 2013). Prezygotic isolation through mate choice on coloration has been observed in neotropical butterflies (Jiggins et al., 2001, Kronforst et al., 2006) and neotropical frogs (Reynolds and Fitzpatrick, 2007, Maan and Cummings, 2008, Maan and Cummings, 2009). Both of these groups possess aposematic coloration, which likely evolved in the context of ecological adaptation as an anti-predator strategy (Venesky and Anthony, 2007, Blount et al., 2009), and thereinafter was employed for sexual selection (Jiggins et al., 2001, Nokelainen et al., 2012, Crothers and Cummings, 2015).

For an animal seeking to reproduce, a first step is to attract the attention of a potential mate. Besides coloration, emitting sound is a prominent feature to approach this task. Depending on properties of the habitat, such as lighting conditions, plant cover, background noise and population density, song might be more effective than coloration in order to draw attention. In anuran amphibians, advertisement calls are crucial for mate choice (Ryan and Rand, 1993, Pröhl et al., 2007, Gerhardt and Huber, 2002, Gerhardt, 2005, Erdtmann and Amezquita, 2009). The dominant frequency of the call provides information about size, and endurance in calling reflects the overall fitness of a male (Pröhl, 2003, Ryan, 1980) and genetic quality (Welch et al., 1998). In amphibians, the evolution of mating calls has been driven by both intra- and intersexual selection (Gerhardt and Huber, 2002, Ryan and Rand, 1993).

The study model of this investigation is the strawberry poison frog (*Oophaga pumilio*), which produces signals of several modalities, including visual and acoustic signals (Meuche et al., 2012, Pröhl et al., 2007, Summers et al., 2003). The calls of *O. pumilio* vary geographically among populations and between two existing genetic lineages (Hagemann and Pröhl, 2007, Pröhl et al., 2007, Summers et al., 2003, Brown et al., 2010). Among populations, skin coloration and patterning is highly diverse, and more than 15 distinct, mostly geographically separated color morphs have been described (Pröhl et al., 2013, Summers et al., 2003, Daly and Myers, 1967). Most color morphs, including aposematic and cryptic colors, occur at the islands and adjacent mainland of the Bocas del Toro Archipelago in Panama, in spite of the very recent formation of the Archipelago within the last 10,000 years (Summers et al., 1997, Reynolds and Fitzpatrick, 2007).

The ecology and behavior of this species is well studied (Donnelly, 1989, Saporito et al., 2004, Pröhl and Ostrowski, 2011, Richards-Zawacki et al., 2012) and many others), with a majority of studies focused on intra- and interspecific communication. The extreme color diversity in this species enabled researchers to investigate whether females prefer males with the local coloration (Summers et al., 1999, Reynolds and Fitzpatrick, 2007, Maan and Cummings, 2008) or males with brighter coloration (Maan and Cummings, 2009) in laboratory experiments. The data suggested that females in most populations preferred males of the local color morph over males of nonlocal color morphs. Further studies evaluated how coloration of this toxic species might affect predation risk and confirmed the aposematic function of the conspicuous coloration of the strawberry poison frog (Maan and Cummings, 2012). Besides predation, further environmental parameters may also affect the evolution of coloration in strawberry poison frogs. As poison frogs acquire their skin toxins through consumption of toxic prey items, such as mites and ants (Saporito et al., 2007b, Saporito et al., 2004), shortage in these food sources may confine toxicity of a population. Because toxicity in *O. pumilio* is positively correlated with their conspicuousness (Maan and Cummings, 2012) this limitation might also affect divergence in coloration. Interestingly, differently colored frogs from one polymorphic green and orange population on Bastimentos were equal in their toxicity, which furthermore supports the idea of a habitat driven effect on toxicity (Maan and Cummings, 2012).

Finally, color divergence among populations of *O. pumilio* seems to be mediated by both sexual and natural selection (see review (Gehara et al., 2013)), and not via genetic drift

(Brown et al., 2010, Rudh et al., 2007, Summers et al., 1997, Wang and Summers, 2010). Among populations of strawberry poison frogs color divergence proceeds much faster than expected under neutral, random processes such as genetic drift (Brown et al., 2010). While the roles of natural and sexual selection have been confirmed several times (Maan and Cummings, 2008, Maan and Cummings, 2012, Pröhl and Ostrowski, 2011), the relative importance of each form of selection is not yet clear. Furthermore, these previous studies were important to evaluate the influence of natural and sexual selection on color divergence in this species, but the importance of advertisement calls and prezygotic isolation regarding this trait has received very little attention (but see (Meuche et al., 2013)). The advertisement calls of the two genetic lineages of strawberry poison frogs differ considerable. This might cause prezygotic isolation between the incipient genetic lineages in areas where animals of the two diverging genetic groups became in secondary contact, as described for seabirds (Lopez-Rull et al., 2016). The evaluation of several sexual signals of different sensory modalities, which may interact during the process of sexual selection, can provide important information about mate selection. The relevance of multiple sexual signals has been studied in the context of within-population sexual selection in several animals (Taylor et al., 2007, Vortman et al., 2013, Taff et al., 2012) but insufficiently as drivers of behavioral prezygotic isolation among diverging populations.

1.2 Signals as a mean of communication with conspecific and heterospecific observers

Phenotypic traits and signals are important means for both intra- and interspecific communication, and may inform (or mislead) the receiver about the identity and intentions of the sender. Phenotypic signals used in intraspecific communication (e.g. for mate selection and intrasexual aggressive interactions) are also visible to heterospecific observers, e.g. predators or parasites (Maan and Cummings, 2008, Crothers and Cummings, 2013, Crothers and Cummings, 2015). Hence, as mentioned earlier, the evolution of phenotypic traits is influenced by several selective forces at the same time, including sexual selection and natural selection (Ruxton et al., 2004, Hernandez-Jimenez & Rios-Cardenas, 2012; Morgans et al., 2014; Endler, 1980). In particular, the evolution of conspicuous signals faces a trade-off between producing conspicuous signals for conspecifics or signals which are not easily detected by predators (Nokelainen et al., 2012, Hernandez-Jimenez and Rios-Cardenas, 2012). For example, in the polymorphic aposematic wood tiger moth, *Parasemia plantaginis*, white coloration entails higher mating success, while yellow individuals are better protected against predation (Nokelainen et al., 2012). In the fiddler crab one highly conspicuous feature is the mouthpart which is mainly visible to conspecifics but hidden from avian predators (Cummings et al., 2008). Just as well sexual and natural selection might also act in concert in the evolution of signaling traits, in particular in aposematic animals. This is the case in *Heliconius* butterflies, where the local phenotype is the most successful for both mate attraction and predator deterrence (Finkbeiner et al., 2014). Aposematic animals demonstrate their unprofitability as prey to potential predators through conspicuous features, in most cases through striking coloration, and thus reduce the risk to be attacked by predators (Mappes et al., 2005, Ruxton et al., 2004). The Heliconius butterfly example demonstrates that high levels of detectability may also be advantageous for predator deterrence in aposematic animals (Cummings and Crothers, 2013, Endler and Mappes, 2004). If predators are able to detect a novel prey from a long distance, they would have more time to assess its profitability during a first approach. This may enhance predator learning to avoid highly conspicuous toxic prey after a first unpleasant encounter. Through identification of potential prey at large distances, learnt aversion can be remembered while approaching the same type of prey for a second time (Gamberale-Stille, 2000, Guilford, 1986). Noteworthy, a warning signal may not only consist of conspicuous coloration presenting pronounced brightness or flashy colors (Nokelainen et al., 2012, Darst et al., 2006, Crothers et al., 2016), but may be reinforced by the use of additional sound or odor, creating an efficient multimodal warning signal (Rowe and Guilford, 1999).

Generally, a successful predation event consists of two elements: first, detection of prey, and second, the decision to attack detected prey items (Stuart et al., 2012). As discussed above, display of aposematic signals may reduce the probability of the predator’s decision to carry out an attack after detection of a prey. Crypsis on the other hand prevents successful predation by impeding detection of prey which blend into the background. Depending on local conditions, e.g. habitat heterogeneity, lighting conditions, predation pressure and availability of noxious prey, different anti-predator strategies might be of selective advantage for a species.

In research investigating predation pressure as an indicator of natural selection many studies focus on the second step of a predation event and compare the likelihood of an attack on different prey items without including information about the visual detectability of these prey items on their specific background to the respective predator. A single prey species might be subject to predation pressure caused by different predator taxa, which differ in the receptive capacities of their visual, acoustic and olfactory systems (Mappes et al., 2005). Several studies evaluated predation pressure considering the visual systems of different predators and hence, tried to incorporate both steps of a predation event – detectability and the probability of an attack. For different species of ladybirds visual modeling revealed that chromatic contrast honestly indicates toxicity to avian predators and that survival of ladybird prey covaried with conspicuousness (Arenas et al., 2015). Another interesting study combining visual modeling and clay model experiments showed that in *Oophaga granulifera*, predation caused by two different predator taxa with tetrachromatic vision followed unequal patterns. While lizard attacks were positively correlated with the conspicuousness of model frogs, bird attacks were consistently directed to clay models resembling nonlocal color morphs (Willink et al., 2014). Interestingly, conspicuousness is negatively correlated to toxicity in *O. granulifera* (Wang, 2011), while for *O. pumilio* conspicuousness is an honest indicator for toxicity (Maan and Cummings, 2012). A study conducted for *O. pumilio* investigated, how the conspicuousness of several prey types to a tetrachromatic tropical bird is correlated with the probability of an attack on the respective model. The results showed that high detectability does not entail high attack rates, which implies that bird predators process information about detected prey items and make post-detection decisions on whether to carry out an attack (Stuart et al., 2012). Another interesting study of Prudic et al. (Prudic et al., 2007) investigates, whether brightness contrast may serve as an effective aposematic signal to predators with very restricted or absent color vision. They revealed that brightness contrast alone can actually serve as an aposematic signal to predatory mantis. This indicates that the general understanding of aposematism as a function of both chromatic (i.e. presence of conspicuous hues) and brightness contrast (Ruxton et al., 2004) needs to be rethought and supports the idea, that any signal (color or pattern), which is conspicuous enough to exceed a certain threshold may serve as an aposematic signal (Tazzyman and Iwasa, 2010).

Several species of dendrobatid frogs possess aposematic coloration and some species show high intraspecific variability of coloration among populations (polytypism). Frogs from different populations of *O. pumilio, O. granulifera, Oophaga histrionica, Oophaga sylvatica,* *Dendrobates variabilis, Dendrobates tinctorius* and *Ranitomeya imitator* possess different coloration and/or pattern. Color polytypism among populations of *R. imitator* represents a form of Müllerian mimicry, resembling coloration and pattern of other sympatrically occurring toxic frogs (e.g. *Ranitomeya ventrimaculata* and *Ranitomeya fantastica*) (Symula et al., 2001). For *O. granulifera* both cryptic and conspicuously colored populations have been described (Wang, 2011), which might be the result of differences in predation pressure among populations (Willink et al., 2014).

In *O. pumilio* both the aposematic function of coloration and the importance of coloration for mate selection have been confirmed in several polytypic populations (Saporito et al., 2007c). Frog coloration probably impacts close-range mate choice and facilitates assortative mating in this species (Maan and Cummings, 2008, Summers et al., 1999). Advertisement calls on the other hand are detectable over larger distances and might allow for long-distance mate attraction (personal observation and (Pröhl, 2005)). While visual and acoustic signals may serve intraspecific communication, both of these signals may be detectable to heterospecific observers - including predators - over large distances as well. Depending on the local composition of predator communities and further geographically variable ecological parameters (e.g. availability of noxious prey) natural selection may cause purifying selection constraining phenotypic variation or directional selection driving signal evolution towards the development of new properties.

Although several projects investigated the effect of coloration of strawberry poison frogs on probability of an attack by predators it is not yet clear, which animals actually predate on this species. There are scarce predation events on *O. pumilio* which are reported in literature, including one by a tropical bird, the motmot (*Baryphthengus martii*) (Alvarado et al., 2013), tropical snakes (i.e. *Rhadinaea decorata*) (Saporito et al., 2007c, Lenger et al., 2014) as well as anecdotal descriptions about spiders (Santos and Cannatella, 2011) see supplementary material)). Further potential predators may be certain species of crabs and lizards (Maan and Cummings, 2012, Willink et al., 2014). These different predators might perceive visual signals from different body regions of their prey (e.g. terrestrial crabs or lizards compared to aerial birds) (Mappes et al., 2005, Cummings et al., 2008, Siddiqi et al., 2004). More importantly, due to differences in their visual systems these predators probably perceive coloration of the frogs very differently (Maan and Cummings, 2012), and traits conspicuous to one predator may be rather cryptic to another (Mappes et al., 2005). Visual models allow for the calculation of the visual conspicuousness of an object, taking particular visual sensitivities of hetero- or conspecific observers into account. The models incorporate information about different classes of photoreceptors and their ratio in the retina of the observing animal species as well as the existence of oil droplets and double cones in their respective visual systems. Incorporating these models in studies of communication with heterospecific and conspecific observers may facilitate to identify the mechanism which drive divergence in phenotypic traits – including the effect of natural selection by predation and sexual selection through preferential mate selection of females.

1.3 Evaluation of the role of natural selection on the evolution of phenotypic signals

In addition to visual modeling I conducted experiments using clay models of frogs to evaluate how attack probability on different colors of clay models varies among populations. I aim to determine, how natural selection may have influenced coloration divergence among populations by integrating information about population differences in predation pressure and predator communities and their visual systems.

In research on color evolution driven by natural selection, it is common practice to extrapolate from clay model experiments to the importance of predation pressure on color divergence. Several studies evaluated predation pressure on *O. pumilio* (Hegna et al., 2012, Paluh et al., 2014, Saporito et al., 2007c, Stuart et al., 2012) and other dendrobatid frogs (Chouteau and Angers, 2011, Chouteau and Angers, 2012, Willink et al., 2014) through clay model experiments. The use of clay models resembling the study species allows to investigate predation pressure and the composition of predator communities because by means of characteristic teeth or claw imprints preserved in the clay attacks can be assigned to different predator classes. Applying this method it was shown that strong homogenizing selection caused by birds favored the survival of the local aposematic morph over novel aposematic color morphs in two polytypic frog species (*R. imitator* and *D. tinctorius*) (Chouteau and Angers, 2011, Noonan and Comeault, 2009). In *Ranitomeya imitator* the survival for the local aposematic morph was higher, especially in monomorphic populations (Chouteau and Angers, 2012). In the previously mentioned study in *O. granulifera* clay model experiments revealed that color dependency of predation pressure varies among different predator taxa, with lizards consistently attacking the most conspicuous red clay models, while birds avoided to attack clay models of local coloration (Willink et al., 2014). Hence, in some populations different predator taxa might cause opposing selective advantages on color morphs while in others, congruent selective forces might cause purifying selection and prevent the emergence of novel color morphs.

The aposematic signal function of some conspicuously colored populations of *O. pumilio* has been confirmed by several studies (Hegna et al., 2012, Maan and Cummings, 2012, Saporito et al., 2007c) and conspicuousness of strawberry poison frogs reliably reflects their toxicity level (Maan and Cummings, 2012). In close geographic proximity to conspicuous aposematic frog populations, several populations of cryptic dull coloration exist (e.g. green frogs from Isla Colón) (Wang and Shaffer, 2008). Cryptic and aposematic frogs of different populations across the Bocas del Toro Archipelago were furthermore found to show significant differences in their behavior, showing cryptic or bold behavior, respective to their coloration (Pröhl and Ostrowski, 2011, Galeano and Harms, 2016, Rudh et al., 2013, Rudh et al., 2011). This indicates that cryptic and aposematic anti-predator strategies consist of several complementing elements. One further project using clay model frogs encountered red clay models to be less attacked than brown models (Saporito et al., 2007). This pattern was furthermore enhanced by the use of moving red and brown clay models (Paluh et al., 2014). However, in one cryptic population of *O. pumilio* (Pröhl and Ostrowski, 2011), clay model frogs resembling the local color morph were more frequently attacked than nonlocal aposematic ones and brown controls (Hegna et al., 2012). Hegnas results support the idea that some conspicuous colors, e.g. red and yellow, might generally act as aposematic colors and deter predators.

Due to differing environmental conditions among geographically isolated populations (Hegna et al., 2012, Brown et al., 2010, Ruxton et al., 2004), in different populations opposing expressions of a signal may be of selective advantage, e.g. cryptic vs. conspicuous coloration (Rundle and Nosil, 2005, Maan and Seehausen, 2011). Strawberry poison frogs possess varying coloration, including aposematic and cryptic color morphs, which resemble two images of contrary anti-predator strategies, which might be reinforced by bold or conspicuous behavior, respectively (Pröhl and Ostrowski, 2011). Due to this fact, I propose that it is very likely that predation pressure, as a mean of natural selection, varies among populations and affected the development of color variety in this species. As conspicuousness in this species is an honest indicator of toxicity (Maan and Cummings, 2012) I expect to measure high levels of attack probabilities in areas where conspicuous coloration of strawberry poison frogs has evolved. Because cryptic species are little toxic (Maan and Cummings, 2012) and skin toxins in this species are mainly sequestered from noxious prey (Saporito et al., 2007b, Saporito et al., 2004) I suggest that elaborate cryptic anti-predator strategies which become manifest in coloration and behavior (Pröhl and Ostrowski, 2011) might have evolved in populations with equally high levels of predation pressure, but low availability of toxic food items. Therefore, I expect lower predation pressure to be present at populations of intermediate conspicuousness.

1.4 The importance of sexual selection for divergence of phenotypic signals

Pre-mating sexual selection often acts on male signals used in intra- and inter-sexual interactions. Males may signal to other males their defensive capacities (Jones and Ratterman, 2009) or display them during courtship to increase their mating success (Uy et al., 2009). Sexual signals often vary among populations of the same species or closely related species and strong preferences for local signals might restrict gene flow through behavioral prezygotic isolation and therefore finally facilitate speciation (Butlin et al., 2012, Smadja et al., 2004, Grace and Shaw, 2012, Brambilla et al., 2008, Panhuis et al., 2001, Maan and Seehausen, 2011, Wilkins et al., 2013, Mendelson and Shaw, 2005). If sexual selection affected ongoing divergence of signals in a species, I expect that the local trait is adapted to the preference of the choosing sex. I furthermore expect mate selection to prevent hybridization among populations that differ in the expression of their sexual signals (Grace and Shaw, 2012, Brambilla et al., 2008, Mendelson and Shaw, 2005). The hypothesis that female preferences cause male trait evolution through sexual selection is generally accepted, and research providing empirical evidence to support this assumption is growing. One study on collared lizards revealed that both intra- and intersexual selection actually affected the evolution of body size and coloration in this species to different degrees among populations (Baird et al., 1997). Quickly evolving species of Hawaiian crickets have diverged regarding their male courtship calls and the respective female preferences support the hypothesis of sexual selection to facilitate rapid speciation in these arthropod genus (Mendelson and Shaw, 2005). In jumping spiders, sexual selection exerted by female spiders caused recent divergence of males` sexual traits among populations, including courtship behavior and physical phenotypic characteristics, which may indicate incipient speciation (Masta and Maddison, 2002). Mate selection in bowerbirds is based on complex bowers elaborated by male bowerbirds. The design of bowers differs geographically among populations and females prefer local bowers over nonlocal ones, which provides evidence of sexual selection to act on the evolution of bower architecture (Uy and Borgia, 2000).

Coloration has been shown to underlie assortative mating not only in cichlid fish (Pauers et al., 2010, Couldridge and Alexander, 2002, Seehausen and van Alphen, 1998) and frogs (Reynolds and Fitzpatrick, 2007, Maan and Cummings, 2008, Maan and Cummings, 2009), but also in neotropical butterflies (Jiggins et al., 2001, Kronforst et al., 2006, Chamberlain et al., 2009). In recently diverged sister species of Heliconius butterflies (*Heliconius melpomene*, *Heliconius cydno* and *Heliconius pachinus*) assortative mate preferences facilitated reproductive isolation among incipient species (Jiggins et al., 2001, Kronforst et al., 2006), although wing color divergence has been initiated by means of natural selection (Kronforst et al., 2006). Similarly, reproductive isolation through assortative mate selection based on coloration was pivotal for speciation in cichlid fish (Couldridge and Alexander, 2002, Seehausen and van Alphen, 1998, Seehausen, 1997). In strawberry poison frogs female mate choice based on color patterns has been broadly investigated. The studies reported different patterns and degrees of assortative mating by spotting pattern and coloration in different populations (Summers et al., 1999, Maan and Cummings, 2009, Richards-Zawacki and Cummings, 2011, Reynolds and Fitzpatrick, 2007, Maan and Cummings, 2008). Females of most but not all populations preferred males of their own color morphs, however successful interbreeding between color morphs was also possible (Dugas and Richards-Zawacki, 2015). These studies were conducted under artificial laboratory conditions and evaluated general preference functions. However, mate preferences measured under laboratory conditions do not necessarily reflect mate selection under natural conditions (Jennions and Petrie, 1997, Gerhardt and Huber, 2002). Therefore, the validity of these preferences should be verified under natural field conditions (Formica et al., 2016). Richards-Zawacki et al. approached this task with a genetic (pedigree) analysis in a polymorphic population of Bastimentos. They discovered that red females mated assortatively, while yellow ones were less selective (Richards-Zawacki et al., 2012). This result is consistent with the general expectation that the rarer morphotype ought to be less discriminating than the more frequent one (Gröning and Hochkirch, 2008, Wirtz, 1999).

Besides coloration, assortative mate selection might also be based on acoustic cues, and its importance for prezygotic isolation between diverging populations and incipient species has been described for different animal taxa including insects, birds, primates and several anuran species (Wilkins et al., 2013, Braune et al., 2008, Boul et al., 2007, Noh and Henry, 2009, Uy et al., 2009, Mendelson and Shaw, 2005). Two sympatric species of mouse lemurs are characterized by species-specific male advertisement calls, which play a role for prezygotic reproductive isolation and thus, might have played a role during speciation of these species (Braune et al., 2008). In the túngara frog *Physalaemus pustulosus*, females prefer local male advertisement calls over nonlocal ones, which suggests for assortative mate preferences, and consequentially for reproductive isolation among populations (Ryan et al., 2007, Pröhl et al., 2006). In one group of insect species, the green lacewings (*Chysoperla carnea group)*, prezygotic isolation based on differences in songs and the respective assortative mate preferences caused rapid separation of five sister species (Noh and Henry, 2009). The strength of sexual selection by female choice differs among these species, which is supposed to depend on encounter probabilities with heterospecific males (Noh and Henry, 2009). Similarly, gene flow among different populations of Hawaiian crickets is limited by prezygotic isolation based on divergence in pulse rate of male songs, which prevents hybridization between diverging lineages (Grace and Shaw, 2012, Mendelson and Shaw, 2005). In one small passerine species, reproductive isolation between the Moltoni's warbler *Sylvia cantillans moltonii* and the currently originating subspecies *Sylvia cantillans cantillans* is also mediated through differences in male songs. Females prefer respective songs not only in sympatric areas, but also in areas, where only one subspecies is present. The authors therefore propose that the subspecies have reached species status (Brambilla et al., 2008) and hence represent an example of speciation mediated through prezygotic isolation based on acoustic signals.

The importance of acoustic signals for mate selection in frogs is largely recognized (Gerhardt and Huber, 2002, Pröhl et al., 2007, Pröhl, 2003, Ryan, 1980) and in *O. pumilio*, advertisement calls may attract female frogs over longer distances. However, the importance of acoustic signals for mate choice in strawberry poison frogs has received very little attention, especially in the context of population divergence. In one exceptional study, Meuche et al. investigated the influence of several call parameters on mate choice in one population of the monomorphic red Northern genetic group. She discovered that in her study population, females did not select their mates based on differences in calling parameters like call rate or dominant frequency, but selected the closest loudspeaker during playback experiments. Selection of the closest male was furthermore corroborated by an analysis of the sampling tactic of receptive females based on field observations of mate choice behavior of these females under natural conditions (Meuche et al., 2013). Meuche argues that the non-selective sampling tactic in this population might be a consequence of the operational sex ratio being strongly female biased and due to the fact that males defending territories in this population are generally of high fitness (Meuche et al., 2013). Differences in population structure and density, as well as variability in phenotypic traits might lead to other mating sampling strategies in other populations of *O. pumilio* (Meuche et al., 2013, Pröhl, 2002).

1.5 Multimodal signaling in studies of animal behavior and sexual selection

Sexual signals serve as indicators of species identity and mate quality (Pröhl et al., 2007), and their role during mate attraction and mate selection emphasizes the importance of sexual signals for reproductive isolation and thus species diversification (Erdtmann and Amezquita, 2009). Unimodal sexual signals have been tested for their role in sexual selection and prezygotic (behavioral) isolation within and among populations, evolving lineages or closely related species (Ptacek, 2000). During the last decade, research of animal communication has begun to increasingly integrate signal modalities (vision, hearing, behavior, smell, tactile information) and to evaluate their interactions (Candolin, 2003, Selz et al., 2014, Taylor et al., 2007, Kodric-Brown and Strecker, 2001, Kodric-Brown and Nicoletto, 2001, Partan and Marler, 2005, Zeyl and Laberge, 2011, Kozak and Uetz, 2016). Despite all these efforts, multimodal mate choice experiments have often been limited to laboratory conditions or single populations. Although the outcome of mate choice experiments under optimal ’signaling’ conditions in the laboratory may deviate from those under complex field conditions (Gerhardt, 1982, Dyson and Passmore, 1988), the assessments of multiple sexual signals under field conditions are still scarce (but see (de Luna et al., 2010, Narins et al., 2003)) and the role of multiple signals for behavioral isolation via mate choice in wild populations remains largely unexplored. Here I take advantage of the divergence in visual and acoustic signals between genetic lineages and populations in the aposematic and color diverse neotropical poison frog *O. pumilio* to evaluate, whether visual-acoustic interactions affect assortative mate selection and thus prezygotic reproductive isolation among populations.

According to Candolin (Candolin, 2003), multimodal signals may act in a multiplicative or hierarchical manner. In the first case, single signals might differ in the reaction they evoke in the presence of certain other cues, because these signals reinforce or deplete each other. One example of multiplicative interactions has been described for two anuran species (*Bombina orientalis* (Zeyl and Laberge, 2011) and *Allobates femoralis* (de Luna et al., 2010, Narins et al., 2003)), where both acoustic and visual signals need to be perceptible to induce a behavioral response in conspecifics. Similary, in two incipient species of flycatchers (*Monarcha castaneiventris*) on the Solomon Islands, both plumage coloration and song played a role in prezygotic reproductive isolation and affected intrasexual territorial responses of males (Uy et al., 2009). Female wolf spiders (*Schizocosa ocreata*) integrate visual and vibratory sexual signals, however, each signal is sufficient to induce female attendance when presented alone (Kozak and Uetz, 2016). The hierarchical use of multimodal signals on the other hand describes different signals which are used in a sequential order, implying that the second signal is only regarded if the first one meets the required criteria (Candolin, 2003). In *Pundamilia nyererei*, a cichlid fish species, acoustic signals reinforced existing preferences for visual or olfactory stimuli, while they did not evoke a behavioral response in two-choice playback experiments when presented alone (Estramil et al., 2013). In several birds, multimodal visual and acoustic signals are also used in a sequential order during mate selection and male-male interactions, with visual cues usually being of primary importance (Ratcliffe and Grant, 1985, Uy et al., 2009).

Geographically separated populations of strawberry poison frogs have diverged in several signals of different modalities, including coloration, advertisement calls and behavior (Pröhl and Ostrowski, 2011, Rudh et al., 2011, Rudh et al., 2013) and both acoustic and visual signals play a role during mate choice in this species (Meuche et al., 2013, Reynolds and Fitzpatrick, 2007, Maan and Cummings, 2008, Summers et al., 1999). Populations also differ in the quantity and composition of their skin toxins (Saporito et al., 2007a, Maan and Cummings, 2012), which might cause differences in olfactory cues as well. Perceptible to potential mates, these diverged signals may be subject to sexual selection, irrespective of whether natural selection, sexual selection or other influences initiated divergence in these traits (Kronforst et al., 2006).

As described above, for this project I integrate signals of two modalities in order to investigate the importance of each signal modality and how these signals interact during mate selection. Because the results of mate choice experiments under laboratory conditions not always reflect preference functions present under field conditions (Dyson and Passmore, 1988, Gerhardt, 1982) I conducted two-choice playback experiments under natural conditions directly in the habitat of each individual frog. During the experiments advertisement call playbacks and self-manufactured frog models (see Fig. 1, adapted from (Dreher and Pröhl, 2014)) of local or nonlocal origin were presented in five types of experiments (see Fig. 2 for an illustration of the experimental types, taken from (Dreher and Pröhl, 2014)). Presented calls resembled average call parameters of the study population ('local calls') or one of the genetic groups ('Southern group call' and 'Northern group call'). In each populations and for each experiment, I tested at least 15 females for their preference, adding up to a total number of 452 individual females evaluated. Unlike previous studies, I tested exclusively receptive females in these playback experiments to assure that the female choice during these experiments reflected mate selection preferences.

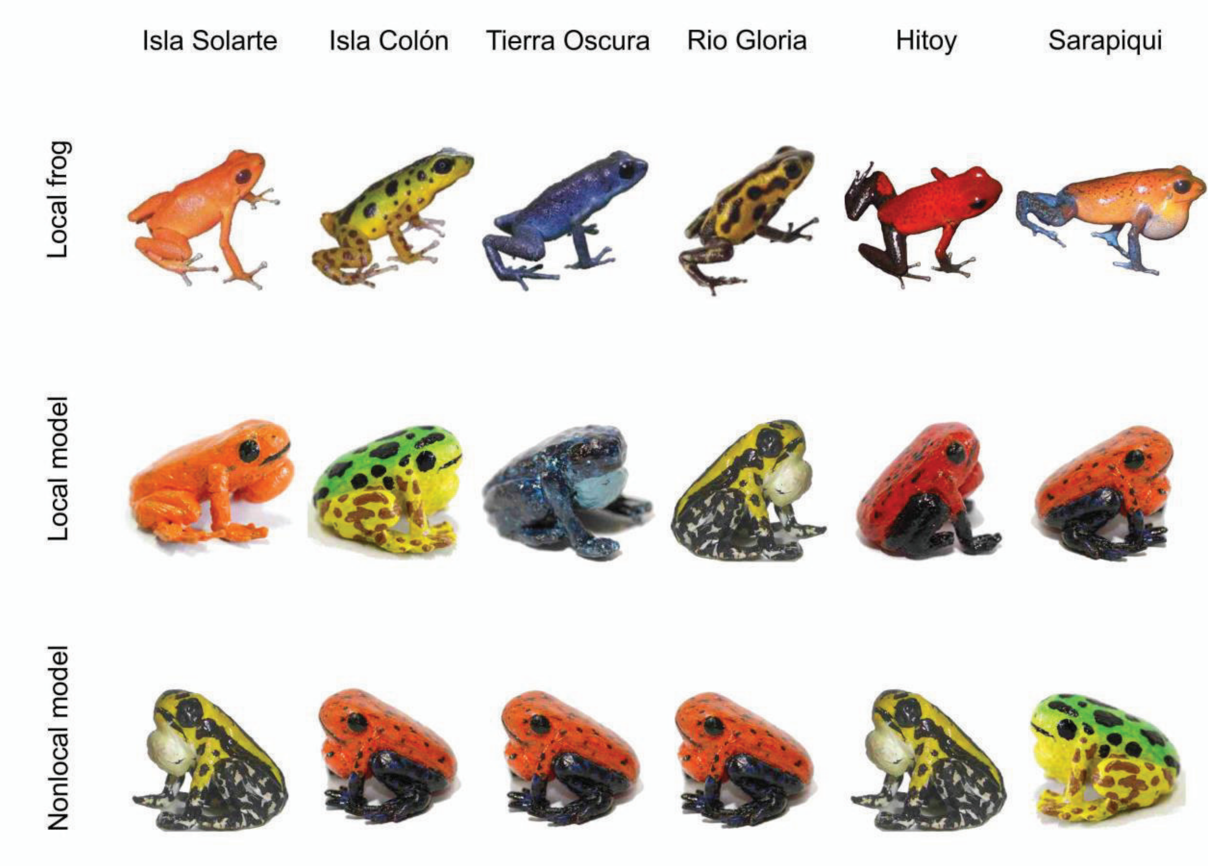

Fig. 1: Pictures of one specimen of *O. pumilio* and frog models used in bimodal playback-experiments for each population. First line: picture of a representative frog of each population; second line: local frog model; third line: alternative frog model presented in bimodal mate choice experiments. (adapted from Dreher and Pröhl 2014, Frontiers in Ecology and Evolution)

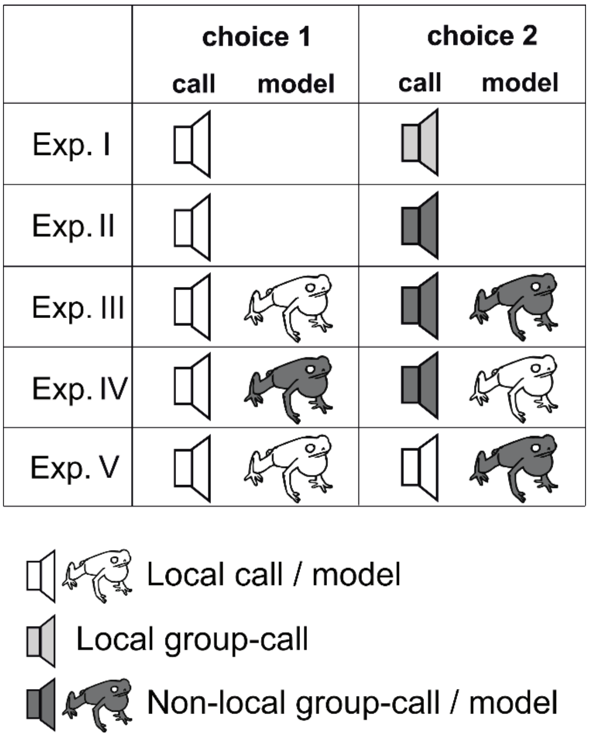

Fig. 2: Experimental types of playback experiments:
Experiment I+II: unimodal experiments: The average call sequence of the local population ('local call') was presented against the average call of the local genetic group (the 'local group call', experiment I) and against the average call of the alternative genetic group (the 'nonlocal group call', experiment II)
Experiment III – V: bimodal experiments combining advertisement calls and frog models: In experiment III one choice resembled local traits (including the local call and the local frog model), while the alternative side presented nonlocal traits (including the nonlocal group call and a nonlocal model). In experiment IV the model frogs were interchanged compared to experiment III. One choice now combined the local call with the nonlocal model and the other choice consisted of the nonlocal group call and the local model. In experiment V the local call was broadcasted on both sides to evaluate, how presentation of local and nonlocal models affected female choices when the acoustic trait was identical. (taken from Dreher and Pröhl 2014, Frontiers in Ecology and Evolution)

1.6 Aims and hypotheses of the study

One of the main aims of the presented study is to investigate, how two signal modalities, including aposematic coloration and acoustic mating signals as the major sexual signals in anuran amphibians, interact in mate choice as an indicator of sexual selection. Connecting these results with an analysis of predation pressure on local and nonlocal colors I aim to clarify how natural selection and sexual selection on several signals of different modalities may have acted in concert to facilitate rapid divergence among populations of strawberry poison frogs in the Bocas del Toro Province.

As proposed earlier, predation pressure as a mean of **natural selection** is very likely to vary among populations (Brown et al., 2010, Maan and Cummings, 2012). Among-population differences in the composition of predator communities and the attack probability on different colors of frogs are a necessary prerequisite for predator selection to affect phenotypic divergence in this species. I therefore examined predation pressure on *O. pumilio* in six study populations, including populations of aposematic and cryptic coloration. Two populations were located in Costa Rica and belong to the Northern genetic group and four study populations were located in Panama, including two mainland and two island populations. Populations of the Northern genetic group had red bodies with the final parts of their limbs colored in blue and black (Sarapiquí) or black (Hitoy Cerere). Populations belonging to the Southern genetic group in Panama were polytypic. Frogs from the mainland location Río Gloria usually had a yellow background coloration and big black spots on their back and white venters, while individuals from Tierra Oscura typically possessed a dark blue coloration all over their body. On Solarte island, frogs were colored in bright orange and frogs belonging to the population of the island of Colón had a green background coloration with black and brown big spots on their back and white and yellow venters (Fig. 3, taken from (Dreher and Pröhl, 2014)).

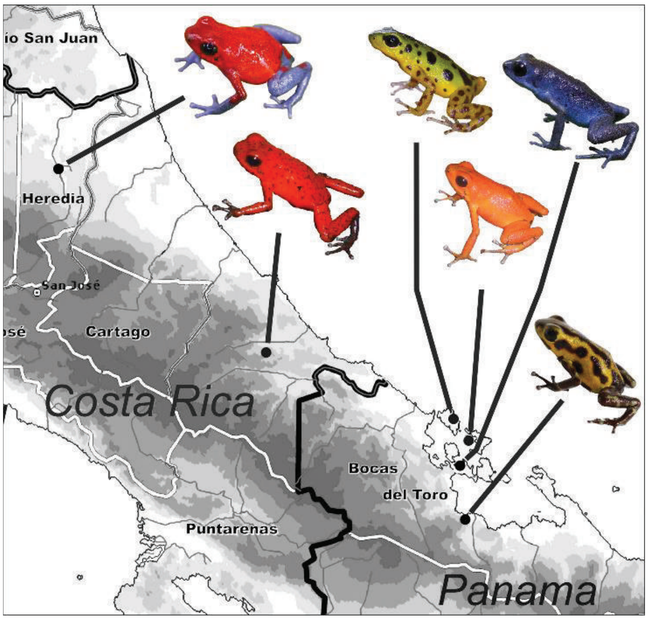

Fig. 3: Geographic distribution of the six investigated populations of *O. pumilio* including one representative frog per population. Two populations were located in Costa Rica: Sarapiquí: red with blue legs; Hitoy: red morph. The other four populations were located in Panama with two mainland population: Río Gloria: yellow-black morph and Tierra Oscura: blue morph and two island populations: Isla Colón: green-black morph and Isla Solarte: orange morph. (taken from Dreher and Pröhl 2014, Frontiers in Ecology and Evolution)

In these populations along the distribution range of *O. pumilio* I recorded predation rates on four different colors of clay model frogs, including clay frogs resembling the main body coloration of frogs of local origin to answer the following questions: (1) are there differences in overall predation pressure among populations? (2) is the probability of an attack dependent on the coloration of the clay models and in particular on the origin of the clay model frog? (3) which animals may be major predators of strawberry poison frogs? (4) does the composition of the predator community vary among geographically separated populations?

Given that red coloration is considered to be an inherently effective aposematic signal (Richards-Zawacki et al., 2013), I expect red clay model frogs to be highly protected, in particular in red populations, where predators are familiar with red poisonous frogs. Less attacks on red clay models in red populations would corroborate the aposematic function of the visual signal (Saporito et al., 2007c). However, predators may perceive coloration of the frogs differently depending on their visual system. Crabs possess mono- or dichromatic visual systems (Horch et al., 2002, Jordao et al., 2007, Maan and Cummings, 2012) while many snakes are trichromatic (Macedonia et al., 2009) and vision in birds is characterized by four different cone types, including one photoreceptor perceiving ultraviolet light (Hart et al., 1998, Bowmaker et al., 1997). Furthermore, a high ratio of double cones is present in avian retinae and oil droplets narrow the absorbance spectra of bird photoreceptors (Hart et al., 1998, Bowmaker et al., 1997). Visual modeling is a mean of calculating conspicuousness of certain object to a specific observer. The method includes information about the composition of photoreceptors in the respective retinae of the observer and thus allows for an estimation of how conspicuous or cryptic certain objects may appear to this viewer. In preparation of the visual models, I conducted reflectance measurements of frogs and their individual substrates. Additionally, irradiance measurements were included to provide information about the particular light environment in the habitat and to adjust the sensitivity of the photoreceptors in the visual model. Using these measurements, I calculated color contrast (DeltaS) and brightness contrast (DeltaL) of frogs for the visual systems of a tetrachromatic bird, a dichromatic crab and a trichromatic snake predator in order to evaluate whether frogs of local coloration are particularly conspicuous or cryptic to certain predators. I furthermore connected these results to the results of clay model experiments to test, whether probability of an attack by these predators covaries with the level of conspicuousness of the frogs to different visual systems, i.e. if attack probability depends directly on the ease of detection by different predators. Finally, I asked how conspicuousness values among different color morphs differ for heterospecific observers in comparison with those calculated for the visual system of a conspecific *O. pumilio* observer as an indicator of sexual selection.

If differences in predation pressure had contributed substantially to color divergence among geographically separated populations of *O. pumilio* – as proposed in literature (Brown et al., 2010, Maan and Cummings, 2012) - I expect that the level of predation pressure varies among populations. Considering the aposematic function of conspicuous coloration (Saporito et al., 2007c) and the honest signal content of these visual signals (Maan and Cummings, 2012), I furthermore hypothesize that conspicuously colored clay model frogs are less prone to be attacked than those of cryptic coloration, in particular in conspicuous populations of strawberry poison frogs.

In agreement with the theory presented above, I expect that the contribution of **sexual selection** to phenotypic divergence should become manifest through differences in mate selection preferences among populations. Here, I expect females as the choosing sex in this species to exert assortative mate preferences according locally evolved sexual signals like mating calls and coloration.

I tested this hypothesis using playback experiments to investigate female mate selection under conditions as natural as possible, directly in their habitat. Using unimodal acoustic playback experiments preference functions for mating calls were investigated, while bimodal playback experiments aimed to disentangle possible interactions between acoustic and visual properties. During preliminary experiments in the field, female strawberry poison frogs were indifferent to the presentation of both visual and acoustic male sexual signals, unless they were receptive. Based on this observation, I propose that studies of sexual selection in strawberry poison frogs should be conducted exclusively with receptive females. Therefore, I selected only females for the experiment, which demonstrated their receptivity undoubtedly by being involved in courtship with a male.

The specific hypotheses which were verified with playback experiments are described in the following. I anticipate that I) mate selection in strawberry poison frogs is affected in a multiplicative way with both advertisement calls and coloration of the frogs to play a role for sexual selection; II) preferences for a local trait should be reinforced by the presentation of another local trait, while presentation of a supplementary non-corresponding (nonlocal) trait should weaken preferences; III) female preference functions concerning the relative importance of both signals may vary among population, because their evolution might have been shaped by natural selection – which probably varies among geographically separated population - as well. I predict that females of the Southern genetic group, where color divergence is strong among populations, might pay more attention to colors while females from the Northern genetic group, where advertisement call variance among population is high, might focus on acoustic cues during mate selection; IV) a positive phonotaxis of receptive females should be induced by presentation of acoustic cues alone, as under natural conditions sight of males might be hindered by obstacles, while advertisement calls transmit over longer distances; V) with an equal call sequence at both loudspeakers, females are expected to show a preference for the local model frog.

For these experiments I decided to use model frogs as opposed to live males for three reasons. First, during preliminary experiments receptive female frogs paid equal attention to model frogs and live male frogs kept under a daylight transparent glass. Second, during preliminary experiments males showed a more pronounced courtship behavior to females of their own coloration, which is problematic because the behavior of a male during courtship can influence the preference of the female (Andersson, 1994, Wong et al., 2011). Third, I could not control for general differences in morphology (body size, vocal sac size), activity (movement, calling behavior) and further potential differences (e.g. olfactory cues) among males of different populations.

In two populations of the Southern genetic group of *O. pumilio*, Isla Solarte and Isla Bastimentos, sexual dimorphism in brightness has been reported, and laboratory experiments revealed that females of these populations show preferences for brighter males (Maan and Cummings, 2009). These findings point to directional selection toward brighter males in these aposematic populations. Building on these results, I measured the spectral reflectance of the skin of male and female frogs which were encountered during courtship in three of my six study populations (Isla Colón, Sarapiquí and Hitoy Cerere). In these populations I conducted the same measurements for male and female frogs which were found in the habitat without being involved in courtship activities. Including two populations of the monomorphic Northern genetic group (Sarapiquí and Hitoy Cerere) I took advantage of the possibility to measure coloration and brightness of mates selected under undisturbed natural conditions to verify whether a preference for brighter animals is a general trend. Using this exceptional data, I explore if the evolution of color divergence of a larger than the previously known set of two island populations of the polytypic Southern genetic group (Maan and Cummings, 2009) may be driven by directional selection on brightness.

2. Publication I

An analysis of predator selection to affect aposematic coloration in a poison frog species

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Author contributions: C.E.D. and H.P.: designed research, C.E.D. performed research, C.E.D, M.E.C. and H.P. analyzed data and wrote the manuscript. All authors revised the manuscript critically, improved the content and approved the final version. They all agree to be responsible for all aspects of the work.

The authors declare no conflict of interest.

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Abstract

Natural selection is widely noted to drive divergence of phenotypic traits. Predation pressure can facilitate morphological divergence, for example the evolution of both cryptic and conspicuous coloration in animals. In this context Dendrobatid frogs have been used to study evolutionary forces inducing diversity in protective coloration. The polytypic strawberry poison frog (*Oophaga pumilio*) shows strong divergence in aposematic coloration among populations. To investigate whether predation pressure is important for color divergence among populations of *O. pumilio* we selected four mainland populations and two island populations from Costa Rica and Panama. Spectrometric measurements of body coloration were used to calculate color and brightness contrasts of frogs as an indicator of conspicuousness for the visual systems of several potential predators (avian, crab and snake) and a conspecific observer. Additionally, we conducted experiments using clay model frogs of different coloration to investigate whether the local coloration of frogs is better protected than non-local color morphs, and if predator communities vary among populations. Overall predation risk differed strongly among populations and interestingly was higher on the two island populations. Imprints on clay models indicated that birds are the main predators while attacks of other predators were rare. Furthermore, clay models of local coloration were equally likely to be attacked as those of non-local coloration. Overall conspicuousness (and brightness contrast) of local frogs was positively correlated with attack rates by birds across populations. Together with results from earlier studies we conclude that conspicuousness honestly indicates toxicity to avian predators. The different coloration patterns among populations of strawberry poison frogs in combination with behavior and toxicity might integrate into equally efficient anti-predator strategies depending on local predation and other ecological factors.

Introduction

A successful predation event consists of two elements: detection of prey and realization of an attack [1]. Aposematism and crypsis are two anti-predator strategies, which hinder successful predation on prey at different stages of the predation event: crypsis aims to prevent successful detection of prey by blending into the background [2], while aposematism signals unprofitability of prey to a predator via conspicuous traits associated with unpalatability [3, 4]. Depending on local conditions (e.g. habitat heterogeneity, predation pressure, and availability of noxious prey), different anti-predator strategies might be of selective advantage for a species. Furthermore, phenotypic signals, which are perceptible to heterospecific observers like predators might also be used in intraspecific communication (e.g. in mate selection and intrasexual aggressive interactions), and their evolution might be affected by several selective forces simultaneously [4-9].

Environmental conditions are likely to differ among geographically isolated populations, and in different populations opposing expressions of a signal may be of selective advantage. Interactions of natural selection with sexual selection and stochastic processes might furthermore contribute to divergent evolution of phenotypic traits among different populations of a species. For example, in several fish species (*Poecilia reticulata, Xiphophorus helleri, Alticus arnoldorum*), the evolution of elaborate signals face a trade-off, since they attract both mates and increase the risk of attacks by predators [7, 9, 10]. In the aposematic wood tiger moth, *Parasemia plantaginis*, the maintenance of polymorphism is the result of a trade-off between predator selection and mating success: white males had higher mating success while yellow males survived better when confronted with predators [11]. On the other hand, natural and sexual selection might act in concert on the evolution of phenotypic features, e.g. in *Heliconius* butterflies coloration and spotting pattern seem to be optimized for both predator deterrence and mate attraction [6]. Some species of aposematic dendrobatid frogs show high variability of coloration among populations. In *Ranitomeya imitator*, frogs of different populations use Müllerian mimicry and resemble several other sympatrically occurring toxic frog species in coloration and pattern (e.g. *R. ventrimaculata* and *R. fantastica*) [12]. In the polytypic species *Oophaga granulifera,* rather cryptic and rather conspicuously colored populations have been described [13], whose divergence might have been facilitated by natural selection via differences in predation pressure among populations [14].

In strawberry poison frogs (*Oophaga pumilio*), skin coloration and patterning is highly diverse among populations, comprising more than 15 distinct, mostly geographically separated, color morphs [15-17]. Most color morphs are located at the islands and adjacent mainland of the Bocas del Toro Archipelago in Panama, despite the very recent formation of the Archipelago in the last 10,000 years [18]. Strawberry poison frogs are a popular model organism for research addressing questions about intra- and interspecific communication. They produce signals of several modalities, including visual and acoustic signals which vary geographically [16, 19]. Color divergence among populations of *O. pumilio* has been proposed to be driven by both sexual and natural selection [see reviews 5, 20].

As the aposematic function of conspicuously colored populations of *O. pumilio* has been confirmed by several experiments, predation was suggested to have played a substantial role in this divergence [21-23]. Although the conspicuousness of several polytypic populations reliably indicates their toxicity to predators [22], several populations of cryptic dull coloration exist [24]. In two populations of *O. pumilio*, the cryptic and aposematic anti-predator strategy was further supplemented by the respective inconspicuous or bold behavior of the frogs [25]. If natural selection exerted by predators has been important for the formation of color morphs in *O. pumilio*, we expect predation pressures to vary among populations [21, 26]. Several previous projects used clay model frogs to evaluate predation pressure on *O. pumilio* [1, 21, 23, 27] and other dendrobatid frog species [14, 28, 29]. In *Ranitomeya imitator* and *Dendrobates tinctorius*, predation caused by birds was significantly higher on novel aposematic morphs compared to local aposematic morphs, which exerts strong homogenizing selection in favor of local color morphs [28, 30], in particular in monomorphic populations [29]. Red coloration is considered to be an effective aposematic signal [31], and red clay models were less attacked than brown controls in a red population of *O. pumilio* [23]. To the opposite, in a cryptic population [25], clay models of local coloration were more attacked than non-local aposematic ones and brown controls [21]. Despite these previous studies it is still unknown how predation pressure and conspicuousness of the frogs to a variety of predators with different visual systems interact on a larger geographic scale covering multiple frog color morphs. Here we use two approaches to understand the interplay between predation pressure and the conspicuousness of the frogs. First, we applied visual modeling to explore if frogs of local coloration are particularly conspicuous or particularly cryptic to certain predators in comparison to their conspicuousness to conspecifics which indicates their importance for sexual selection. Second, using clay model frogs of four different colors (including the color of the local frog population) we investigated 1) which animals may be major predators of strawberry poison frogs 2) if predation pressure and the composition of predator communities vary among populations, 3) whether predation differs among clay frogs of different colors, especially among local and non-local colors. We furthermore interpret our predation data to tackle the question whether conspicuousness of the live frogs and attacks by (certain) predators co-vary. Thus our study combines information about predation pressure caused by local predators and the visual conspicuousness of living specimens of local strawberry poison frogs to different predator classes.

Material and Methods

Reflectance Measurements and Visual Modeling

Field work was conducted between December 2008 and June 2011 in six populations of strawberry poison frogs, two populations in Costa Rica and four populations in Panama. To assess the conspicuousness of the frogs on their specific substrate for conspecific and several heterospecific observers we took reflectance measurements. We measured the spectral reflectance of the skin of a total 255 frogs in Sarapiquí (n = 40 red frogs; 10° 28.227 'N; 84° 0.553 'W; 44 m.a.s.l.), Hitoy Cerere (n = 52 red frogs; 9° 37.819 'N; 83° 0.879 'W; 270 m.a.s.l.) (both Costa Rica), Río Gloria (n = 38 yellow frogs,; 8° 59.100 'N; 82° 13.916 'W; 24

m.a.s.l.), Tierra Oscura (n = 47 blue frogs; 9° 11.776 'N; 82° 14.976 'W; 7 m.a.s.l.), Isla Colón (n = 40 green frogs; 9° 23.170 'N; 82° 15.941 'W; 35 m.a.s.l.) and Isla Solarte (n = 38 orange-red frogs; 9° 19.946 'N; 82° 12.939 'W; 4 m.a.s.l.) (Panama). Additionally we measured the reflectance of the specific substrate (e.g. leaves, trunks) on which each individual frog was found. Reflectance spectra of the skin and substrate were taken at a distance of 2mm using an Ocean Optics HR2000+ Spectrometer, an Ocean Optics bifurcal optic fiber (R-200-7-UV/VIS) with a fixed outer sleeve to control for the 2mm distance and a deuterium-tungsten lamp (DT-Mini-2-GS). To account for lamp drift we calibrated the measurements with a white standard (WS-1-SS) every other frog. Illumination of the habitat (Irradiance) was measured using an optic fibre (QP400-2-UV-BX) with an Ocean Optics cosine adaptor-head (CC-3UV). Irradiance spectra were taken at the places where we found the frogs and at times when the frogs showed most activity (between 7am and 12noon in Costa Rica; and between 8am and 1pm in Panama) on two to three different days. The population-specific average irradiance was calculated for each population, including between 192 and 396 irradiance spectra per population.

We calculated average reflectance spectra for dorsal and ventral regions for each frog. For dorsal reflectance spectra four reflectance measurements were averaged (two of which were taken on the head between the eyes, and two on the middle of the dorsum). For frogs from the population of Río Gloria and on Isla Colón, where frogs possess a dark spotting pattern on a yellow or green background color on their dorsums, we included two measurements of the dorsal background color and the two head measurements for dorsal average spectra. We neglected the influence of dark spots, because spotting pattern did not affect predation risk on clay models of *O. pumilio* in an earlier study [32], and bird predators were found to base attack decisions on coloration cues rather than on contrasting patterns [33-35]. For calculations of ventral measurements we averaged two reflectance curves taken on the belly. We did not include measurements taken from the throat region in order to avoid the darker coloration of the throat of males to impact the results. Because there is no general sexual dimorphism described in this species [36], and our study does not focus on differences between males and females, we analyzed males and females together. An equal number of males and females was measured in each population.

Visual models were calculated according to Maan & Cummings and Crothers & Cummings [22, 37] using average dorsal and ventral reflectance spectra from each frog, from the specific substrate of each frog and the population-specific average irradiance. For the trichromatic visual model of a conspecific viewer, microspectrophotometric data on the visual sensitivity of cones of *O. pumilio* [38] were used. For avian, crab, and snake visual models we used data of visual sensitivity of the European Starling (*Sturnus vulgaris*, [39]), the fiddler crab (*Uca tangeri*, [40-42]) and the Coachwhip (*Masticophis flagellum*, [43, 44]). These predators differ in their visual systems, including dichromatic, trichromatic and tetrachromatic vision. The crab vision model was calculated for the dichromatic visual system of *Uca tangeri* [41, 42]. The trichromatic visual models for snakes and *O. pumilio* include information about the spectral sensitivity of *Masticophis flagellum* [43] and one specimen of *O. pumilio* [38]. The avian visual model we used is based on a tetrachromatic system with UVS-cones and brightness contrast of this model was calculated using the spectral sensitivity of double cones with oil droplets (as in [39]). For these four observers, we calculated brightness contrast (ΔL) and color contrast (ΔS) for each frog on its specific substrate. Brightness contrast yields negative results, if frogs are darker than their specific background. Since we are interested in their conspicuousness in terms of how they contrast to their substrate rather than if they are brighter or darker than the substrate, we use the absolute values of brightness contrasts. Finally we estimated the dorsal and ventral overall conspicuousness of the frogs on their substrates for the visual models of birds, snakes and crabs. Overall conspicuousness between frog and background was calculated as the Euclidean distance ($OC = \sqrt{\Delta S^2 + \Delta L^2}$) based on the average color and brightness contrast for every population as proposed by Cummings & Crothers [5].

Our study species is not a species protected by the laws of Panama or Costa Rica. However it is on the CITES Appendix II colourful dendrobatid species are often collected illegally for commercial reasons. *Oophaga pumilio* is abundant across its range and listed as a species of "least concern" by the IUCN. All sampling and measuring methods are described in detail above. No animals have been sacrificed and all individuals have been released at their capture site after measuring. Approval by an ethics committee was not necessary since all sampling methods and manipulation of the frogs is part of the evaluation of the study by the local authorities (SINAC and ANAM) that authorize the research permits.

Data Analysis of Visual Modeling

Brightness and color contrast (ΔL and ΔS) of the frogs from the study populations were calculated for potential predators and conspecifics. As absolute values of color and brightness contrasts are not comparable among different visual models, i.e. different observers, we normalized the results of each calculated model and calculated means and standard deviation of color and brightness contrasts (ΔS and ΔL) for dorsal and ventral measurements for each population. We applied ANOVAs to test if contrasts differ significantly among populations for different (conspecific or heterospecific) observers. ANOVAs indicated differences in color and brightness contrasts of both body regions among populations for all observers. Therefore, we applied Tukey post-hoc tests to localize the differences among populations. We furthermore visually checked the residuals to justify the use of ANOVAs. Statistical analyses were conducted using STATISTICA 6.0.

Predation experiments

To evaluate whether frogs of local or nonlocal (i.e. novel) colors are more or less prone to an attack, we conducted predation experiments with clay model frogs. The use of clay models as specimen replicates facilitates assigning damaged models to different predator categories, according to teeth marks or other characteristic imprints preserved in the clay [1]. Previous to the experiments, we measured the spectral reflectance of the clay colors used for clay models and compared the reflectance spectra of the clay models to those of real strawberry poison frogs of red, yellow, green and blue coloration, respectively. We considered the achieved accordance as sufficient for the experiments and used standard clay colors. Furthermore, we modeled one exemplary clay model frog, which was used to manufacture silicon molds. With these molds we were able to make model frogs of standardized shape in high numbers from non-toxic standard clay in four colors (red, yellow, green and blue). The experiments were conducted over time periods of 20 consecutive days, which were divided into four time intervals of five days. Per time interval, we placed 400 clay model frogs in the habitat of the frogs, resulting in a total number of 1600 clay frogs in each population. 400 clay frogs were put out simultaneously, in assorted groups of four frogs containing one frog of each coloration. In each population we selected two representative areas of the frog habitat, and placed 50 clay frog groups in consecutive transects of approximately 50 m in both areas. At the end of each time interval – after five days - the clay model frogs were controlled, and all damaged or missing frogs were replaced with new clay frogs. All predation marks were documented with pictures. Each clay model frog was scored as attacked or not-attacked and the attacked models were furthermore assigned to one of the following categories: bird marks (U- and V-shaped imprints), holes and scratches, rodent marks, snake marks, crab marks, lizard marks, missing models and unknown predation marks. Attacks assigned to the category holes and scratches were scored as potentially caused by birds, as they looked like the damages on clay frogs of the category bird marks, but missed the typical U- and V-shaped imprints [32]. Examples of attack marks assorted to the categories birds, holes & scratches, rodents, snakes, crabs, lizards and unknown predation marks are presented in Fig. S1. We furthermore applied visual modeling to calculate color and brightness contrasts of the different colors of clay model frogs on typical substrates of each population. In these visual models, we included reflection of the clay model frogs, the population specific irradiance and the substrates of the living frog specimens of the respective population. All field experiments were conducted in accordance with the laws and ethical standards of Costa Rica and Panama. Research permits were obtained before starting field work from the local authorities.

Data Analysis of Predation Experiments

There was no difference in frequency of predation among the consecutive time intervals of the experiment. Hence, for the analyses we summarized the attacks of all intervals. To analyze whether risk of an attack was predicted by the population, the coloration of the clay model frog and/or by its origin (local or non-local), we used Generalized Linear Models (GLM´s), assuming binomial-error distribution and a logit-link-function. Conducting deviance analyses with Chi-square statistics for the GLM´s we tested whether population, coloration and/or origin of the clay model were significant predictors for general risk of attack, and whether predation risk within each population was predicted by the latter two variables. Furthermore, we applied Tukey post-hoc tests to localize between which populations or clay model colors predation pressure differed. All analyses were calculated using R [45].

Considering the assignation of different predation marks to predator categories, our data – as well as previous studies [1, Maan & Cummings 2012, 23] - strongly suggest, that birds are the main predators of *O. pumilio*. We therefore repeated the analyses considering only damages caused undoubtedly by birds (category ‘bird marks’: U- and V- shaped marks) and damages probably caused by birds (summing up the results of the categories ‘bird marks’ and ‘holes &

scratches' to a new category of 'potential bird marks'). The assignment of 'holes & scratches' to 'potential bird marks' follows Hegna et al. [32] and is supported by the observations of Willink et al. [14], who observed birds causing similar attack marks. Because the majority of predation marks are assigned to this category of 'potential bird marks' and the low numbers of attacks for all other predator classes did not allow for statistical tests in these categories, we summarized all other predation marks to a new category 'non-bird predation' and ran the analyses for this category as well.

We furthermore tested, if predation pressure is correlated with high levels of conspicuousness (color and brightness contrasts and overall conspicuousness) of the local frogs, which would indicate that natural selection contributed to the evolution of aposematic coloration in this species. Correlation analyses were conducted with STATISTICA 6.0.

Results

Visual Modeling

Visual modelling calculations are based on dorsal and ventral reflectance spectra of differently colored frogs. Average reflectance spectra for each studied population are depicted in Fig. S2 (Note the high spectral reflectance of the white ventral regions of some populations (e.g. Sarapiqui and Río Gloria) exceeded that of the lambertian white standard (Spectralon)).

Color Contrast (ΔS)

Dorsal contrasts: Visual modeling revealed that frogs from Isla Solarte had the highest values of dorsal color contrast (Fig. 1). These contrasts were significantly higher than contrasts from all other populations for all observers, except for contrasts of frogs of Tierra Oscura for snake and crab vision (Table S1). The dorsal color contrast of frogs from Sarapiquí, Hitoy Cerere, Río Gloria and Isla Colón were similar and lower than those from Isla Solarte and Tierra Oscura (Fig. 1, Table S1).

Ventral contrasts: For avian and conspecific vision, the frogs of Isla Solarte showed the highest values for ventral color contrast, which significantly differed from contrasts of frogs of most other populations, followed by contrasts of frogs from Tierra Oscura and Isla Colón. For avian and conspecific vision, frogs from Río Gloria had the lowest contrasts which were significantly different from most other populations. For crab and snake vision frogs from Isla Colón had the highest color contrasts, followed by frogs from Tierra Oscura and Isla Solarte, while frogs from Hitoy Cerere had the lowest contrasts (Fig. 1, Table S1).

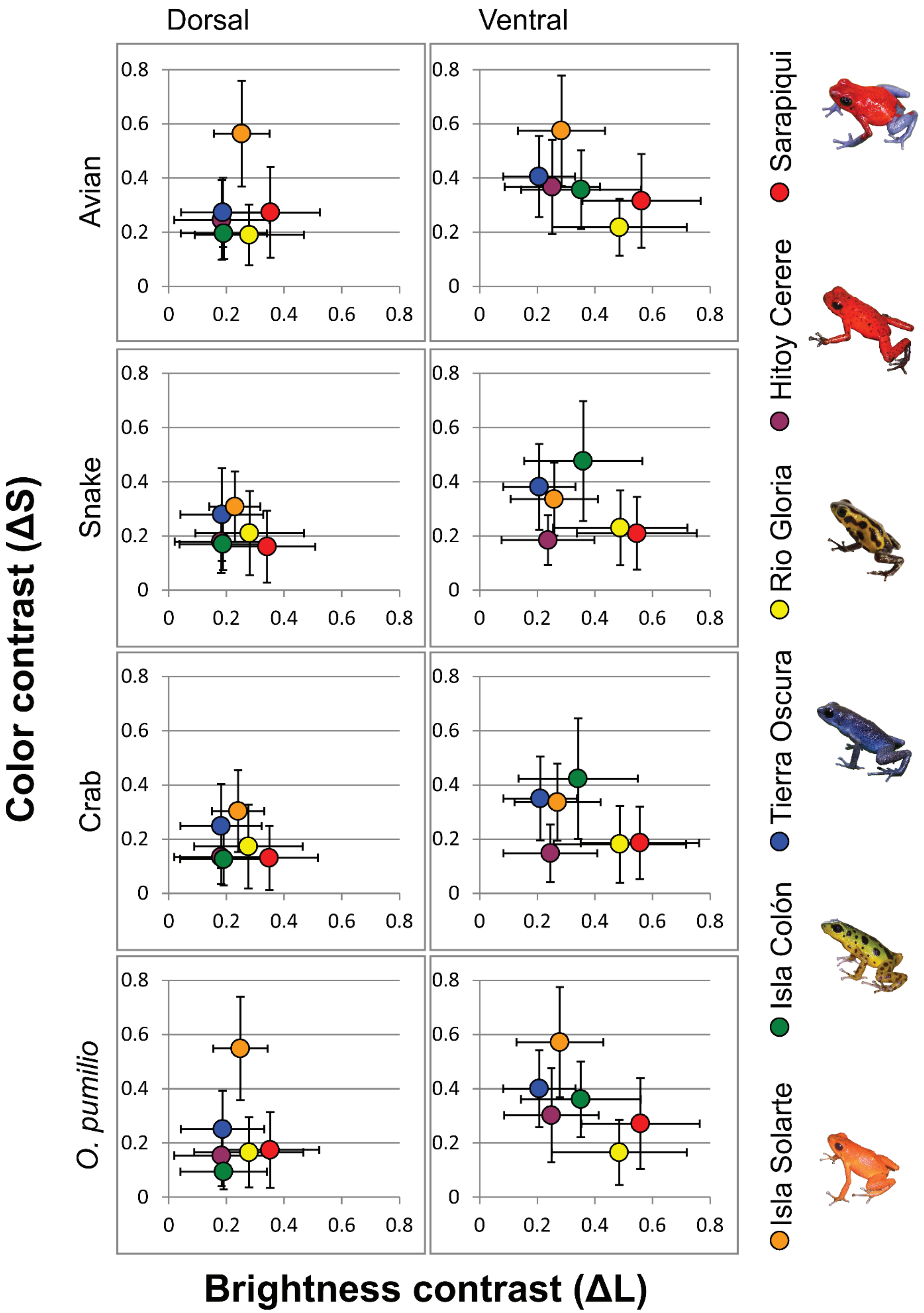
Dorsal
Ventral
Avian
Snake
Crab
O. pumilio
0
0.2
0.4
0.6
0.8
Color contrast (ΔS)
Brightness contrast (ΔL)
● Sarapiqui
● Hitoy Cerere
● Rio Gloria
● Tierra Oscura
● Isla Colón
● Isla Solarte

Fig. 1. Visual conspicuousness of *O. pumilio* from the study populations for three different heterospecific (avian, snake and crab) and a conspecific observer. Graphs show average color and brightness contrasts of dorsal and ventral measurements of reflectance, normalized for each observer. Circles indicate mean values for brightness and color contrasts, bars show standard deviation of means for each population.

Brightness Contrast (ΔL)

Dorsal contrasts: Frogs of Sarapiquí displayed the highest dorsal brightness contrasts for all observers, and were significantly brighter than frogs from Hitoy Cerere, Tierra Oscura and Isla Colón, marginally brighter than frogs from Isla Solarte, but not significantly brighter than frogs from Río Gloria (Fig. 1).
Ventral contrasts: Similar to their dorsal measurements, frogs from Sarapiquí had the highest ventral brightness contrasts for all observers, followed by frogs from Río Gloria. For all observers, brightness contrasts of frogs from Sarapiquí and Río Gloria were significantly higher than contrasts from all other populations. The frogs from Hitoy Cerere, Tierra Oscura and Isla Solarte displayed the lowest ventral brightness contrasts (Fig. 1, Table S2).

Overall conspicuousness

Overall conspicuousness represents the combined contrast of ΔL and ΔS in a brightness and color contrast space (the Euclidean distance, see Fig. 1). Dorsally the red (-orange) and yellow populations were more conspicuous than the blue and green ones for all observers. One exception was Hitoy Cerere where the frogs were red but duller than the red frogs from Sarapiquí and Isla Solarte. Ventrally the trend of conspicuousness was the same but the bright yellow ventral regions of the frogs from Isla Colón was equally or more conspicuous than the ventral regions of frogs from Isla Solarte.

Contrasts of clay frogs

As for the live frogs the red and blue clay models showed a particularly high color contrast while the yellow models showed the highest brightness contrast for most predator - population combinations (Fig. S3).

Predation experiments

We found a highly significant difference in overall attack rate among populations and a marginally significant effect of clay model color, while we did not detect any significant effect of the origin (local or non-local) of the model frogs (Table 1, Table S3). Overall attack rate on clay models in both island populations in Panama (Isla Colón and Isla Solarte) was significantly higher than overall attack rate in Sarapiquí, Hitoy and Tierra Oscura mainly due to attack by ‘potential bird marks’ (Fig. 2). Attack rate on blue clay frogs was significantly higher than on yellow frogs (Fig. 3). In Sarapiquí, attack rate on blue frogs was higher than on yellow and on green frogs (Table S4b).

	Overall attack rate	bird marks	potential bird marks	non-bird predation
Population	**0.0000**	**0.0000**	**0.0000**	**0.0108**
Clay model color	**0.0162**	**0.0029**	**0.0298**	0.1760
Origin	0.9214	0.8195	0.5476	0.0857

Table 1. Effects of frog population, clay frogs coloration and origin on the attack rate of different predator on clay frogs. Results (P-values) of generalized linear models (GLM´s), which tested whether there was an effect of population, coloration of clay frogs or clay frog origin (local versus non-local coloration) on the attack rate of different predators.

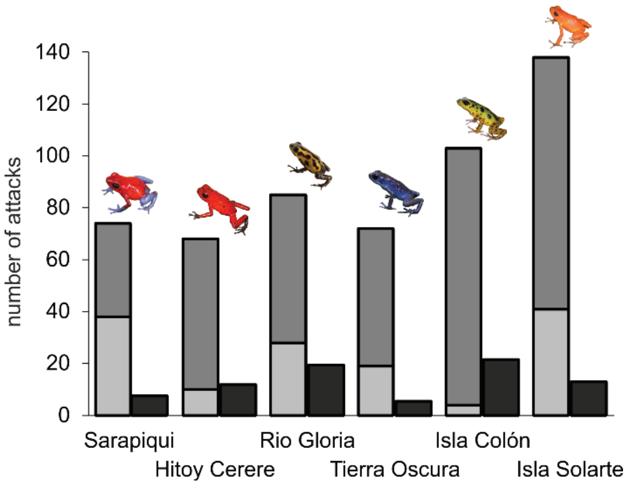

Fig. 2. Number of predation events in each population. Bar coloration indicates the predator category the attack was assigned to. Light grey: U- and V-shaped attack marks caused by birds; grey: holes and scratches potentially caused by birds; and black: non-bird predation. Isla Colón and Isla Solarte are island populations, while all others are located on the main land.

The results strongly suggest that birds are the main predators of *O. pumilio* (Fig. 2, Table S3). In the category ‘bird marks’ population and clay model color were significant predictors of attack rate (Table 1). In this category more attacks on clay frogs were observed in the yellow to red populations Río Gloria, Isla Solarte and Sarapiquí (but not on clay frogs in Hitoy Cerere). Attack rate in Isla Colón was significantly lower than in Sarapiquí, Río Gloria, Tierra Oscura and Isla Solarte. Furthermore attack rate in Hitoy was significantly lower than in Sarapiquí, Río Gloria and Isla Solarte (Table S4c). We observed more ‘bird marks’ on blue than on yellow or green clay frogs in *O. pumilio*, i.e. across all populations, and in Sarapiquí (Fig. 3, Table S4d).

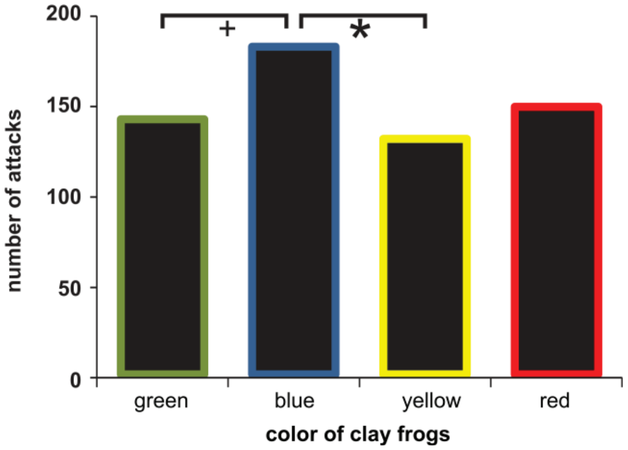

Fig. 3. Predation on clay frogs of different colors. Overall number of attacks on clay frogs from all six studied populations of *O. pumilio*. + $P < 0.10$; * $P < 0.05$.

The analysis of ‘potential bird marks’ yielded similar results. Overall there was a highly significant effect of population and a marginally significant effect of clay model color on predation risk (Table 1). Predation risk on Isla Solarte was significantly different from all other populations except from Isla Colón (Table S4e). Overall predation rate on blue clay frogs was significantly higher than on yellow and green frogs (Table S4f).

There were low numbers of attacks, which were assigned to other predators like crabs, snakes and lizards or as ‘missing’ and ‘unknown’ (Table S3). All these attacks were analyzed together in the category ‘non-bird predation’ (Fig. 2). There was no detectable effect of clay model color or origin on attack rate (Table 1, Table S4h). While there was an overall effect of population on attack rate (Table 1), pairwise comparisons of populations only detected a trend for a difference in attack rate between Isla Colón and Tierra Oscura (P=0.08) (Table S4g). Even though the number of non-bird predation events is very low, Isla Colón is the only population with 4 documented snake and 2 documented crab attacks (Hitoy Cerere: two lizards and one crab; Isla Solarte: two snakes). In Tierra Oscura no recorded attack was assigned to non-bird predation.

Correlation between conspicuousness of local frogs and risk of attack

Across populations, the dorsal brightness contrast of the local frogs for bird eyes was highly, almost significantly correlated with the total number of attacks by birds on clay frogs (r=0.79, P=0.06) (Table S5). Total number of attacks by birds includes attacks on all clay model colors of variable conspicuousness to bird predators, and thus estimates avian predation pressure in each population._Importantly the only highly significant correlation between attack rates on clay frogs and conspicuousness of the local frogs was between the dorsal overall conspicuousness for the bird visual model and number of bird attacks (r= 0.94, P=0.0048; N=6, Fig. 4). Dorsal color contrast, ventral color and brightness contrasts as well as ventral overall conspicuousness of frogs did not show any association with attack rates (data not shown).

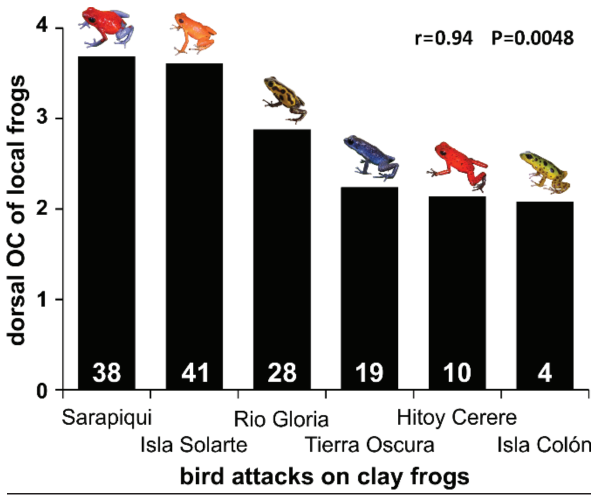

Fig. 4. Correlation between dorsal overall conspicuousness of local frogs for avian eyes and avian predation on clay frogs across frog populations. The correlation is highly significant (r = Spearman rank correlation coefficient).

Discussion

Our study yielded several interesting results. Strawberry poison frogs from populations differed in their coloration and conspicuousness, i.e. in color and brightness contrast as well as in overall conspicuousness. In predation experiments with clay model frogs, predation risk varied among frog populations and was higher on island than on mainland populations. Birds were the main predators, while attacks of other predators like lizards, snakes, rodents and crabs were rare. Unlike other studies, our results do not provide evidence that the local color morph was better protected than other color morphs. However bird predation was highest on blue frog models (a non-local color morph in five of the six populations of our study). Our finding of higher attacks on blue frog models than on yellow or green models might indicate that frogs of high conspicuousness (yellow) or low conspicuousness (green) were better protected than intermediate color morphs (blue). Moreover, in populations where avian predation on clay frogs was higher, local frogs were dorsally brighter and more conspicuous, which suggests that predation exerted by birds primarily selects for higher conspicuousness in the local prey.

Conspicuousness and toxicity

The results of visual modeling demonstrate that contrasts calculated for each population are very similar among different observers especially between snake and crab and between avian and *O. pumilio* visual models for the specific background where each frog was found. For all observers, including the conspecific viewer, frogs from the population of Isla Solarte possess the highest dorsal color contrasts on their substrates from all studied populations but also possess a high brightness contrast and overall conspicuousness. Previous studies revealed that Isla Solarte is one of the most toxic populations of *O. pumilio* [22]. Being highly toxic and conspicuous [22], Isla Solarte frogs employ an aposematic anti-predator strategy, which is also reflected in their bold behavior [25, 36]. The study of Maan and Cummings (2012) revealed a positive correlation between brightness and overall conspicuousness to bird eyes and the noxiousness of frogs in 10 different Panamanian populations [22], i.e. toxicity seems to be honestly signaled by the dorsal coloration of the frogs. A theoretical framework presented by Holen and Svennungsen [46] suggests that honest signaling in the tradition of the handicap theory can potentially be achieved by two mechanisms: the “go slow” behavior of predators or the “resource allocation trade-off” in prey. In “go slow” predators are reluctant when tasting conspicuous and well defended prey. Survival of conspicuous prey is therefore enhanced. The “resource allocation trade-off” hypothesis instead proposes that the same resource is involved in the expression of the warning signal and the defense (e.g. toxicity). In case the resource is limited only individuals with access to a high resource quantity can afford to develop conspicuous signals. Conspicuous warning signals improve predator learning and as a consequence the number of attacks decreases. Currently it is unknown whether one of these mechanisms applies to the evolution of aposematism in our study model.

An additional possibility is the involvement of sexual selection. Our data reveal that avian predators and conspecifics, i.e. potential mating partners, perceive frogs as similarly conspicuous when evaluating contrasts against the specific backgrounds and irradiance conditions in which the frogs were captured. While sexual selection often favors high conspicuousness, it entails easy detection by predators. This might imply that sexual selection for brighter colors in strawberry poison frogs evolved in concert with higher toxicity reinforcing aposematism in this species. The interaction between natural and sexual selection for the divergence in warning colors in this and other taxa warrants further study [5].

Predation risk

Our experiments with clay model frogs showed, that predation pressure significantly varies among populations with higher predation rates on islands (Isla Solarte and Isla Colón) compared to mainland populations. These results are contrary to the general assumption of relaxed predation pressure on islands [47, 48]. For spiny-tailed iguanas [49] and nest predation of several bird species experiments confirmed less predation on island populations compared to mainland populations [49-51], however, one study conducted in Sweden also found predation pressure on nests of two bird species to be equal for island and mainland study sites [52]. In this regard our study does not support the hypothesis, that relaxed predation pressure in the Bocas del Toro region allowed the evolution of a high diversity of warning signals in strawberry poison frogs, as proposed by Hegna et al. [21]. However, our results strongly suggest that birds are the main predators of *O. pumilio*. Damages caused by crabs or snakes or other unidentified animals were rare. Most predation events on Isla Solarte clearly resulted from birds, but on Isla Colón more attacks were assigned to the category “holes and scratches”. In this regard more research is necessary to confirm the true nature of these predators and to differentiate predator composition among populations. One previous study using clay model frogs to investigate predation pressure in *O. pumilio* on Isla Colón confirmed that even though overall attack rate was high on the local frogs, unambiguous bird predation was low on this island compared to mainland populations [21]. We obtained lower overall predation rates (4.8-7.7%) in comparison to similar experimental studies with clay frogs in this species (7.5-12.6%) [21, 31, 32], which might be due to differences in localities, duration of the experiments as well as shape and color of clay frogs.

Predation on local versus non-local coloration

Our data does not provide direct evidence that model frogs of local coloration are better protected than model frogs of non-local coloration. The color which was most attacked in all four mainland populations (Sarapiquí, Hitoy, Río Gloria and Tierra Oscura) was blue, which is a non-local color for all populations except Tierra Oscura. In Tierra Oscura, where local frogs are blue and possess moderate toxicity levels (Aguacate population [5, 22], local predators also attacked the blue morphs significantly more than green and yellow. Only in the population of Río Gloria the number of (bird) attacks was lowest on the yellow clay frogs of local origin. The low attack rate on yellow clay models might be due to a generally low number of attacks on yellow coloration – which was also found in Sarapiquí, Hitoy Cerere and Isla Solarte, rather than the local origin of the yellow coloration in this population. Since the yellow clay frogs are brighter than clay frogs of other coloration it is possible that predators - in this case mainly birds - avoid attacking the bright yellow objects since brightness reliably indicates toxicity in our study species. Similar studies in *O. pumilio* found that attack rates on red and yellow clay frogs were not affected by the local frog coloration [31] or found clay frogs of local coloration to be more attacked than novel brown or red models in one of our study populations (Isla Colón) [21]. On the contrary, studies of predation pressure on clay frog models of local or non-local origin in two other dendrobatid frog species (*Dendrobates tinctorius* and *Ranitomeya imitator*) reported that predation rate on local color morphs was lower than on a (conspicuous) novel color morph [29, 30]. An explanation of these different findings might be that in the latter studies predation experiments were finished after 72 hours. Nonetheless the study on *R. imitator* showed that the initial advantage of the local color morphs disappeared during the experiment and after 72 hours all frog morphs in the experiment were equally attacked. Thus the apparent local color morph advantage is of short duration, which makes a long-term evolutionary effect unlikely.

Overall, our data do not provide evidence for substantial differences of predation pressure on clay model frogs as a function of the local coloration of strawberry poison frogs. Three possible explanations for this result are as follows: First, predation pressure might not be the major force driving divergence in coloration among populations and its contribution to divergence in coloration might be concealed by the impact of other forces (i.e. toxicity levels and sexual selection). Second, selection for divergence in color patterns cannot be examined properly as predator communities may have changed since color morphs diverged among different populations in the Bocas del Toro archipelago [31] and elsewhere, as proposed by Alcover and McMinn [53]. Third, optimal local protection is not achieved by coloration alone but via a combination of coloration, behavior, body size and toxicity. This idea is supported by the finding that besides being less toxic, cryptic morphs are also smaller and use other anti-predator and reproductive strategies than more conspicuous morphs in strawberry poison frogs [25, 36, 54]. The differential availability of toxic food items (small insects that contain toxic alkaloids [55] among populations might also influence the evolution of conspicuous or cryptic coloration in *O. pumilio*.

Correlation between conspicuousness of local frogs and attack rate

High conspicuousness (e.g. through high brightness contrast) can facilitate detection of prey by predators, but it might also provide protection through predator learning. Interestingly the rate of attacks which were unambiguously caused by birds is highly correlated with brightness contrast and overall conspicuousness of the local frogs for avian eyes. This indicates that bird predation selects for a bright, conspicuous coloration in local prey because the conspicuousness itself facilitates the education of birds for predator avoidance. For instance, unpalatable prey of higher brightness contrast provided greater predator aversion learning in the Chinese mantid than prey of lower brightness contrast [56]. However, we did not find the same correlation for ‘potential bird marks’. In this regard we need more clarity about the animal species, whose attacks have been categorized to this group. An erroneous classification of some damaged clay models as “potentially caused by birds” (e.g. in the category “holes & scratches”) might explain the missing correlation of “potential bird marks” and the conspicuousness of local frogs. Despite this finding conspicuous colors like orange, red and yellow, may generally function as effective aposematic signals for predator deterrence, even when predators are not familiar with frogs of these colors [31, 57].

Comparison of predation risk among *Oophaga granulifera*, *O. pumilio* and other dendrobatid frog species

Oophaga granulifera is another dendrobatid frog species which shows color polytypism ranging from green to red populations in natural lowland forests along the pacific coast of Costa Rica and Panama [13]. This species is very closely related to *O. pumilio* and exhibits high similarity in its ecology, behavior and morphology. Research on *O. granulifera* explores the relation between predation pressure, toxicity and conspicuousness to the visual systems of birds with similar methods as in our study [13, 14, 58].

Contrary to *O. pumilio* [22], toxicity levels and visual conspicuousness were inversely related among populations of *O. granulifera*, i.e. green frogs were more cryptic but more toxic than red frogs [13], while the predator community was more diverse consisting of birds, lizards and crabs of similar magnitude [14]. Willink’s study revealed that predation in *O. granulifera* follows a specific pattern: birds avoided attacking clay models reproducing the local coloration of the frogs (i.e. red in populations of red frogs and green in populations with green frogs), while lizards mostly attacked red clay models which mimic the highly conspicuous but less toxic red frogs.

Another study, investigating the link among predator avoidance, visual conspicuousness and toxicity in three species of the genus *Epipedobates*, proposed that there might be a trade-off between conspicuousness and toxicity in some dendrobatid frogs [59]. A similar mechanism might apply to *O. granulifera* and different predator taxa might cause opponent selective advantages for phenotypic traits. For *O. pumilio* there is evidence that conspicuousness is an honest indicator of toxicity [22] for its main predator group. A more general avoidance of bright coloration might override the avoidance of the local frog coloration, since local frogs are not always highly toxic. The establishment of an honest signaling system might be facilitated when it is directed towards the sensory system of a single predator group. However, if various predator groups are involved alternative strategies might be favored which may even include a trade-off between conspicuousness and toxicity. Different predation patterns among *O. granulifera* and *O. pumilio* might furthermore be caused by differences in the ecology of these two species. *Oophaga granulifera* inhabits more natural, undisturbed habitats [60], where predator communities may not have changed in the last millennia. *Oophaga pumilio*, however, populates primary and secondary habitats, including abandoned agricultural areas (e.g. cocoa plantations).

Future work

For a real understanding of the impact natural selection may have on color divergence of *O. pumilio*, further research is necessary. A persisting problem is the lack of knowledge about the actual predators of this species, which is essential for the evaluation of the importance of natural selection for color divergence [61]. Further studies including video traps [14], will help to evaluate, which animal taxa actually predate on strawberry poison frogs, and facilitate interpretation of damages inflicted on clay models. Afterwards, the evaluation of the importance of additional features (e.g. internal black spotting patterns, olfactory cues and movement) for prey detection and avoidance learning can be evaluated for different predator classes. To address the question of whether abundance of toxic prey might facilitate or constrain ongoing divergence of coloration in strawberry poison frogs, we recommend examining whether the availability of toxic prey differs among populations of strawberry poison frogs. We furthermore suggest comparing toxicity levels among different species of poison frogs. A comparison of the toxicity of *Oophaga granulifera* and *Oophaga pumilio* would allow setting the results of predation experiments and visual modeling in relation to toxicity levels. This may allow insights in trade-offs and the interplay between conspicuousness, toxicity and behavioral strategies and how these interactions may have affected color divergence of poison frogs. Finally, similar studies on other aposematic animals would help to achieve a better understanding of the relationship between “honest” or “dishonest signaling” and predator and prey ecology.

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2.1 Correction

Correction: An Analysis of Predator Selection to Affect Aposematic Coloration in a Poison Frog Species

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The email address for the first author, Corinna E. Dreher, is incorrect. The correct email address is: corinnadreher@gmail.com. The publisher apologizes for this error.

Fig 4 is incorrect. The authors have provided a corrected version here.

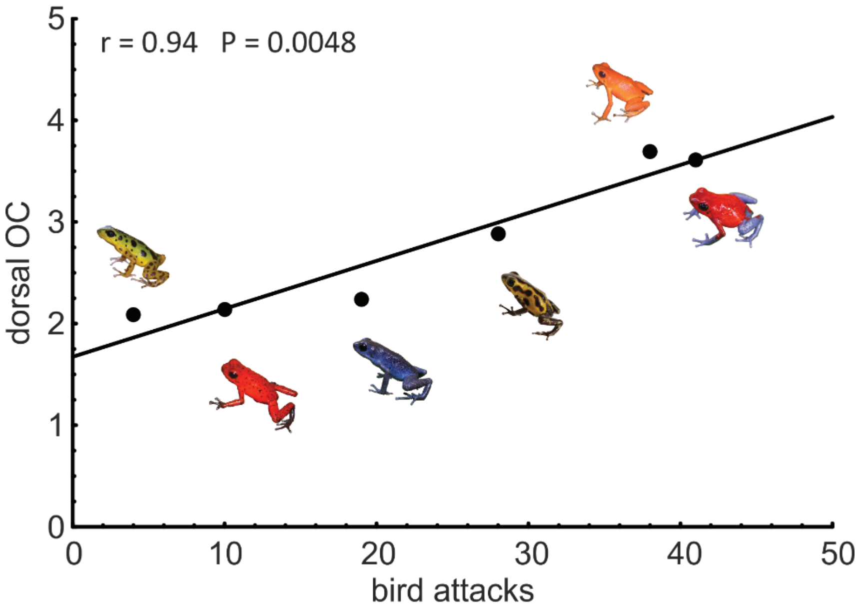

Fig 4. Correlation between dorsal overall conspicuousness of local frogs for avian eyes and avian predation on clay frogs across frog populations. The correlation is highly significant (r = Spearman rank correlation coefficient).

2.2 Supplementary material

Fig S1. Examples of predation marks assigned to different categories: (a-c) birds marks; (d-e) holes & scratches: (d) holes, (e) scratches; (f) rodent marks; (g) snake marks; (h) crab marks; (I) lizard marks; (j-l) unknown predation marks.

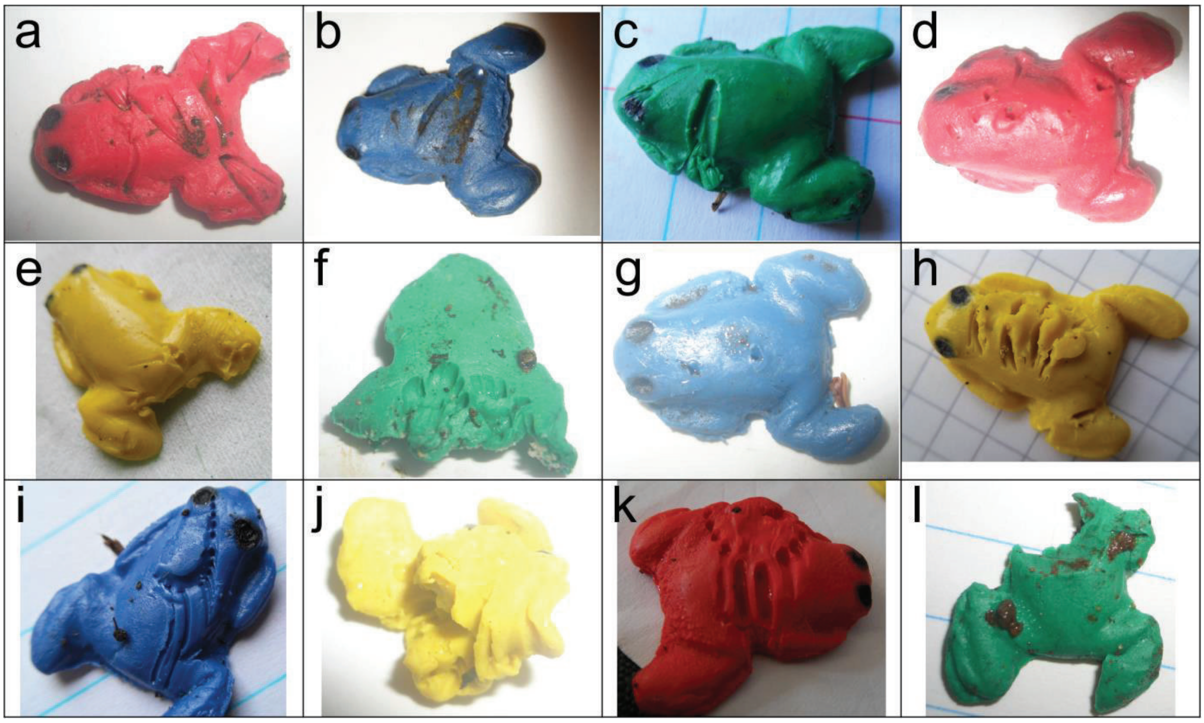

Fig S2. Mean dorsal and ventral reflectance spectra including standard deviation of *Oophaga pumilio* specimens from six study populations. Mean reflectance curves average spectra of 38 to 52 individuals (Sarapiquí: 40; Hitoy: 52; Río Gloria: 38; Tierra Oscura: 47; Colón: 40; Isla Solarte: 38). Reflectance curves show reflection of the frogs relative to white standard. Reflectances over 100% emerge due to the brilliant surface of the frogs´ skin, while the used white standard has a dull surface. In order to verify accuracy of reflectance curves, all spectra were visually controlled for oversaturation.

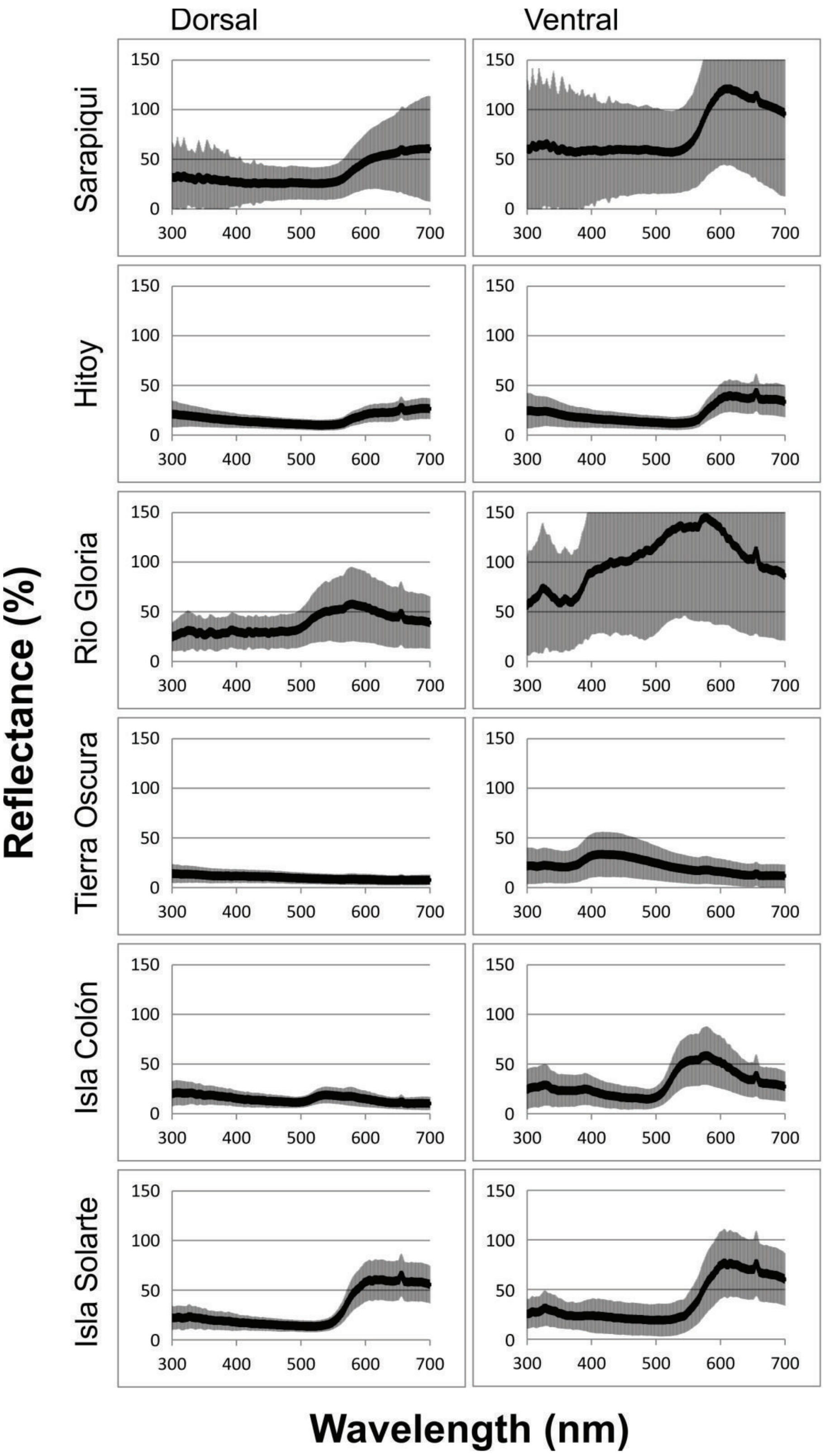

Fig S3. Conspicuousness of clay model frogs on local substrates for four different observers. We calculated conspicuousness measurements (color and brightness contrast) of blue, red, green and yellow clay model frogs on all measured substrates of the respective population for four different observers (avian, snake, crab, *O. pumilio*). Circles of different colors represent means of calculated conspicuousness measurements of each clay color in each population for the respective observer.

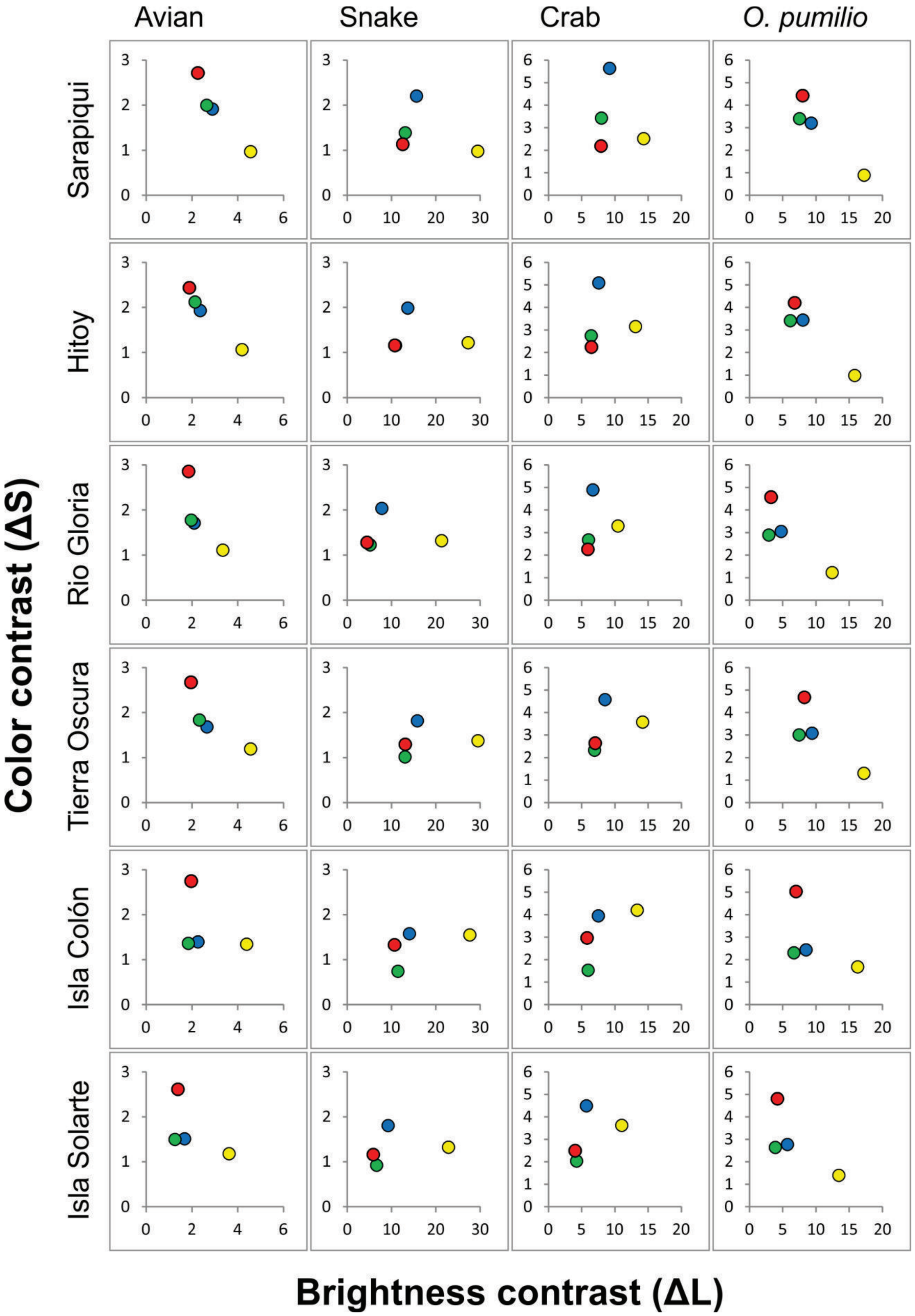

Table S1. Differences in color contrast between populations for four different observers. Differences in color contrast between populations were localized using Tukey post-hoc test. P-values below 0.05 indicate that color contrasts between the respective two populations differ for the indicated observer.

DORSAL

	Sarapiqui	Hitoy C	Río Gloria	T.Oscura	I.Colón	I.Solarte
	Avian vision					
Sarapiqui		0.942	0.113	1.000	0.153	**0.000**
Hitoy C	0.942		0.473	0.930	0.570	**0.000**
Río Gloria	0.113	0.473		0.088	1.000	**0.000**
T. Oscura	1.000	0.930	0.088		0.122	**0.000**
I.Colón	0.153	0.570	1.000	0.122		**0.000**
I.Solarte	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	
	Snake vision					
Sarapiqui		0.988	0.590	**0.001**	1.000	**0.000**
Hitoy C	0.988		0.888	**0.003**	1.000	**0.000**
Río Gloria	0.590	0.888		0.182	0.777	**0.021**
T. Oscura	**0.001**	**0.003**	0.182		**0.002**	0.929
I. Colón	1.000	1.000	0.777	**0.002**		**0.000**
I. Solarte	**0.000**	**0.000**	**0.021**	0.929	**0.000**	
	Crab vision					
Sarapiqui		1.000	0.718	**0.000**	1.000	**0.000**
Hitoy C	1.000		0.721	**0.000**	1.000	**0.000**
Río Gloria	0.718	0.721		0.088	0.612	**0.000**
T. Oscura	**0.000**	**0.000**	0.088		**0.000**	0.378
I. Colón	1.000	1.000	0.612	**0.000**		**0.000**
I. Solarte	**0.000**	**0.000**	**0.000**	0.378	**0.000**	
	Frog vision					
Sarapiqui		0.979	1.000	0.083	0.081	**0.000**
Hitoy C	0.979		0.999	**0.004**	0.279	**0.000**
Río Gloria	1.000	0.999		**0.039**	0.179	**0.000**
T. Oscura	0.083	**0.004**	**0.039**		**0.000**	**0.000**
I. Colón	0.081	0.279	0.179	**0.000**		**0.000**
I. Solarte	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	

VENTRAL

	Sarapiqui	Hitoy C	Río Gloria	T.Oscura	I.Colón	I.Solarte
Sarapiqui		0.648	0.084	0.101	0.862	**0.000**
Hitoy C	0.648		**0.000**	0.852	1.000	**0.000**
Río Gloria	0.084	**0.000**		**0.000**	**0.002**	**0.000**
T. Oscura	0.101	0.852	**0.000**		0.731	**0.000**
I.Colón	0.862	1.000	**0.002**	0.731		**0.000**
I.Solarte	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	
Sarapiqui		0.968	0.991	**0.000**	**0.000**	**0.003**
Hitoy C	0.968		0.712	**0.000**	**0.000**	**0.000**
Río Gloria	0.991	0.712		**0.000**	**0.000**	**0.024**
T. Oscura	**0.000**	**0.000**	**0.000**		**0.036**	0.740
I. Colón	**0.000**	**0.000**	**0.000**	**0.036**		**0.000**
I. Solarte	**0.003**	**0.000**	**0.024**	0.740	**0.000**	
Sarapiqui		0.838	1.000	**0.000**	**0.000**	**0.000**
Hitoy C	0.838		0.905	**0.000**	**0.000**	**0.000**
Río Gloria	1.000	0.905		**0.000**	**0.000**	**0.000**
T. Oscura	**0.000**	**0.000**	**0.000**		0.222	0.998
I. Colón	**0.000**	**0.000**	**0.000**	0.222		0.117
I. Solarte	**0.000**	**0.000**	**0.000**	0.998	0.117	
Sarapiqui		0.945	**0.040**	**0.003**	0.125	**0.000**
Hitoy C	0.945		**0.001**	0.029	0.499	**0.000**
Río Gloria	**0.040**	**0.001**		**0.000**	**0.000**	**0.000**
T. Oscura	**0.003**	0.029	**0.000**		0.867	**0.000**
I. Colón	0.125	0.499	**0.000**	0.867		**0.000**
I. Solarte	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	

Table S2. Differences in brightness contrast between populations for four different observers.
Differences in brightness contrast between populations were localized using Tukey post-hoc test. P-values below 0.05 indicate that brightness contrasts between the respective two populations differ for the indicated observer.

	DORSAL						VENTRAL					
	Sarapiqui	Hitoy C	Río Gloria	T.Oscura	I.Colón	I.Solarte	Sarapiqui	Hitoy C	Río Gloria	T.Oscura	I.Colón	I.Solarte
	Avian vision											
Sarapiqui		**0.000**	0.291	**0.000**	**0.000**	0.053		**0.000**	0.438	**0.000**	**0.000**	**0.000**
Hitoy C	**0.000**		**0.049**	1.000	1.000	0.299	**0.000**		**0.000**	0.817	0.096	0.965
Río Gloria	0.291	**0.049**		0.077	0.124	0.979	0.438	**0.000**		**0.000**	**0.016**	**0.000**
T. Oscura	**0.000**	1.000	0.077		1.000	0.386	**0.000**	0.817	**0.000**		**0.003**	0.378
I. Colón	**0.000**	1.000	0.124	1.000		0.488	**0.000**	0.096	**0.016**	**0.003**		0.563
I. Solarte	0.053	0.299	0.979	0.386	0.488		**0.000**	0.965	**0.000**	0.378	0.563	
	Snake vision											
Sarapiqui		**0.000**	0.496	**0.000**	**0.000**	**0.016**		**0.000**	0.726	**0.000**	**0.000**	**0.000**
Hitoy C	**0.000**		**0.031**	1.000	1.000	0.694	**0.000**		**0.000**	0.960	**0.017**	0.994
Río Gloria	0.496	**0.031**		**0.047**	0.082	0.697	0.726	**0.000**		**0.000**	**0.022**	**0.000**
T. Oscura	**0.000**	1.000	**0.047**		1.000	0.760	**0.000**	0.960	**0.000**		**0.001**	0.776
I. Colón	**0.000**	1.000	0.082	1.000		0.838	**0.000**	**0.017**	**0.022**	**0.001**		0.140
I. Solarte	**0.016**	0.694	0.697	0.760	0.838		**0.000**	0.994	**0.000**	0.776	0.140	
	Crab vision											
Sarapiqui		**0.000**	0.292	**0.000**	**0.000**	**0.023**		**0.000**	0.538	**0.000**	**0.000**	**0.000**
Hitoy C	**0.000**		**0.045**	1.000	1.000	0.466	**0.000**		**0.000**	0.929	0.119	0.989
Río Gloria	0.292	**0.045**		0.052	0.133	0.915	0.538	**0.000**		**0.000**	**0.006**	**0.000**
T. Oscura	**0.000**	1.000	0.052		1.000	0.483	**0.000**	0.929	**0.000**		**0.010**	0.662
I. Colón	**0.000**	1.000	0.133	1.000		0.698	**0.000**	0.119	**0.006**	**0.010**		0.500
I. Solarte	**0.023**	0.466	0.915	0.483	0.698		**0.000**	0.989	**0.000**	0.662	0.500	
	Frog vision											
Sarapiqui		**0.000**	0.293	**0.000**	**0.000**	**0.039**		**0.000**	0.476	**0.000**	**0.000**	**0.000**
Hitoy C	**0.000**		**0.047**	1.000	1.000	0.358	**0.000**		**0.000**	0.860	0.084	0.976
Río Gloria	0.293	**0.047**		0.075	0.124	0.961	0.476	**0.000**		**0.000**	0.015	**0.000**
T. Oscura	**0.000**	1.000	0.075		1.000	0.450	**0.000**	0.860	**0.000**		**0.003**	0.470
I. Colón	**0.000**	1.000	0.124	1.000		0.562	**0.000**	0.084	0.015	**0.003**		0.491
I. Solarte	**0.039**	0.358	0.961	0.450	0.562		**0.000**	0.976	**0.000**	0.470	0.491	

Table S3. Number of non-attacked and attacked clay model frogs of different coloration in six study populations. Attacked clay model frogs were assigned to one of the categories 'bird marks', 'holes and scratches', 'rodent', 'snake', 'crab', 'lizard', 'missing' or 'unknown'. For statistical analyses we summarized the attacks of the categories 'bird marks' and 'holes and scratches' to the category 'potential bird marks' and all attacks of further categories to the category 'non-bird predation'.

Population	clay model color	origin	non-attacked	Attack-ed	bird	holes & scratches	Potential bird marks	Rodent	Snake	crab	lizard	Missing	Unknown	non-bird predation
Sarapiqui (red)	red	local	382	18	11	7	18	0	0	0	0	0	0	0
	blue	non-lo.	367	33	19	13	32	1	0	0	0	0	0	1
	yellow	non-lo.	385	15	5	6	11	1	0	0	0	0	3	4
	green	non-lo.	385	15	3	10	13	1	0	0	0	0	1	2
Hitoy Cerere (red)	red	local	381	19	2	14	16	2	0	0	0	0	1	3
	blue	non-lo.	375	25	5	17	22	0	0	0	2	0	1	3
	yellow	non-lo.	382	18	0	13	13	1	0	1	0	1	2	5
	green	non-lo.	383	17	3	14	17	0	0	0	0	0	0	0
Rio Gloria (yellow)	red	non-lo.	373	27	5	18	23	2	0	0	0	2	0	4
	blue	non-lo.	366	34	13	13	26	4	0	0	0	3	1	8
	yellow	local	381	19	3	13	16	2	0	0	0	1	0	3
	green	non-lo.	377	23	7	13	20	1	0	0	0	1	1	3
Tierra Oscura (blue)	red	non-lo.	382	18	4	11	15	0	0	0	0	1	2	3
	blue	local	379	21	4	17	21	0	0	0	0	0	0	0
	yellow	non-lo.	382	18	6	10	16	0	0	0	0	0	2	2
	green	non-lo.	380	20	5	15	20	0	0	0	0	0	0	0
Isla Colón (green)	red	non-lo.	371	29	0	21	21	0	2	2	0	0	4	8
	blue	non-lo.	368	32	1	25	26	2	2	0	0	1	1	6
	yellow	non-lo.	371	29	0	26	26	1	1	0	0	0	1	3
	green	local	367	33	3	27	30	0	0	0	0	1	2	3
Isla Solarte) (red)	red	local	360	40	14	23	37	2	0	0	0	1	0	3
	blue	non-lo.	360	40	11	24	35	3	2	0	0	0	0	5
	yellow	non-lo.	366	34	12	21	33	1	0	0	0	0	0	1
	green	non-lo.	364	36	4	29	33	3	0	0	0	0	0	3

Table S4a-g. Pairwise differences in attack rates among frog populations, clay frogs of different colors and clay frogs of local and non-local coloration.

Table S4a. Differences in for overall attack rate between populations. Results (P-values) of Generalized linear models (GLM). P-values < 0.05 indicate differences between populations.

	Sarapiqui	Hitoy Cerere	Río Gloria	Tierra Oscura	Isla Colón	Isla Solarte
Sarapiqui						
Hitoy Cerere	1					
Río Gloria	0.5524	0.4467				
Tierra Oscura	0.9995	1.0000	0.3509			
Isla Colón	**0.0301**	**0.0185**	0.7371	**0.0113**		
Isla Solarte	**<0.001**	**<0.001**	0.261	**<0.001**	0.5239	

Table S4b. Differences in 'overall attack rate' between different colors of clay models and between clay models of local and non-local coloration for all populations (*O. pumilio*) and for each population. P-values (Tukey-Test) below 0.05 indicate that attack rate differed between the two compared colors, or models of local and non-local origin (last column).

	blue-yellow	blue-green	blue-red	yellow-green	yellow-red	green-red	local-nonlocal
O.pumilio	**0.014**	0.0906	0.2773	0.9029	0.6964	0.9728	0.921
Sarapiqui	**0.044**	**0.0441**	0.1392	1	0.951	0.9507	0.553877
Hitoy Cerere	0.6935	0.5875	0.7896	0.9982	0.9983	0.9863	0.8416
Río Gloria	0.151	0.4345	0.7877	0.921	0.6199	0.9368	0.11456
Tierra Oscura	0.9609	0.9985	0.9609	0.9873	1	0.9873	0.63707
Isla Colón	0.9784	0.9992	0.9784	0.9522	1	0.9522	0.62593
Isla Solarte	0.8845	0.9631	1	0.9945	0.8845	0.9631	0.62058

Table S4c. Differences attack rate among populations in the category 'bird marks'. Results (P-values) of generalized linear models (GLM). P-values < 0.05 indicate differences between populations.

	Sarapiqui	Hitoy Cerere	Río Gloria	Tierra Oscura	Isla Colón	Isla Solarte
Sarapiqui						
Hitoy Cerere	**0.00173**					
Río Gloria	0.81145	**0.04838**				
Tierra Oscura	0.1128	0.5576	0.74964			
Isla Colón	**<0.001**	0.6135	**0.00311**	**0.04948**		
Isla Solarte	0.99931	**<0.001**	0.60529	0.05042	**<0.001**	

Table S4d. Differences in attack rate in the category 'bird marks' on different colors of clay models and on clay models of local and non-local coloration for summarized results of all populations (*O. pumilio*) and for each population. P-values (Tukey-Test) below 0.05 indicate that attack rate differed between the two compared colors, or models of local and non-local coloration (last column).

	blue-yellow	blue-green	blue-red	yellow-green	yellow-red	green-red	local-nonlocal
O. pumilio	**0.0135**	**0.0108**	0.3162	0.9996	0.682	0.6644	0.819

Sarapiqui	**0.0326**	**0.0128**	0.443	0.8915	0.4391	0.1746	0.57017
Hitoy Cerere	1	0.8757	0.644	1	1	0.9641	0.7151
Río Gloria	0.0916	0.5315	0.2495	0.597	0.8933	0.9367	0.0917
Tierra Oscura	0.9215	0.9871	1	0.9903	0.9215	0.9871	0.69
Isla Colón	1	0.7306	1	1	1	1	0.05662
Isla Solarte	0.9966	0.2918	0.9279	0.2129	0.9781	0.1101	0.1741

Table S4e. Differences attack rate among populations in the category ‘potential bird marks’. Results (P-values) of generalized linear models (GLM). P-values < 0.05 indicate differences between populations.

	Sarapiqui	Hitoy Cerere	Río Gloria	Tierra Oscura	Isla Colón	Isla Solarte
Sarapiqui						
Hitoy Cerere	0.9956					
Río Gloria	0.9452	0.7161				
Tierra Oscura	1.0000	0.9995	0.8823			
Isla Colón	0.2224	0.6898	0.7624	0.1482		
Isla Solarte	**<0.001**	**<0.001**	**0.0038**	**<0.001**	0.1755	

Table S4f. Differences of attack rate in the category ‘potential bird marks’ on different colors of clay models and on clay models of local and non-local coloration for summarized results of all populations (*O. pumilio*) and for each population. P-values (Tukey-Test) below 0.05 indicate that attack rate differed between the two compared colors, or models of local and non-local coloration (last column).

	blue-yellow	blue-green	blue-red	yellow-green	yellow-red	green-red	local-nonlocal
O.pumilio	**0.0089**	**0.0239**	0.17826	0.97569	0.55161	0.79582	0.546
Sarapiqui	0.4133	0.8452	0.7526	0.8796	0.942	0.998	0.8907
Hitoy Cerere	0.3947	0.7996	0.971	0.9041	0.6619	0.9655	0.7748
Río Gloria	0.3947	0.7996	0.971	0.9041	0.6619	0.9655	0.1789
Tierra Oscura	0.8355	0.9985	0.7381	0.9042	0.9978	0.8244	0.4043
Isla Colón	1	0.9455	0.8764	0.9455	0.8764	0.565	0.3183
Isla Solarte	0.9943	0.9943	0.9947	1	0.959	0.959	0.6073

Table S4g. Differences attack rate among populations in the category ‘non-bird marks’. Results (P-values) of generalized linear models (GLM). P-values < 0.05 indicate differences between populations.

	Sarapiqui	Hitoy	Río Gloria	Tierra Oscura	Colón	Solarte
Sarapiqui						
Hitoy Cerere	0.9343					
Río Gloria	0.3072	0.8309				
Tierra Oscura	0.9912	0.6689	0.1207			
Isla Colón	0.2244	0.7280	1	0.0824		
Isla Solarte	0.8614	0.9999	0.9147	0.547	0.8377	

Table S4h. Differences of attack rate in the category 'non-bird predation' on different colors of clay models and on clay models of local and non-local coloration for summarized results of all populations (*O. pumilio*) and for each population. P-values (Tukey-Test) below 0.05 indicate that attack rate differed between the two compared colors, or models of local and non-local coloration (last column).

	blue-yellow	blue-green	blue-red	yellow-green	yellow-red	green-red	local-nonlocal
O.pumilio	0.952	0.286	0.995	0.952	0.286	0.995	0.102
Sarapiqui	0.5453	0.9292	1	0.8247	1	1	0.9986
Hitoy Cerere	0.8762	1	1	1	0.8762	1	0.8614
Río Gloria	0.4579	0.458	0.6605	1	0.9812	0.9812	0.4166
Tierra Oscura	1	1	1	1	0.9625	1	0.9978
Isla Colón	0.7538	0.7538	0.949	1	0.457	0.4571	0.3068
Isla Solarte	0.4433	0.8927	0.8927	0.7695	0.7695	1	1

Table S5. Correlation between risk of an attack on clay model frogs and the conspicuousness of local specimens of strawberry poison frogs. Correlational analyses were calculated to test for a relationship of 'overall attack rate', 'bird marks', 'potential bird marks' and 'non-bird predation' with the conspicuousness (color and brightness contrast) of local, living specimens of *O. pumilio*.

	avian ΔS	avian ΔL	crab ΔS	crab ΔL	snake ΔS	snake ΔL
overall attack rate	.6288 p=.181	-.0143 p=.979	.4256 p=.400	-.1005 p=.850	.4764 p=.340	-.0457 p=.932
bird marks	.6373 p=.173	.7919 p=.060				
potential bird marks	.7539 p=.083	.0179 p=.973				
non-bird predation			-.3050 p=.557	-.1271 p=.810	-.2931 p=.573	-.1194 p=.822

3. Publication II

Multiple sexual signals: Calls over colors for mate attraction in an aposematic, color-diverse poison frog

Running Title: Mate choice in *Oophaga pumilio*

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Abstract

Sexual signals indicate species identity and mate quality, and their importance for mate attraction is largely recognized. Recently, research in animal communication has started to integrate multiple signal modalities and evaluate their interactions. However, mate choice experiments across animal taxa have been limited to laboratory conditions, and assessments of multiple sexual signals under field conditions are still lacking. We take advantage of the divergence in visual and acoustic signals among populations of the Neotropical poison frog *Oophaga pumilio* to evaluate the importance of male advertisement calls and color patterns in female mate selection. Previous mate choice experiments in this species suggested color-assortative female mate preferences across many populations. Nevertheless, acoustic signals are crucial for sexual selection in frogs, and males of *O. pumilio* use advertisement calls to attract females. We hypothesize that both advertisement calls and coloration affects female mate selection in *O.pumilio*. To test this hypothesis we tested 452 receptive females from six populations in Costa Rica and Panama in their natural home ranges for preferences regarding local vs. non-local advertisement calls and color patterns. Overall, the calls overrode the effect of coloration, whereby most females preferred local over non-local calls. We found a tendency to prefer brighter (but not necessarily local) males in two populations. Furthermore the strength of preferences varied geographically, and thus might be involved in prezygotic isolation among populations. The stronger effect of calls on mate attraction is associated with acoustic divergence between genetic groups in the species, while colour pattern diversity is mostly located within one genetic group, i.e. not linked to large-scale population structure. Finally our data highlights the importance to consider an array of signal modalities in multiple wild populations in studies of behavioral isolation.

1. Introduction

A prerequisite for speciation to proceed is ongoing diversification (phenotypically or genetically) between different groups in a single species. Diversification can be promoted by natural selection (e.g. predation pressure), sexual selection or genetic drift (Panhuis et al., 2001; Sobel et al., 2010; Butlin et al., 2012) and their interactions, while habitat heterogeneity and geographic isolation generally contribute to trait divergence (Rundle and Nosil, 2005; Maan and Seehausen, 2011). All agents of diversification also affect sexual signals, like mating calls or color patterns, which are important for intrasexual communication and mate selection (Uy et al., 2009). Divergence in male sexual signals acting in concert with female preferences may lead to assortative mate-choice, which triggers (incomplete) reproductive isolation and constrains gene flow between diverging subpopulations or incipient species (Panhuis et al., 2001; Maan and Seehausen, 2011). Strong preferences for certain signals can ultimately cause complete prezygotic behavioral isolation and generate new species. Prezygotic or behavioral isolation seems to evolve earlier and induce the speciation process, and might contribute more to reproductive isolation than postzygotic isolation (Coyne and Orr, 2004; Butlin et al., 2012). For example, acoustic signals in insects, birds and anuran species are involved in assortative mate choice, causing prezygotic isolation between genetically divergent populations or incipient species (Wilkins et al., 2013).

Mate choice is a complex task and decisions are often based on multiple sexual traits of one or different sensory modalities (Ptacek, 2000; Candolin, 2003; Taylor et al., 2007). Recently, studies investigating the influence of sexual traits on mate choice have moved to an integrated approach, incorporating signals of different modalities (e.g. visual, acoustic, behavioral, chemical) to study the interactions between signals and their importance for mate choice and sexual selection (Kodric-Brown and Strecker, 2001; Candolin, 2003; Partan and Marler, 2005). The two most common forms of interactions between traits are (a) the sequential (hierarchical) use of different traits and (b) multiplicative interactions (both signals are required, reinforce or deplete each other) (Candolin, 2003). Several studies in birds show that visual and acoustic signals are used in a sequential order, with visual traits being more important than acoustic signals for male aggression and mate choice (Ratcliffe and Grant, 1985; Uy et al., 2009). Whereas for two anuran species, *Bombina orientalis* (Zeyl and Laberge, 2011) and *Allobates femoralis* (Narins et al., 2003; de Luna et al., 2010), both acoustic and visual signals are necessary to evoke a behavioral response in conspecifics; thus, the interactions are multiplicative. The importance of multimodal signals for prezygotic isolation is well investigated in fish in laboratory experiments. In the *Cyprinodon* species complex mate selection in two sympatric species was found to be either affected by visual and olfactory cues or by olfactory cues alone (Kodric-Brown and Strecker, 2001). For *Pundamilia nyererei* acoustic signals alone did not induce phonotactic response in females, however presentation of acoustic signals reinforced preferences for visual stimuli (Estramil et al., 2013). These studies indicate, how different species may use single or multiple cues of different modalities for mate selection. However, research assessing the importance of multiple traits for sexual selection in the context of prezygotic isolation in the wild is scarce.

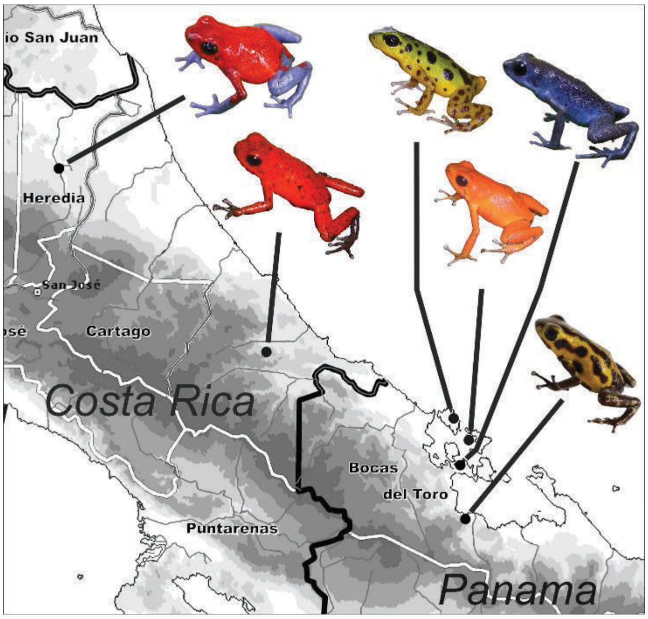

Fig. 1: Geographic distribution of the studied populations of *O. pumilio* in Costa Rica (Sarapiquí: red with blue legs; Hitoy: red morph) and Panama (Mainland: Río Gloria: yellow-black morph; Tierra Oscura: blue morph; Islands: Isla Colón: green-black morph; Isla Solarte: orange morph) with one representative frog for each population.

The Neotropical poison frog species *Oophaga pumilio* is distributed across the Caribbean slope of Nicaragua, Costa Rica and Panama and has diverged into two genetic groups, a Northern group and a Southern one (Hauswaldt et al., 2011). Panamanian populations belong to the Southern genetic group, while Costa Rican and Nicaraguan populations belong to the Northern genetic group (hereafter called Southern and Northern genetic groups). The species shows considerable divergence in both body coloration (Daly and Myers, 1967) and acoustic signals (Pröhl et al., 2007) among geographically separated populations. Color morph variability is mainly located at the Bocas del Toro Archipelago and adjacent mainland in Panama (Southern genetic group, Fig. 1), despite the recent formation of the Archipelago in the last 10,000 years (Summers et al., 1997). The aposematic coloration of this toxic species has been shown to be involved in sexual selection. In previous studies, female strawberry poison frogs were tested for their preference for males with local coloration or spotting pattern in mate choice experiments under laboratory conditions. The results showed that females prefer their local color morphs in some but not all populations (Summers et al., 1999; Reynolds and Fitzpatrick, 2007; Maan and Cummings, 2008). These studies were highly important to identify general female preferences for diverging visual sexual signals. However, previous studies neglected the impact of acoustic signals although advertisement calls are involved in mate or species recognition in almost all anuran amphibians (Ryan and Rand, 1993; Gerhardt and Huber, 2002). In strawberry poison frogs most variation in advertisement calls resides between the Northern and Southern genetic groups (Pröhl et al., 2007). If both visual and acoustic traits are subject to female preference in strawberry poison frogs, assortative mate choice based on multimodal traits could restrict gene exchange between populations or genetic groups and promote the evolution of prezygotic isolation. Here we integrate visual and acoustic signals to identify the contributions of these signals for mate recognition under natural conditions. Hereby we aim to identify the role of sexual selection for progressive divergence in these traits, which may cause prezygotic reproductive isolation and facilitate speciation.

Given the presented theory and earlier findings we hypothesize that 1) both advertisement calls and colors should impact mate selection in strawberry poison frogs; 2) females preference functions may vary among populations, because divergence in traits may be affected by natural selection as well. Populations of the Northern genetic group (were calls vary substantially among populations) may show particular preferences for calls, while populations of the Southern genetic group may pay more attention to colors; 3) presentation of advertisement calls should be sufficient to induce an approach of receptive females to the loudspeaker (as in natural situations calling males may be hidden behind obstacles in the habitat); 4) potential preferences for local calls should be enhanced by presentation of the respective local model, while interchange of local and non-local frog models should mitigate preferences; 5) presenting the same sound on both sides, we expect females to show preferences for the local model.

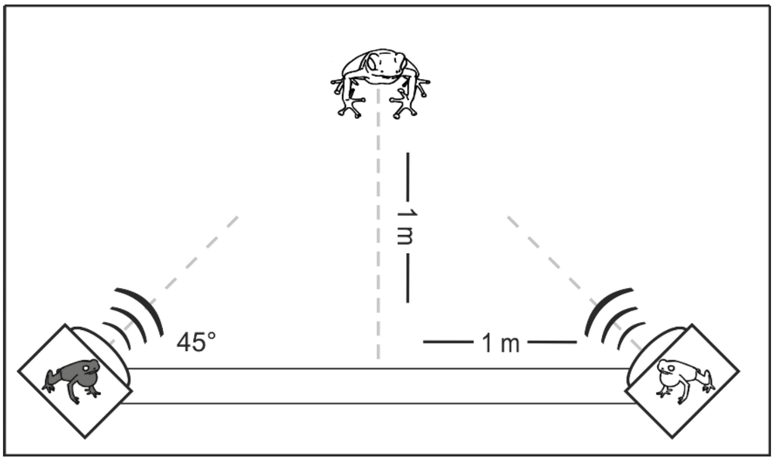

Fig. 2: Experimental setup for two-choice playback experiments. Model frogs positioned on top of the loudspeakers were only present in bimodal experiments (exp. III – V; Fig. 3).

To address these issues we conducted playback experiments to test female preferences for local and non-local traits in two populations of the Northern genetic group and four populations of the Southern genetic group (Fig. 1 & 2). Assuming that i) the complexity of the tropical habitat of strawberry poison frogs, in which both sexes possess large territories or home ranges (Pröhl and Berke, 2001; Meuche et al., 2011), has consequences for female choice behavior, and that ii) preferences found under laboratory conditions do not necessarily coincide with preferences in natural situations (Gerhardt, 1982; Dyson and Passmore, 1988) we tested females directly in their natural habitat. Furthermore, we tested only females that were found in courtship with males to assure that their choice was related to mate selection. By testing receptive females directly in their home ranges we created experimental choice settings mimicking natural mate choice situations.

2. Material and Methods

2.1. Study system

Strawberry poison frogs are distributed across the Caribbean slope of Central America in tropical lowland forests. The frogs are abundant, diurnal, and both sexes are territorial and provide parental care. Since female care is more time-intensive than male care, the operational sex ratio is male biased (Pröhl, 2002) and females have been observed to be choosy about their mates (Pröhl and Hödl, 1999). Once initiated courtship, females seem to abandon courting males occasionally (< 20% of courtships) (Pröhl and Hödl, 1999; Meuche et al., 2013). The species shows a complex phylogenetic history with admixture between evolving linages and former dispersion of frogs from Northern populations into the South (Hauswaldt et al., 2011; Galindo Uribe et al., 2014). Within the species two recent genetic groups have been identified: microsatellite analyses roughly assign Costa Rican populations to a Northern group and Panamanian populations to a Southern group (Hauswaldt et al., 2011). Advertisement calls of males, which are used to attract females and defend territories against intruders, vary in all call parameters among populations and between the two genetic groups (Pröhl et al., 2007). The frogs are toxic and mostly aposematically colored. Throughout Costa Rica and in some Panamanian locations, strawberry poison frogs have a bright red coloration, while in the province of Bocas de Toro, Panama, an extremely diverse series of mostly non-syntopic color morphs on different island and mainland localities has evolved, with coloration ranging from cryptic green, over blue, black-white to conspicuous orange or yellow populations (Daly and Myers, 1967; Summers et al., 2003).

2.2. Study sites and duration of field study

We conducted mate-choice experiments in six populations. Two populations were located in Costa Rica and belong to the Northern genetic group (Sarapiqui: 10° 28.227 'N; 84° 0.553 'W; 44 m.a.s.l. and Hitoy Cerere: 9° 37.819 'N; 83° 0.879 'W; 270 m.a.s.l.) and four were located in Panama and belong to the Southern genetic group. Two Panamanian populations were located on the mainland (Rio Gloria: 8° 59.100 'N; 82° 13.916 'W; 24 m.a.s.l. and Tierra Oscura: 9° 11.776 'N; 82° 14.976 'W; 7 m.a.s.l.) and two on different islands (Colón: 9° 23.170 'N; 82° 15.941 'W; 35 m.a.s.l. and Solarte: 9° 19.946 'N; 82° 12.939 'W; 4 m.a.s.l.). Mate Choice experiments were conducted in three study periods, lasting from August 2008 to December 2009, from April 2010 to November 2010 and from April 2011 to October 2011. During this time we tested a total amount of 452 different receptive females for their mate preferences.

2.3. Mate choice experiments

Playback experiments (see Meuche et al., 2013) were conducted between 6 am and 5 pm, with most of the experiments taking place during the morning hours when courtship activity was at its peak. In preliminary experiments we found that only females showing courtship behavior towards a male responded to and approached sexual signals (calls sequences emitted via loudspeakers and model frogs of different coloration). Therefore, we selected courting females that followed a calling male and interacted with him for the experiments to assure that the females’ response to visual and acoustic signals was related to mate choice. That courting females were receptive was furthermore corroborated by the fact that most of these females (87%) lost unfertilized eggs when held in captivity until the next day after the experiments, while non-courting females never laid eggs. Before starting playback-experiments with receptive females the male of the couple, as well as males calling in the immediate vicinity, were captured to avoid interference.

To generate the acoustic signals we analyzed 2930 advertisement call sequences from 293 males of 23 different populations of strawberry poison frogs of the Northern and Southern genetic group (Pröhl et al., 2007). We analyzed five call parameters (dominant frequency, call rate, call duration, duty cycle and pulse rate), adjusted them for temperature and calculated average values for each population and the two genetic groups (see Table S1 in Supporting Information). Out of the recorded call sequences we chose the call that fitted the average values of dominant frequency, call duration and pulse rate best. We then added a silent interval of a population-specific duration to create call sequences with the required call rate and duty cycle. By amplification of the selected call with its specific silent interval we generated call sequences for each population (“local calls”) and for both genetic groups (“Southern group call” and “Northern group call”)(see Table S1).

These sound sequences were used in two-choice playback-experiments. In five experiments we tested receptive female strawberry poison frogs for their preferences for unimodal or bimodal signals. In experiment I and II females had to choose from two call sequences (Fig. 3: exp. I & II), and in three further experiments we used bimodal stimuli, combining two calls with two plasticine model frogs of different color patterns (see Fig. 3: exp III, IV & V; and Figure S1). Experiment I tested for female preferences when the local call was presented against the average call of the local genetic group ("local group call"). Experiment II presented the local call against the average call of the non-local genetic group ("non-local group call"). The remaining experiments used bimodal stimuli, presenting the calls in combination with two model frogs painted to mimic the local and a non-local color morph (see Figure S1). Experiment III presented the local call in combination with the local model on one side vs. the non-local group call with the non-local model on the other side. In Experiment IV the model frogs were switched, (combining local call/non-local model and non-local group call/local model). For Experiment V both loudspeakers broadcast the local call in combination with one of the two different model frogs (Fig. 3). We tested a minimum of 15 receptive females in each of the five experiments in each population, resulting in a total of 452 different females. To assure that each female was tested only once – and to avoid problems with pseudoreplication – photographs were taken and we applied toe-clipping where no individual spotting pattern allowed identification of the females. The study was conducted in accordance with German, Costa Rican and Panamanian laws and followed the "Guidelines for the treatment of animals in behavioural research and teaching" and the "Guidelines for use of live amphibians and reptiles in field research".

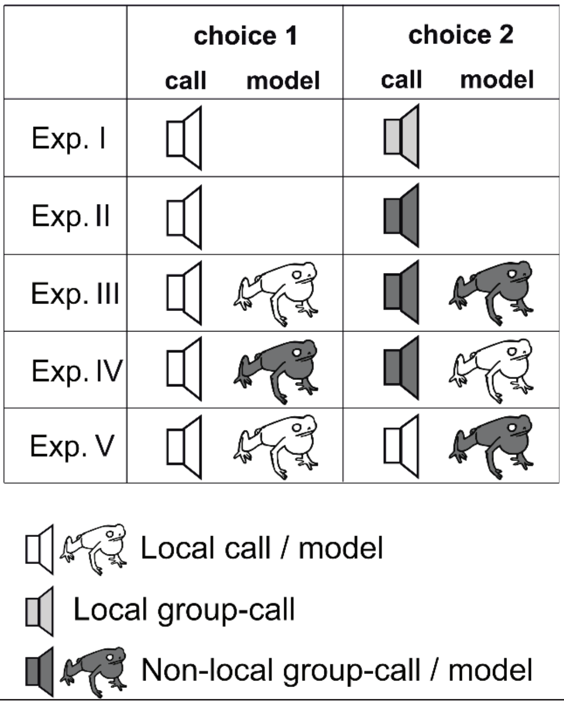

Fig. 3: Experimental types used for playback experiments: type I & II unimodal experiments with different call sequences (indicated by loudspeaker symbol) on both sides; type III – V bimodal experiments with different combinations of calls sequences and local and non-local model frogs (indicated by frog symbol). (White: local call/ local model; light grey: local group call; dark grey: non-local group call/ non-local model).

During the playback experiments the calls were presented via Apple® Ipods® and loudspeakers (Visaton FRS 10 WP), which were connected to an amplifier (Basetech AP-2100 Mini-Verstärker) and a 12V battery. The loudspeakers were fixed at the ends of a wooden plank at an angle of 45°, ensuring that the calls were broadcast toward the female when the middle of the plank was placed at a distance of 1 m from the receptive female before starting the experiment (Fig. 2). The sound pressure level in both loudspeakers was set to 72 dB at a distance of 50 cm, which corresponds to the average natural sound pressure level of advertisement calls at this distance. The experimental setup was examined for linear presentation of the frequency range of the frogs' advertisement calls: power spectra of the playback calls resembled natural calls up to 10 kHz. For experiments with bimodal stimuli, we used handmade plasticine model frogs, which were modeled with STAEDTLER® Fimo®, hardened in an oven and later painted with acrylic paint. To mimic the brilliance of living frogs, we applied a layer of clear coat on the painted models. In bimodal experiments, two model frogs (one local model and one non-local model) were placed on top of the loudspeakers since males usually call from elevated perches. The criterion for the selection of the alternative model presented in experiments with bimodal stimuli, was to be substantially different in coloration from the local model. Since red populations are prevalent in this species, we always combined one red model with one non-red model. In red populations we combined the local red model with green or yellow models (Fig. S1).

Upon approaching one loudspeaker to a distance less than 20 cm, which took on average between 29 minutes and 1:15 hours in different populations, the female was considered to have a preference for this choice and the female was captured. We selected 20 cm as cut-off distance because male *O. pumilio* stay stationary and emit advertisement calls until the female gets into close proximity. At these distances (~ 20-30 cm) the males orient towards and approach the female and start to interact with it. During close-range interactions males switch from advertisement calls to courtship calls. With this experimental procedure we intended to cover the mate attraction phase in which both signals – advertisement calls and colors – could be perceived and evaluated by the female in a natural situation. At larger distances the visual signals is probably not perceptible while at closer distances the very low courtship calls replace the advertisement calls.

For several reasons we decided to use model frogs instead of live males in our experiments. In preliminary experiments we found receptive females to pay equal attention to model frogs and live male frogs kept under a daylight transparent glass. Conducting the experiments with live males, we expected the following problems: 1) the behavior of a male during courtship can influence the preference of the female in mate choice experiments (Andersson, 1994; Wong et al., 2011) and we could not control for motivation of a male to court females of local or non-local origin, including calling behavior (with vocal-sac inflation probably acting as an additional visual cue); 2) advertisement calls emitted by living males interfere with presentation of local or non-local sound sequences and 3) we could not control for body or vocal sac size of the animals (average weight of males differs between 0.38 and 0.99g among study populations).

2.4. Data analysis

We conducted global binomial tests to test for preferences in each experiment. For this analysis we included the results of all investigated populations (N ≈ 90 females per experiment). Furthermore, we applied binomial tests to analyze general preferences in each genetic group (North: N ≈ 30, South: N ≈ 60 females per experiment). To test for population specific preferences, we applied binomial tests to analyze preferences in each experiment separately for each of the six populations. All binomial tests were conducted with IBM® SPSS.

To test if presentation of assorted or interchanged model frogs affects preferences in females, we used Generalized Linear Models (GLMs) for pairwise comparisons. We tested if results of the following pairs of experimental types deviated significantly from each other: I & II (are preferences for the local call more pronounced when tested against the non-local group call or the local group call?); II & III (are preferences more pronounced when calls are combined with the respective models?); II & IV (are preferences for calls altered when calls are combined with non-local models?); III & IV (are there differences in the results depending on the position of the models?) and III & V (are preferences for the local call/ local model combination altered, when the non-local model is also presented with the local call?). These pairwise comparisons were conducted on species, group and population levels. Depending on the size of the data sets, GLMs were conducted assuming quasi-binomial or binomial error distributions. All GLMs were conducted with R (R Developmental Core Team, 2012).

3. Results

All statistical tests were conducted at the species level (all females included in the analysis, N ≈ 90 females per experiment), group level (all females in a single genetic group included in the analysis, Northern group: N ≈ 30 females, Southern group N ≈ 60 females per experiment), and population level (N ≈ 15 females for each experiment) (see Table 1).

3.1. Female preferences for local versus non-local calls and coloration:

In experiment I female strawberry poison frogs did not discriminate between the local call and the local group call on species, group or population level (see Fig. 4 & 5 and Table 1). However, in experiment II we found a highly significant preference for the local call over the non-local group call on species and group level and in nearly all populations. This preference was also present when the calls were presented together with either matching or non-matching model frogs (exp. III and IV). No preference was found for either the local or the non-local color morph at any level (see Fig. 4 & 5 and Table 1).

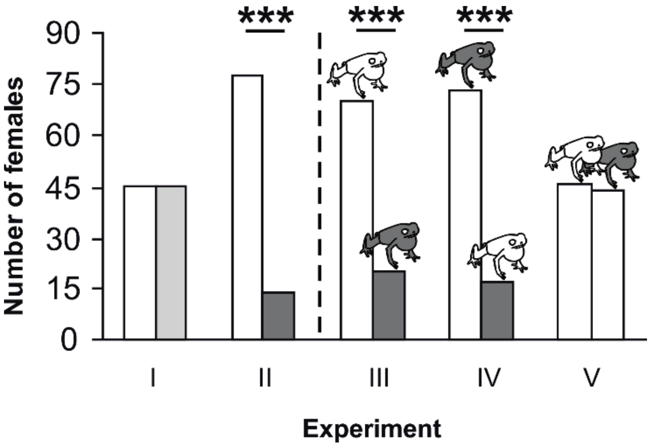

Fig. 4: Global results of playback experiments for all tested populations: The bars present the number of females which selected one or the other signal or signal combination. Bar coloration indicates call type, model frog coloration indicates model type presented in the experiments (white: local call/ local model; light grey: local group-call; dark grey: non-local group-call/ non-local model). Asterisks indicate significant preferences for one choice over the other (***, $P < 0.001$; Table S1).

Females in the two genetic groups differed in their preferences for sexual signals. In the Southern genetic group, females showed a highly significant preference for the local call when presented against the non-local group call, independent of the presentation or assignment of model frogs (see Table 1: exp. II, III and IV). However, in the Northern genetic group, the acoustic signal did not override the effect of the visual signal as clearly as in the Southern genetic group. Though females of the Northern genetic group also showed a preference for the local call when presented against the non-local group call (exp. II), this preference was no longer significant when the calls were presented with either matching (exp. III) or non-matching model frogs (exp. IV). When broadcasting the local call on both sides (exp. V) in combination with a local or non-local model, we did not find significant preferences for any presented model in either genetic group (see Fig. 5 and Table 1).

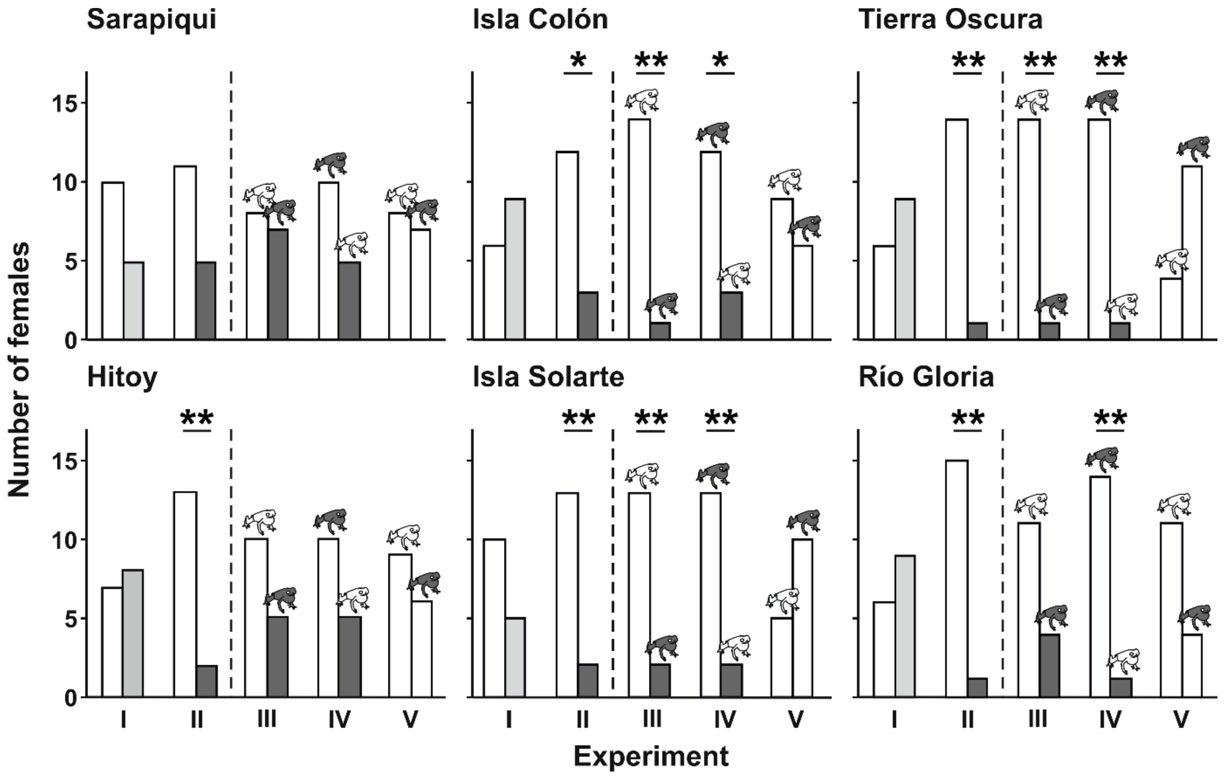

Fig. 5: Results of playback experiments for each population. The bars present the number of females which selected one or the other signal or signal combination. Bar coloration indicates call type, model frog coloration indicates model type presented in the experiments (white: local call/ local model; light grey: local group-call; dark grey: non-local group-call/ non-local model). Preferences for one choice over the other are indicated with asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001; Table S1).

Furthermore, females of different populations in the Northern genetic group differed in their responses to sexual signals. Frogs from the Sarapiquí population did not show a significant preference for any of the signals or signal combinations. In contrast, females from Hitoy Cerere significantly preferred the local call over the non-local group call, but only when presented without model frogs (exp. II)(see Fig. 5 and Table 1). In the Southern genetic group, females from most populations preferred the local call when presented against the non-local group call, independent of the presence and allocation of model frogs (exp. II, III and IV). In two populations of the Southern genetic group, 11 out of 15 females preferred one of the color morphs over the other when both were combined with the local call: at Río Gloria 11 females chose the local (yellow) color morph, while only 4 chose the non-local (red) morph. In Tierra Oscura we found the opposite pattern: 11 females selected the non-local (red) morph and 4 females selected the local (blue) color morph. Even though these results point to preferences for colors (73% versus 27% choices) the results were not significant (see Fig. 5 and Table 1).

For additional information about the behavior of females after approaching the loudspeakers to a distance of 20 cm please see Supplementary Information 1.

	experiment I	**experiment II**	**experiment III**	**experiment IV**	**experiment V**
O. pumilio	1.000 (92)	**0.000** (90)	**0.000** (90)	**0.000** (90)	0.916 (90)
North	0.585 (31)	**0.003** (30)	0.362 (30)	0.099 (30)	0.585 (30)
Sarapiqui	0.302 (16)	0.210 (15)	1.000 (15)	0.302 (15)	1.000 (15)
Hitoy Cerere	1.000 (15)	**0.007** (15)	0.302 (15)	0.302 (15)	0.607 (15)
South	0.699 (61)	**0.000** (60)	**0.000** (60)	**0.000** (60)	0.897 (60)
Rio Gloria	0.607 (16)	**0.001** (15)	0.118 (15)	**0.001** (15)	0.118 (15)
Tierra Oscura	0.607 (15)	**0.001** (15)	**0.001** (15)	**0.001** (15)	0.118 (15)
Colón	0.607 (15)	**0.035** (15)	**0.001** (15)	**0.035** (15)	0.607 (15)
Solarte	0.302 (15)	**0.007** (15)	**0.007** (15)	**0.007** (15)	0.302 (15)

Table 1: Results (P-values) of binomial tests including sample sizes (N). Tests were conducted for each experiment on species-level (result for *O. pumilio* summarizing the results of all six study populations), group-level (summarizing the results of all populations belonging to the Northern or Southern genetic group) and on population level. Bold numbers indicate significant preferences for one signal combination.

3.2. Differences in female behavior between experiments:

I & II: *Are preferences for the local call more pronounced when tested against the non-local group call than against the local group call?* – At the species level, for the Southern genetic group as well as for Tierra Oscura and Rio Gloria, the results of experiments I & II were significantly different, i.e. females discriminated significantly against the non-local group call, but not the average call of their own genetic group (see Table 2).

II & III: *Are preferences more pronounced when calls were combined with the respective models?* – None of the pairwise comparisons was significant, i.e. the preferences for the local calls were not enhanced by a combination with a model frog of the local coloration (see Table 2).

II & IV: *Are preferences for calls altered when calls are combined with non-local models?* – None of the pairwise comparisons was significant, i.e. the preference for the local call was not affected by a combination with a non-local color morph (see Table 2).

III & IV: *Are there differences in the results depending on the position (matching or non-matching) of the models?* – The presence of a model of the local versus the non-local color morph did not affect the preference for the local call in any of the comparisons (see Table 2).

III & V: *Are preferences for the local call & local model combination (vs. non-local group-call & non-local model) still present when the alternative choice consists of the same local call combined with the non-local model?* – The results of experiments III & V were significantly different at the species level, for the Southern genetic group as well as for Solarte and Tierra Oscura, i.e. the choice of females did depend on the call the non-local model was presented with (see Table 2).

	I & II	II & III	II & IV	III & IV	III & V
O. pumilio	**0.001** *-3.829*	0.780 *-0.945*	0.969 *-0.504*	0.980 *0.446*	**0.015** *-2.954*
North	0.143 *-2.086*	0.264 *-1.780*	0.662 *-1.141*	0.929 *0.655*	0.995 *-0.321*
Sarapiqui	1.000 *-0.124*	0.834 *-0.876*	1.000 *-0.124*	0.899 *0.743*	1.000 *0.000*
Hitoy Cerere	0.112 *-2.182*	0.570 *-1.259*	0.570 *-1.259*	1.000 *0.000*	0.989 *-0.378*
South	**0.000** *-4.012*	0.998 *-0.245*	1.000 *0.000*	0.998 *0.245*	**0.001** *-3.752*
Rio Gloria	**0.037** *-2.621*	0.4948 -1.369	1.000 *0.000*	0.4948 *1.369*	1.000 *0.000*
Tierra Oscura	**0.036** *-2.621*	1.000 *0.000*	1.000 *0.000*	1.000 *0.000*	**0.009** *-3.072*
Colón	0.116 *-2.150*	0.710 *1.027*	1.000 *0.000*	0.710 *-1.027*	0.189 *-1.923*
Solarte	0.569 *-1.259*	1.000 *0.000*	1.000 *0.000*	1.000 *0.000*	**0.0268** *-2.739*

Table 2: Results (P-values) of pairwise comparisons among different experiments including test statistics (z-values) in italics. Bold numbers indicate significant differences among results of different experiments.

4. Discussion

Mate choice decisions are crucial for the formation of isolation barriers among emerging lineages or species. Within the framework of prezygotic behavioral isolation the importance of different sexual traits is still understudied, especially under field conditions. In this study we conducted mate choice experiments in six natural populations belonging to two genetic groups across the distribution range of the strawberry poison frog. We demonstrate that i) acoustic signals exceed visual signals in their importance for mate attraction across populations, and ii) female preferences vary among populations and between genetic groups.

4.1. North versus South

Our 452 playback experiments reveal that preferences of females of the Northern genetic group are less pronounced than those of the Southern group; thus, behavioral isolation is asymmetric between the genetic groups. In the Southern genetic group, where divergence in call parameters is lower among populations (Pröhl et al., 2007), we found a strong pattern of preference for the local call over the non-local group call, independent of the presence and assortment of the visual signal (coloration of model frogs). The Northern group call was less attractive for frogs from the Southern genetic group, while females from the Northern genetic group also chose the Southern group call but not as often as the local call. These differences in preferences could be due to a variety of reasons: For example, the Northern group call has a lower calling rate, which is known to be less attractive to female anurans (Sullivan et al., 1995; Gerhardt and Huber, 2002)(see Table S1). On the other hand, asymmetrical behavioral isolation caused by differences in the strength of discrimination against heterospecific traits between lineages seems to be common in frogs (e.g. Hoskin et al., 2005) and other systems (Kodric-Brown and Strecker, 2001; Panhuis et al., 2001; Berdan and Fuller, 2012). Whether it corresponds to the strength of postzygotic isolation or hybrid fitness as found in some of these earlier studies is not known for strawberry poison frogs.

4.2. Impact of calls and colors

Pairwise comparisons showed that females discriminate between calls of different genetic groups but not among different calls of their own group (exp. I & II) (see Table 2), suggesting that call-based prezygotic isolation is present between genetic groups but absent among populations within genetic groups. Acoustic signals are involved in assortative mate choice and contribute to prezygotic isolation between incipient species or diverging populations across different animal taxa (e.g. Boul et al., 2007; Noh and Henry, 2009; Uy et al., 2009); therefore, this result is not surprising. More importantly, female preferences for local calls override the possible effect of model frog coloration in our experiments, since exchanging model frogs did not alter female preferences (exp. III & IV), whereas a change in the presented call did affect the results (exp. III & V). This indicates that for strawberry poison frogs, advertisement calls play a more important role in mate attraction than colors, and most females would probably not approach a male emitting the “wrong” call. However, strength of preferences varied among the two genetic lineages. In laboratory experiments investigating mate selection based on visual, olfactory and acoustic signals sympatric sister species of fish showed asymmetrical use of signals of different modalities: while *Cyprinodon maya* used a combination of visual and olfactory cues, *Cyprinodon labiosus* discriminated against heterospecifics based on olfactory cues alone (Kodric-Brown and Strecker, 2001). In one cichlid fish species, visual signals - but not acoustic signals - alone were sufficient for mate attraction. Nevertheless, acoustic signals enhanced female preferences for males when visual signals were combined with conspecific sound (Estramil et al., 2013). Showing that the importance of signals of different modalities for prezygotic isolation might vary not only among sister species but even among populations of a single species, we consider the results of our study to be a valuable addition to existing studies of multimodal signaling.

Although our experiments did not reveal a general pattern of females to prefer the local or non-local color morphs, females among populations differed in their preferences respective to coloration. In experiments with local calls only (exp. V), females from Río Gloria showed a tendency to select the local yellow color morph over the alternative red color morph, while the blue Tierra Oscura females showed a tendency to select the brighter non-local red morph. Earlier studies of sexual selection in strawberry poison frogs recorded different patterns and degrees of color-assortative preferences in different populations (Summers et al., 1999; Reynolds and Fitzpatrick, 2007; Maan and Cummings, 2008). Furthermore, sexual dimorphism in brightness was detected in the population from the island Solarte, and experiments suggested sexual selection for brighter animals (Maan and Cummings, 2009; Dreher et al., 2017). Preferences for brightness have been linked to superior health status in a number of studies (e.g. Milinski and Bakker 1990) and/or may evoke greater sensory stimulation in the choosing sex (Maan and Cummings, 2009). Our findings are consistent with the idea that females prefer brighter males, since in both cases – Rio Gloria and Tierra Oscura – the preferred color morph was brighter than the alternative color morph, regardless of the origin (local or non-local) of the model frog (Dreher, unpublished data). These results might imply that the preference for brighter males is a widespread but not exclusive pattern in strawberry poison frogs.

In the Southern genetic group, in the region of Bocas del Toro populations vary in coloration and advertisement calls (Pröhl et al., 2007). However call variation is lower than in the Northern genetic group and variation of calls within the genetic group does not seem to affect female choice. Here, prezygotic isolation based on color-assortative mating might vary from week to strong depending on the characteristics and ecology of the involved populations (Maan and Cummings, 2008; Richards-Zawacki et al., 2012). For example, in several contact zones between populations of different colors, animals with intermediate color morphs can be observed (e.g. between red and black-white at Rio Cascaje on the Panamanian mainland). Hybridization among color morphs indicates that there is no complete reproductive isolation between different color morphs through color-assortative mating in this species. Taken together, the results of this and previous studies suggest that, when controlling for call variation, preferences for local or bright coloration range from strong and moderate to absent across the species distribution (Summers et al., 1999; Reynolds and Fitzpatrick, 2007; Maan and Cummings, 2008). Ecological correlates to the different patterns of female preferences (e.g. predation pressure, light conditions, level of divergence in coloration and brightness) need further attention. Polymorphic populations or contact zones where the sexes of different and intermediate morphs meet offer the opportunity to study the link between coloration, mate choice and gene flow across natural populations.

Furthermore our and earlier data imply that strawberry poison frogs use acoustic and visual cues in a sequential order, with advertisement calls used to attract potential mates and facilitate selection at a larger distance while visual cues might act later in courtship during close-range assessment. In natural populations, the distance between calling males is usually several meters (Pröhl and Berke, 2001; Pröhl and Ostrowski, 2011), which makes it difficult for females to assess the coloration of two or more males simultaneously, while the advertisement calls of frogs appear to be detectable and assessable from this distance. Receptive females have been observed to react to and approach a male calling from several meters away (Meuche et al., 2013). However, during the prolonged courtship of strawberry poison frogs, a female still has the chance to reject the male after entering the final stage of courtship (which includes direct tactile interaction between the sexes at the oviposition site) (Pröhl and Hödl, 1999). During close-range assessment, coloration, courtship calls, and other behavioral traits of the male or the quality of the oviposition site could play a role for a female’s decision. However, to date, there are no studies investigating the importance of this final courtship stage for female mate choice of strawberry poison frogs. As failure in long-distance attraction would impede the stage of close-range assessment our observations point to a higher importance of acoustic signals for mate choice in strawberry poison frogs.

4.3. Sexual signals, genetic population structure and speciation in *Oophaga*

In *O. pumilio*, variation in the two sexual signals - calls and colors - is differently situated with respect to geography and genetic groups. The vast amount of color diversity is located within the Southern genetic group in a small geographic area in Panama while variation in calls is mainly located between the two genetic groups (Pröhl et al., 2007; Wang and Shaffer, 2008; Hauswaldt et al., 2011). Hence, divergence in calls coincides with divergence in the genetic population structure within the species. In Panama, one other *Oophaga* species (*O. vincentei)* and one separated population of *O. pumilio* on Isla Escudo are genetically closely related to populations of *O. pumilio* from North Costa Rica (Hauswaldt et al., 2011). Several lineages (originating Southern *O. pumilio*, *O. vincentei* and *O. pumilio* from Escudo) probably invaded Panama from the North approximately 2 to 1 million years ago (Galindo Uribe et al., 2014). Differences in call properties among populations of Southern *O. pumilio* and other invading frogs might have prevented mating among genetically distinct animals to prevent hybridization. These circumstances could have contributed to the evolution of pronounced discrimination abilities of females from the Southern genetic group against non-local call attributes. To date, *O. vincentei* and *O. pumilio* from Escudo are bioacoustically clearly differentiated from all (other) *O. pumilio* populations (Jungfer et al., 1996).

We propose the following scenario for sexual signal divergence and associated preferences in strawberry poison frogs: The association between acoustic and genetic divergence suggests sexual selection as the main driver of acoustic divergence. Based on preferences for local calls, prezygotic isolation potentially impedes mating with genetically different males and thus decreases the risk of reproductive failure. This might have been relevant for the divergence between both genetic groups in strawberry poison frogs and among *Oophaga* species. In contrast, the evolution of color diversity appears to be the result of drift and natural selection (predation pressure) since it is coupled with divergence in toxicity and antipredator behavior (Pröhl and Ostrowski, 2011; Maan and Cummings, 2012). Under that scenario the evolution of preferences for local or brighter coloration would have followed ecological adaptation, as previously found in several *Heliconius* species (e.g. Jiggins et al., 2001) and a number of other taxa (Rundle and Nosil, 2005).

Consistent with this idea, several closely related *Oophaga* species that occur in Costa Rica and Panama are clearly differentiated among each other by their advertisement calls (Pröhl et al., 2013), and some of these species (e.g. *O. granulifera* and *O. vincentei*) also show color divergence among populations, mainly into red and green morphs (Brusa et al., 2013)(http://www.dendrobase.de/html/D_oophaga_vicentei.html). As in *O. pumilio,* color divergence is not related to genetic divergence in *O. granulifera* (Brusa et al., 2013). These complex patterns of call and color diversity within several dendrobatid species offer prospects to further evaluate the importance of different mechanisms of speciation mediated through different sensory channels. In the future, multimodal experiments could evaluate the effects of signals of different sensory channels on intra- and intersexual selection. Unimodal and multimodal experiments using an array of intra- and interspecific signals might furthermore help to disentangle how signals of one or more modalities impacted divergence among incipient species.

5. Conclusions

Divergence in the sexual signals of one sex and associated preferences in the other sex contribute to prezygotic isolation, speciation and thus the evolution of species diversity. The complexity of the link between mate choice and speciation increases when several signal modalities – shaped by different evolutionary forces - are involved in mate selection. In the strawberry poison frog, advertisement calls have diverged between two genetic groups while variation in (aposematic) coloration is limited to populations within the Southern genetic group. Testing females for preferences for local or non-local calls and colorations, we found acoustic stimuli outpaced visual stimuli in female attraction. However, the strength of preferences varied between genetic groups. The results suggest that prezygotic isolation based on calls could contribute to ongoing divergence between the genetic groups. They also indicate that sexual signals vary in their relevance for mate attraction in the field, and divergence in coloration might contribute to sexual selection only in areas where color diversity had previously evolved. While our study concentrated on the relative importance of visual versus acoustic signals, further studies should disentangle ecological factors that mediate variations in sexual behavior across species ranges.

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Statement of authorship:

C.E.D. and H.P. designed the experiments, C.E.D. performed the research, C.E.D. & H.P. analyzed the data and wrote the manuscript.

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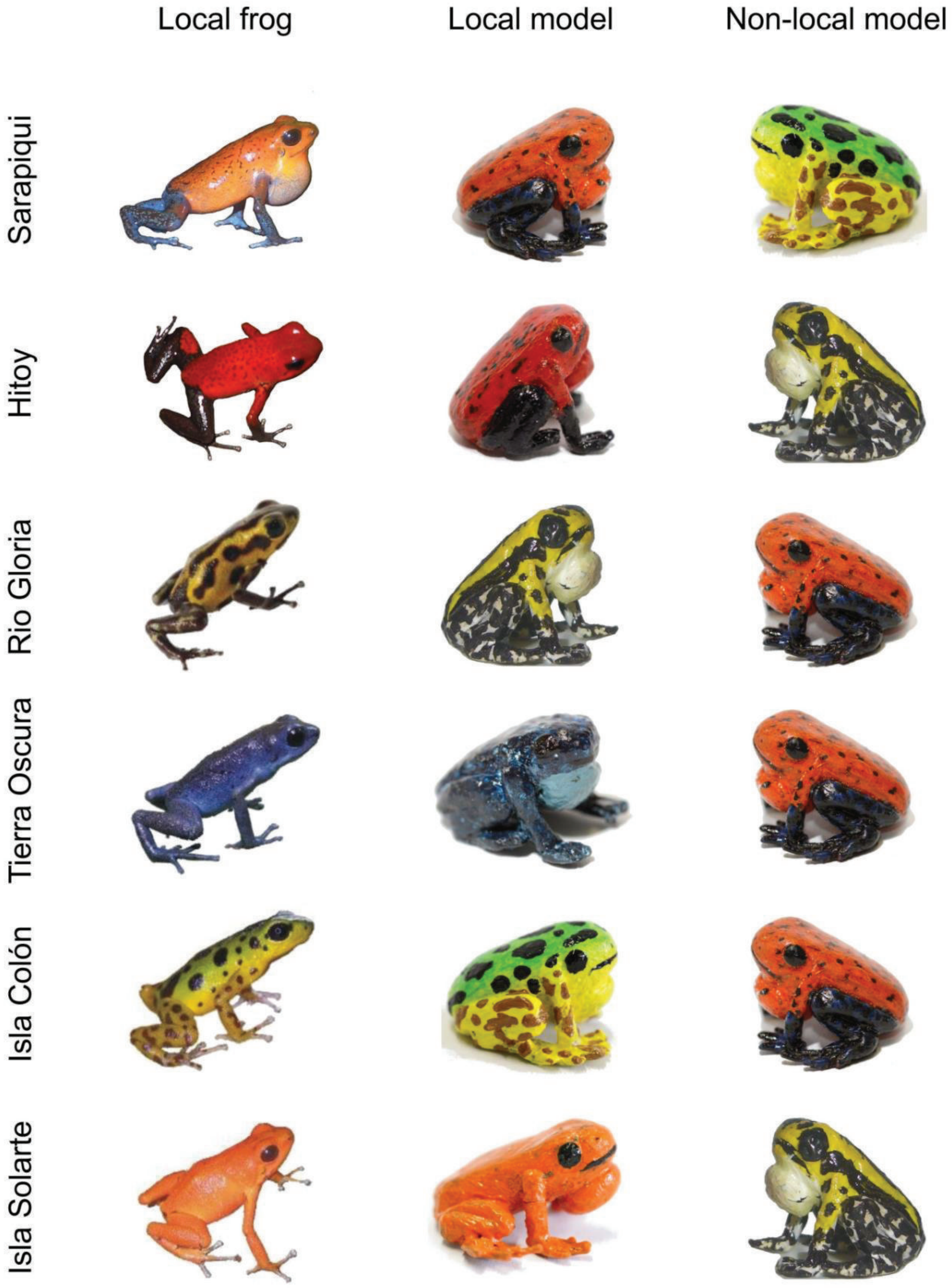
3.1 Supplementary material

3.1.1. Supplementary information

Supplementary Information 1: Behavior of females after entering the 20cm diameter around the loudspeaker. Upon approaching one loudspeaker to a distance less than 20 cm we considered the female to have a preference for this choice and the female was captured. However, we did not capture all of the females immediately after the experiment. Whenever possible without risking to lose the female (e.g. when the female was apparently impatient, or the vegetation was very dense) we observed the behavior of the females after entering the cut-off distance. Females usually kept approaching the loudspeaker until touching it. They frequently walked around the loudspeaker searching for the calling male or climbed the speaker. In normal courtship situations, at these close distances the males had already started to interact with the females. When observing females after the experiments, females very rarely (four times over the whole study period), and only after searching for the chosen male for several minutes, walked over to the other loudspeaker.

3.1.2 Supplementary figures

Figure S1: Pictures of one specimen of *O. pumilio* and frog models used in bimodal playback-experiments for each population. On the left one representative frog of each population, in the middle the local frog model, painted to mimic the local frog coloration. The right column shows the alternative frog model presented in experiment III to V.



3.1.3 Supplementary tables

	Average call parameters (temperature adjusted)				
	Call duration (ms)	Pulse rate (pulses/ms)	Dominant frequency (Hz)	Call rate (calls/s)	Duty Cycle (ms/s)
North	79.02	0.242	4143.0	6.00	468.00
Sarapiqui	69.30	0.231	4618.6	5.96	407.30
Hitoy	66.69	0.246	3964.6	7.64	505.05
South	54.14	0.302	4665.3	7.70	416.40
Rio Gloria	49.56	0.292	4971.2	7.72	379.34
Tierra Oscura	61.46	0.282	4595.0	6.96	424.62
Colón	52.77	0.356	4827.9	8.38	439.23
Solarte	47.99	0.337	4769.4	9.08	434.19

	Parameters of selected call			% deviation selected from average call		
	Call duration (ms)	Pulse rate (pulses/s)	Dominant frequency (Hz)	Call duration	Pulse rate	Dominant frequency
North	78.60	0.242	4149.1	0.5	0.0	0.1
Sarapiqui	67.50	0.237	4656.7	2.6	2.6	0.8
Hitoy	67.10	0.238	4048.4	0.6	2.9	2.1
South	53.10	0.301	4723.0	1.9	0.2	1.2
Rio Gloria	50.75	0.296	5055.2	2.4	1.2	1.7
Tierra Oscura	62.60	0.272	4430.0	0.0	2.7	3.6
Colón	53.40	0.356	4759.3	1.2	0.0	1.4
Solarte	48.20	0.332	4782.5	0.4	1.6	0.3

Table S1: Call properties of advertisement calls. Call parameters of average calls of each genetic group and every study population and the parameters of the selected call for each group or population. Selected calls were added a pause of defined length and used to synthesize call sequences fitting the average call parameters. The last three columns show the percentage of deviation of selected from average calls.

4. Publication III

Mating status correlates with dorsal brightness in some but not all poison frog populations

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Short title: brightness in mating strawberry poison frogs

Abstract

Sexual signals are important for intraspecific communication and mate selection, but their evolution may be driven by both natural and sexual selection, and stochastic processes. Strawberry poison frogs (*Oophaga pumilio*) show strong colour divergence among populations, but colouration also varies among individuals of the same population. The importance of colouration for female mate choice has been studied intensely, and sexual selection seems to affect colour divergence in strawberry poison frogs. However, the effect of colouration on mating success under field conditions has received very little attention. Furthermore, no study to date examines how phenotypic variation among individuals of the same colour morph affects mate selection under natural conditions. We measured the spectral reflectance of courting and non-courting individuals and their background substrates in three geographically separated populations. In one population (Sarapiquí, Costa Rica) we found males and females actively engaged in interactions with mates to have brighter dorsal colouration than single individuals. Our field observations suggest that, in the wild, females prefer brighter males while the reason for the higher courtship activity of brighter females remains unclear. Overall our results imply that brightness differences among individuals of the same colour morph may actually affect reproductive success in some populations of strawberry poison frogs.

Keywords: brightness contrast - colour divergence - dendrobatid frogs - mate choice - sexual selection - visual modelling

INTRODUCTION

Sexual signals such as mating calls, colour patterns or behavioural displays are important for intrasexual communication and mate selection (Gerhardt and Huber, 2002, Setchell et al., 2009). The evolution of these traits may be driven by sexual selection, but may additionally be shaped by natural selection and stochastic processes, e.g. genetic drift (Coyne and Orr, 2004, Richards-Zawacki and Cummings, 2011). The strength of these forces depends on local conditions, hence geographically isolated populations are likely to differ in their sexual signal phenotypes (Gerhardt and Huber, 2002, Pröhl et al., 2013, Rudh et al., 2007, Stuart-Fox and Ord, 2004, Uy et al., 2009). Variation in sexual signals combined with preference functions for these signals can lead to a Fisherian runaway process, causing rapidly diverging traits and accelerated speciation (Fisher, 1930, Iwasa and Pomiankowski, 1995). However, intraspecific divergence in sexually-selected traits is not only expected among populations, but also within populations. Variation in sexual signals can correlate with mate condition, mate quality, and/or serve as a means to exploit perceptual biases of the opposite sex (Ryan and Cummings, 2013, Ryan and Keddyhector, 1992, Zahavi, 1975). Colouration and brightness of colour patterns are used in intraspecific communication, and are known to influence mate choice in a variety of taxa (Maan and Cummings, 2009, Rowland et al., 1991, Setchell et al., 2009). Neotropical poison frogs of the family Dendrobatidae are highly variable in colouration among species and populations (Lötters et al., 2007, Pröhl et al., 2013, Willink et al., 2013), and to a lesser degree within populations of a species (Richards-Zawacki et al., 2013, Brusa et al., 2013). A large body of knowledge has accumulated on visual signalling and other ethological aspects of these frogs currently used as model system to study the evolution of phenotypic diversity in sexual signals (Cummings and Crothers, 2013, Rojas and Endler, 2013).

The Strawberry poison frog (*Oophaga pumilio*) (Schmidt, 1857) is highly diverse in colouration patterns among populations, with more than 15 different colour morphs, mostly located in the Bocas del Toro Province of Panama (Daly and Myers, 1967). Natural selection may have promoted the evolution of colour polytypism in this species (Gehara et al., 2013, Maan and Cummings, 2012, Pröhl and Ostrowski, 2011), but sexual selection is also expected to have played a role (Cummings and Crothers, 2013). Female strawberry poison frogs of several populations show colour-assortative mate preferences (Maan and Cummings, 2008, Reynolds and Fitzpatrick, 2007, Richards-Zawacki et al., 2012, Summers et al., 1999), and in some populations frogs show directional inter- and intra-sexual selection for bright colouration in males (Crothers et al., 2011, Crothers and Cummings, 2013, Maan and Cummings, 2009). On the Panamanian Island Solarte, strawberry poison frogs show sexual dimorphism in coloration, presumably facilitated through female preference for brighter males (Maan and Cummings, 2009). The above mentioned studies investigated female choice in *O. pumilio* under laboratory conditions, and discovered general preference functions, which might underlie mate choice decisions. However, preferences found under laboratory conditions may not completely reflect natural choice situations. It is largely unknown whether 1) observed preferences translate in actual choice during courtship and 2) whether reproductive behaviour observed under artificial laboratory conditions equals the behaviour in the wild where it should be modulated by additional factors and costs related to mate searching (Meuche et al., 2013). For example in a captive breeding experiment males and females from different populations and colour morphs reproduced as successful as within-population pairs (Dugas and Richards-Zawacki, 2015). Even though in this study the frogs were situated in a no-choice situation, it demonstrates that females accept non-assortative partners when no colour-assortative partners are present. Research investigating natural mate choice under field conditions is still scarce (but see Dreher and Pröhl, 2014, Richards-Zawacki et al., 2012) and will be explicitly addressed here.

Our investigation aims to explore whether evidence of sexual selection could be observed in different natural populations of strawberry poison frogs by examining the phenotypic features of reproductively engaged individuals relative to individuals not participating in reproductive interactions under natural field conditions. Specifically, we explore if individuals of *O. pumilio* observed in pairs and engaged in reproductive activities i.e. courtship differ in brightness and colouration from single individuals not engaged in courtship activities. In strawberry poison frogs, reproductive success of males seems to be directly correlated with the number of successful matings (i.e. laid clutches), unless clutch loss is extraordinarily high (Pröhl, 2005). Therefore, differences in the colour phenotype of courting and single individuals in a sampled population could be indicative of differences in the reproductive success of these phenotypes.

MATERIAL AND METHODS

Field work

Spectral reflectance of the skin of male and female strawberry poison frogs was measured under controlled conditions in each study population. Individuals were classified as courted (= ‘courtship’) or isolated (= ‘single’), based on contextual observations. Frogs classified as ‘single’ were randomly selected and observed for a couple of minutes to assure they were not courting at this particular moment. Obviously, these frogs might have been involved in courtship activities before and after; hence ‘single’ might not represent a general mating status. With this method we might risk masking existing differences between reproductively active and non-active animals. However, it is a conservative approach because any significant results will provide evidence of a true difference in colouration between ‘courted’ and ‘single‘ animals. Couples of frogs found in the field were observed until courting behaviour was undoubtedly identified. In an early stage of the prolonged courtship of strawberry poison frogs (Pröhl, 2005) males are walking around and calling to females in their proximity, while the females usually do not show any visible response to male courting behaviour. This early stage of courtship can take up to one hour or more (CE Dreher, personal observation). Later during courtship, the female starts to react to and approach the calling male. Alternately emitting advertisement calls and walking, the male will lead the female towards the oviposition site. We considered a female to have made a decision to mate with the calling male once she was observed to follow him. Courtship in this ultimate stage is rarely interrupted and normally ends in clutch deposition (Meuche et al., 2013, Pröhl and Hödl, 1999). At this point, we captured the couple in order to avoid losing the frogs. Captured courted and single frogs were stored individually in perforated plastic containers with a moist tissue and several leaves. Reflectance measurements were taken in the afternoon under standardized handling procedures and light conditions. In each study population between 39 and 52 frogs were measured. In Costa Rica, we measured 39 individuals in Sarapiquí (10° 28.227 'N; 84° 0.553 'W; 44 m.a.s.l.)(n = 9/10 courted/single males; n = 10/10 courted/single females) and 40 specimens in Hitoy Cerere (9° 37.819 'N; 83° 0.879 'W; 270 m.a.s.l.)(n =10/10 males; n = 10/10 females). In Panama we measured the reflectance of 52 specimens at Isla Colón (9° 23.170 'N; 82° 15.941 'W; 35 m.a.s.l.)(n = 13/13 males; n = 13/13 females).

Additionally we measured the reflectance of the substrate (e.g. leaves, trunks) on which each frog was found. Reflectance spectra of the skin or substrate were taken at a distance of 2mm using an Ocean Optics bifurcal optic fiber (R-200-7-UV/VIS) with a fixed outer sleeve to control the 2mm distance, an Optics HR2000+ Spectrometer, and a deuterium-tungsten lamp (DT-Mini-2-GS). To account for lamp drift we calibrated the measurements with a white standard (WS-1-SS) every other frog. Illumination of the habitat (Irradiance) was measured using an optic fibre (QP400-2-UV-BX) with an Ocean Optics cosine adaptor-head (CC-3UV) attached. Irradiance spectra were taken at the places where we found the frogs and at times when the frogs are reproductively active (between 7am and 12noon in Costa Rica; between 8am and 1pm in Panama) on two to three different days. The population-specific average irradiance was calculated for each population, using between 240 and 396 irradiance spectra per population.

Dorsal reflectance spectra were calculated averaging four reflectance measurements (two of which were taken on the head between the eyes, and two on the middle of the dorsum). Calculations of dorsal average spectra for frogs from the population on Isla Colón, where frogs possess a dark spotting pattern on a green background colour on their dorsum, included two measurements of the green background colour and the two head measurements. Ventral average spectra were calculated averaging two reflectance curves taken on the belly. We did not include measurements taken from the throat region in order to avoid the darker colouration of the throat of males to impact the results.

Visual models were calculated according to Maan & Cummings (Maan and Cummings, 2012) and Crothers & Cummings (Crothers and Cummings, 2013) using average dorsal and ventral reflectance spectra from each frog, the population-specific average irradiance and microspectrophotometric data on the visual sensitivity of cones of *O. pumilio* (Siddiqi et al., 2004). For the calculation of brightness contrast (ΔL) and colour contrast (ΔS) we additionally included reflectance spectra of the individual-specific substrate for each frog. In addition, to account for variation in conspicuousness driven by an animal's specific background rather than the inherent reflectance properties of its body, we calculated a colour (S) and brightness (L) value for each frog independent of background that included only frog reflectance spectra, the population-specific average irradiance and data on visual sensitivity of *O. pumilio*. The (S) and (L) values indicate the intrinsic conspicuousness, i.e. a value that indicates how well the frogs` colouration can be detected by the visual system of the respective observer. For comparison of the overall brightness of individuals, we calculated total reflectance flux, summarizing the recorded reflectance for each nm ($\Sigma R_{(\lambda)}$ for λ=300-700) (Crothers and Cummings, 2013, Maan and Cummings, 2012).

The study was conducted in accordance with German, Costa Rican and Panamanian laws and followed the ‘Guidelines for the treatment of animals in behavioural research and teaching’ and the ‘Guidelines for use of live amphibians and reptiles in field research’.

Data analysis

Measurements of the dorsal and ventral spectral reflectance variables (ΔL, ΔS, L, S, $\Sigma R_{(\lambda)}$) were subjected to separate principal components analysis and the individual scores of the components with eigenvalues < 1 were retained for subsequent analyses. This strategy reduces the dimensionality and collinearity in the original variables while maintaining separate dorsal and ventral PC-derived spectral variables that facilitate biological interpretation of results. We tested the effects of three categorical predictors; locality (Sarapiquí, Hitoy, and Isla Colón); mating condition (courted/single), and sex (male/female); on the spectral reflectance values with a multivariate analysis of variance (MANOVA) using the principal components as dependent variables and allowing for interactions among predictors. Statistically significant multivariate effects (alpha = 0.05) were also inspected with univariate analyses of variance and significant effects were explored further by plotting the 95% confidence intervals of the means of the PC-derived spectral variables, after grouping the data into predictor categories, and with post-hoc pairwise comparisons using the Tukey test. All statistical analyses were conducted with R (R_Core_Team, 2016).

RESULTS

Mean spectra of females and males from all three populations are presented in SM Figure 1. Generally the dorsal and ventral spectral reflectance was highest for frogs from Sarapiquí; in this population some differences between 'courted' and 'single' frogs are more pronounced than in the other two populations (see below).

Individual scores on the first two principal components derived from the dorsal spectral measurements (representing 84.4% of the variance in the data) indicated an overall trend towards higher values in Sarapiquí (SM Table-1, Figure 1a). MANOVA results indicated a significant effect of locality (Wilkinson's $\lambda = 0.39$, $F_{(4,\ 230)} = 34.88$, $p < 0.001$), mating condition (Wilkinson's $\lambda = 0.91$, $F_{(2,\ 115)} = 5.63$, $p = 0.005$) and the interaction between these two predictors (Wilkinson's $\lambda = 0.85$, $F_{(4,\ 230)} = 4.88$, $p < 0.001$). No statistically significant effect of sex or any other interactions among predictors were observed. Univariate analysis of dorsal PC1 values (most correlated with brightness variables, see SM Table-1) indicated statistically significant differences among localities ($F_{(2,\ 116)} = 39.00$, $p < 0.001$) and the interaction between localities and mating condition ($F_{(2,\ 116)} = 4.56$, $p = 0.012$). Statistically significant differences in PC1 values were observed between Sarapiquí and the other two localities and within-locality differences in mating condition were only significant in Sarapiquí where courted individuals exhibit higher values of PC1 (Figure 1b, SM Table 2). Analysis of dorsal PC2 values (most correlated with color variables, see SM Table-1) indicated a statistically significant effect of locality ($F_{(2,\ 116)} = 26.95$, $p < 0.001$), mating condition ($F_{(2,\ 116)} = 11.28$, $p = 0.001$), and the interaction between these two factors ($F_{(2,\ 116)} = 7.62$, $p < 0.001$). Significant differences in dorsal PC2 values were observed between Isla Colón and the other two localities studied and differences in mating condition were only significant among individuals of Sarapiquí where courted individuals exhibit lower values of PC2 (Figure 1b).

Analysis of the ventral measurements indicated a slight differentiation among localities with individuals from Sarapiquí attaining slightly higher values of the first two PC-derived spectral variables (accounting for 85.9% of the total variance) (SM Table-1, Figure 1c). MANOVA results indicated a statistical significant effect of locality (Wilkinson's $\lambda = 0.43$, $F_{(4,\ 230)} = 30.33$, $p < 0.001$) but no effect of mating condition, sex or any other interactions among predictors. Univariate analysis of ventral PC1 values (most correlated with brightness variables, see SM Table-1) showed statistically significant differences among the means of all three localities ($F_{(2, 116)} = 39.00$, $p < 0.001$) (Figure 1d). Mean ventral PC2 values (most correlated with color contrast, see SM Table-1) differed significantly among localities ($F_{(2, 116)} = 8.92$, $p < 0.001$) with individuals from Hitoy showing lower values than those from Sarapiquí (Figure 1d).

DISCUSSION

Despite several in-depth studies (Kapan, 2001), the mechanisms that facilitate and maintain intraspecific colour divergence among aposematic organisms remain an unresolved issue (Schaefer et al., 2002, Hegna et al., 2015). Several studies examining phenotypic variation in the strawberry poison frog have suggested that sexual selection promotes colour divergence (Summers et al., 1999, Maan and Cummings, 2009, Tazzyman and Iwasa, 2010, Richards-Zawacki et al., 2012), but the role of natural selection has also been discussed to drive divergence in this trait (Dreher et al., 2015, Maan and Cummings, 2012, Pröhl and Ostrowski, 2011).The most plausible scenario involves both natural selection (e.g. for initiation of colour divergence), and sexual selection acting at different stages of divergence, and affecting different populations to a different degree (Cummings and Crothers, 2013, Dreher and Pröhl, 2014). Although studies investigating the importance of selection for phenotypic divergence in our study species are numerous, the importance of within-population divergence has received far less attention from the scientific community (but see (Richards-Zawacki et al., 2012) than among-population divergence.

Our results demonstrate that in a Costa Rican population (Sarapiquí) frogs actively engaged in courtship have brighter dorsa, but lower values for colouration measurements. For the males, these results confirm laboratory based estimates of female preferences for brighter males across several Panamanian populations of *O. pumilio* (Maan and Cummings, 2009). These results also suggest that phenotype affects mating success and therefore reproductive success, with sexual selection as a possible driver for divergence in this trait. However, the fact that brightness measurements were significantly higher while colour indices were significantly smaller for courting males needs further attention. Additional studies will help to clarify whether there might be a trade-off between colour and brightness contrasts in poison-dart frog colouration. Interestingly, the absence of an effect of sex on any of these comparisons indicates that differences in colour and brightness also exist between females from Sarapiquí engaged in courtship and those not engaged in courtship.

With their higher parental investment during reproduction, females are suggested to be the choosing sex in strawberry poison frogs (Pröhl and Hödl, 1999). Dorsal colouration is discussed to be the best visual indicator for mate selection (Maan and Cummings, 2008, Tazzyman and Iwasa, 2010), and within some populations, e.g. on Isla Solarte, Panama, females possess a preference for brighter males (Dreher and Pröhl, 2014, Maan and Cummings, 2009). For the same population sexual dimorphism with males being brighter than females has been reported (Maan and Cummings, 2009). Males also pay attention to rival brightness in this population and aggressive behaviour of males is predicted by their own brightness (Crothers et al., 2011). Females might prefer brighter males because of their superior condition, competitive ability or higher breeding experience (Summers et al., 1997, Bitton et al., 2008, Murphy and Pham, 2012). Mechanisms that promote elevated brightness (within populations) are discussed to have the potential to cause shifts in hue as well and might have influenced divergence in colouration among different populations (Maan and Cummings, 2009). In this context sexual selection might be particularly relevant in populations where the variation in brightness is not detectable by the main predator (Crothers and Cummings, 2013).

Female preferences for brighter coloured males has been found in a diversity of invertebrates (butterflies: (Kemp et al., 2014, Kemp et al., 2008); mantis: (Barry et al., 2015)) as well as vertebrates (toads: (Vasquez and Pfennig, 2007); birds: (Loyau et al., 2007); but see (Beausoleil et al., 2012) for no such preferences in red-side dace). Unluckily most studies fail to report the benefit that females gain by mating with brighter coloured males. For the spade food toad (*Scaphiopus couchii*) females seem to use coloration brightness to identify larger and better condition males which also possess larger testes and might better fertilise the eggs of the female (Vasquez and Pfennig, 2007). In a beetle species, higher brightness is correlated with enhanced predator avoidance because brightness indicates better deterrence against predators (Bezzerides et al., 2007).

Many dendrobatid species use aposematic colouration to display their toxicity to potential predators. Across Panamanian populations of strawberry poison frogs in the Bocas del Toro archipelago, including various distinct colour morphs, brightness has been found to be an honest indicator of toxicity (Maan and Cummings, 2012). A recent study (Cummings and Crothers, 2013) suggests that low toxicity levels constrain populations to a cryptic colouration, while high toxicity levels provide protection against predators and therefore allows for colouration to be shaped by sexual selection. Here we show that strawberry poison frogs from the Costa Rican Sarapiquí population have higher dorsal reflectance than those from Hitoy Cerere and Isla Colón (Fig. 1 and Fig. S1) and that couples in the Sarapiquí population show higher colour brightness than single individuals. In the aposematic species *Dendrobates tinctorius* movement behaviour is related to the color pattern of individuals (Rojas et al., 2014). It is therefore possible that in *O. pumilio* brighter males and females, which could be more toxic, exhibit a different behaviour conferring higher mating success than that of duller individuals. Under this rationale, brighter males might be more active, faster, call more, defend better territories and therefore attract more females. In case several females arrive at these territories males might court most intensely the brightest female, while the other females leave his territory without entering courtship. One study found brightness of female birds to be correlated with offspring fitness (owlets with higher mass at fledging; (Aviles and Parejo, 2012) and similar correlations might exist in the Sarapiquí population. Whether an extremly bright and conspicuous coloration as observed in Sarapiquí and Solarte, can evolve might depend on resource availability. Based on some theoretical models Lee et al. (Lee et al., 2011) discuss the possibility of bright conspicuous coloration in combination with high toxicity to emerge in aposematic species when resources (toxins and pigments) are abundant in the habitat and when additionally benefits for bright coloration as such mate choice are present. Future studies testing the relationship between toxicity and colouration brightness across a broader range of strawberry poison frog populations would be an excellent test of this prediction.

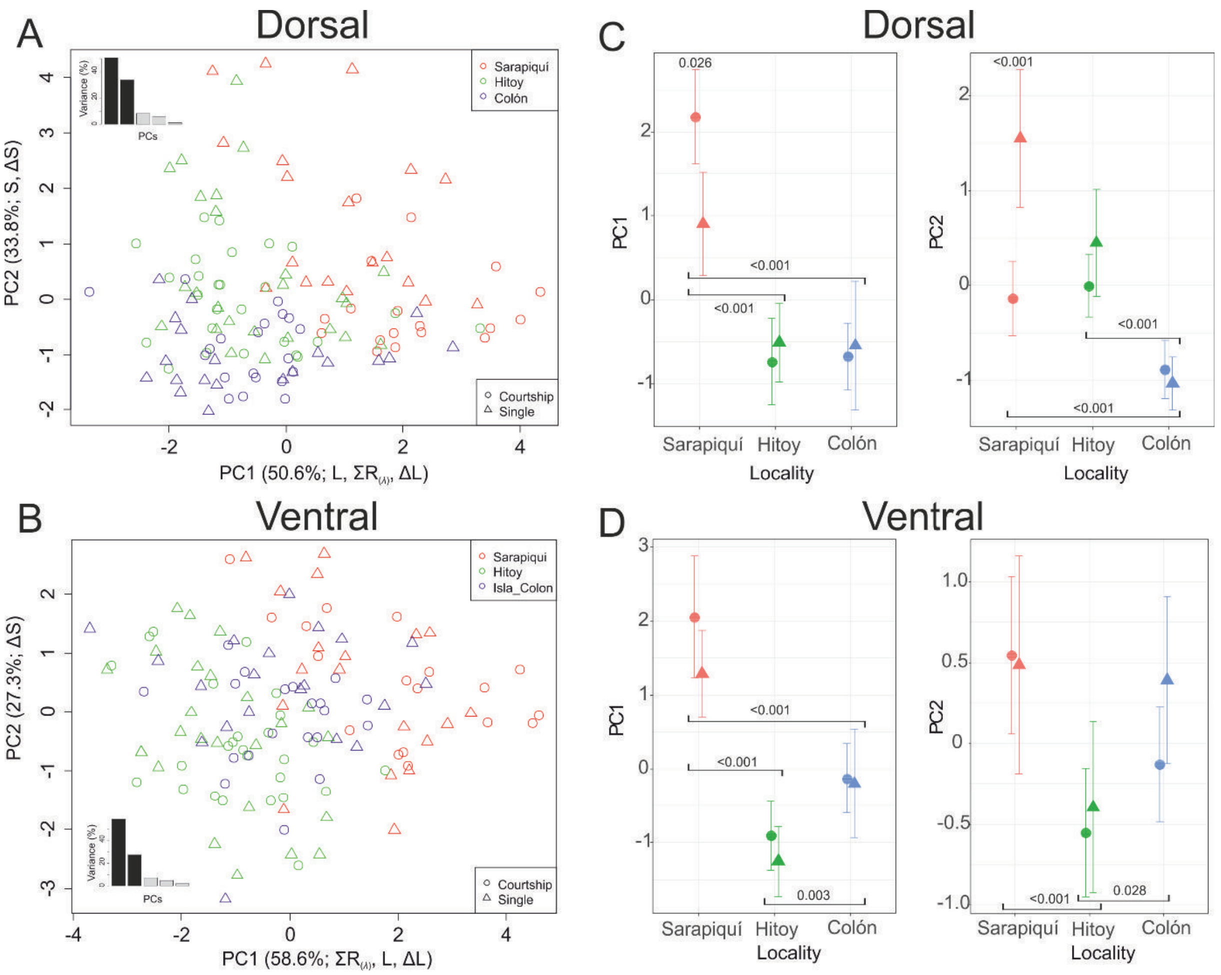

Figure 1. Variation in spectral variables of 128 individuals of *Oophaga pumilio* derived from PCA of dorsal and ventral spectral measurements. Plots on the right show the individual scores of the first two components extracted from five dorsal (A) and five ventral (B) spectral variables with symbols and colors identifying the corresponding locality and observed mating condition; the percent of explained variance and most contributing variables (Pearson's r > 0.7) are indicated on the axes. The inset shows the proportion of total variance explained by each component (black) that is displayed in the plot; see text and SMTable-1 for details on variable abbreviations and description of the principal components. Panels C and D illustrate the means (symbols) and 95% confidence intervals (whiskers) of dorsal (C) and ventral (D) PCs grouped by locality and mating condition. P-values of pairwise Tukey tests are indicated next to horizontal lines (locality comparisons) or on top of locality symbols (mating condition comparisons). Only significant effects in univariate analyses were tested with post-hoc comparisons, for graphic simplicity only significant results are shown (see SM Table for detailed results).

Some animal species are able to undertake rapid colour changes, which may be employed in intra- and interspecific communication, e.g. for predator deterrence, territorial fights and courtship behaviours (Hanlon, 2007, Stuart-Fox and Moussalli, 2008). Beside rapidly changing colour patterns, well-known for cephalopods and chameleons for example, some species temporarily exhibit certain colour patterns during courtship or breeding seasons. In Zebrafish (*Danio rerio*), both sexes change their striping pattern during spawning (Hutter et al., 2012). Body colouration of the male moor frogs (*Rana arvalis*) changes from dull brown to bright blue during the breeding season, resulting in a temporal sexual dichromatism, which probably facilitates sex discrimination in this explosive breeder (Sztatecsny et al., 2012). Whether strawberry poison frogs alter their brightness or colouration during courtship, which could be an alternative explanation for the observed differences between courting and single animals in Sarapiquí, cannot be detected with the method applied in this study and thus warrants further attention. Alternatively, skin brightness could be correlated with reproductive hormone levels. This could explain the observed differences in reproductive behaviour which is strongly dependant on hormonal levels in frogs just as well as in other vertebrates (Schmidt, 1966, Moore et al., 2005).

In this study, we measured the reflectance of courting couples, which were found in a late stage of courtship. Interruption of courtship during this stage is very rare, and probably due to external reasons (e.g. disturbance by other animals, pers. obs.). Once a female follows a calling male to the oviposition site, egg deposition is very likely, and reproductive success seems to be positively correlated with mating frequency under most circumstances (Pröhl, 2005). Our study is the first to provide evidence from the field that brighter individuals might have greater reproductive success than duller ones in some populations of *O. pumilio*.

Our findings from the population in Sarapiquí, together with the sexual dimorphism and preference for brighter males in the population of Solarte (Maan and Cummings, 2009), suggest that sexual selection affects brightness only in some, but not all populations of strawberry poison frogs. Furthermore our results demonstrate that sexual selection is not restricted to Bocas del Toro populations (Southern genetic group), where most colour divergence among populations is located, but also in Costa Rica (Northern genetic group), where all populations are red on the dorsum (Hagemann and Pröhl, 2007). With colour being affected by natural selection (predator avoidance) and sexual selection (intra- and intersexual selection), and females having differential preferences for local colouration and brighter males (Maan and Cummings, 2008, Maan and Cummings, 2009, Reynolds and Fitzpatrick, 2007, Summers et al., 1999), colouration of strawberry poison frogs might thus be a classic magic trait (Noonan and Comeault, 2009, Rudh et al., 2011, Servedio et al., 2011).

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AUTHOR CONTRIBUTIONS

C.E.D. and H.P. designed the research. C.E.D. performed field work, C.E.D., A.R. and M.E.C. analysed the data. C.E.D., H.P and A.R. wrote the paper. All authors revised the manuscript and approved the final version.

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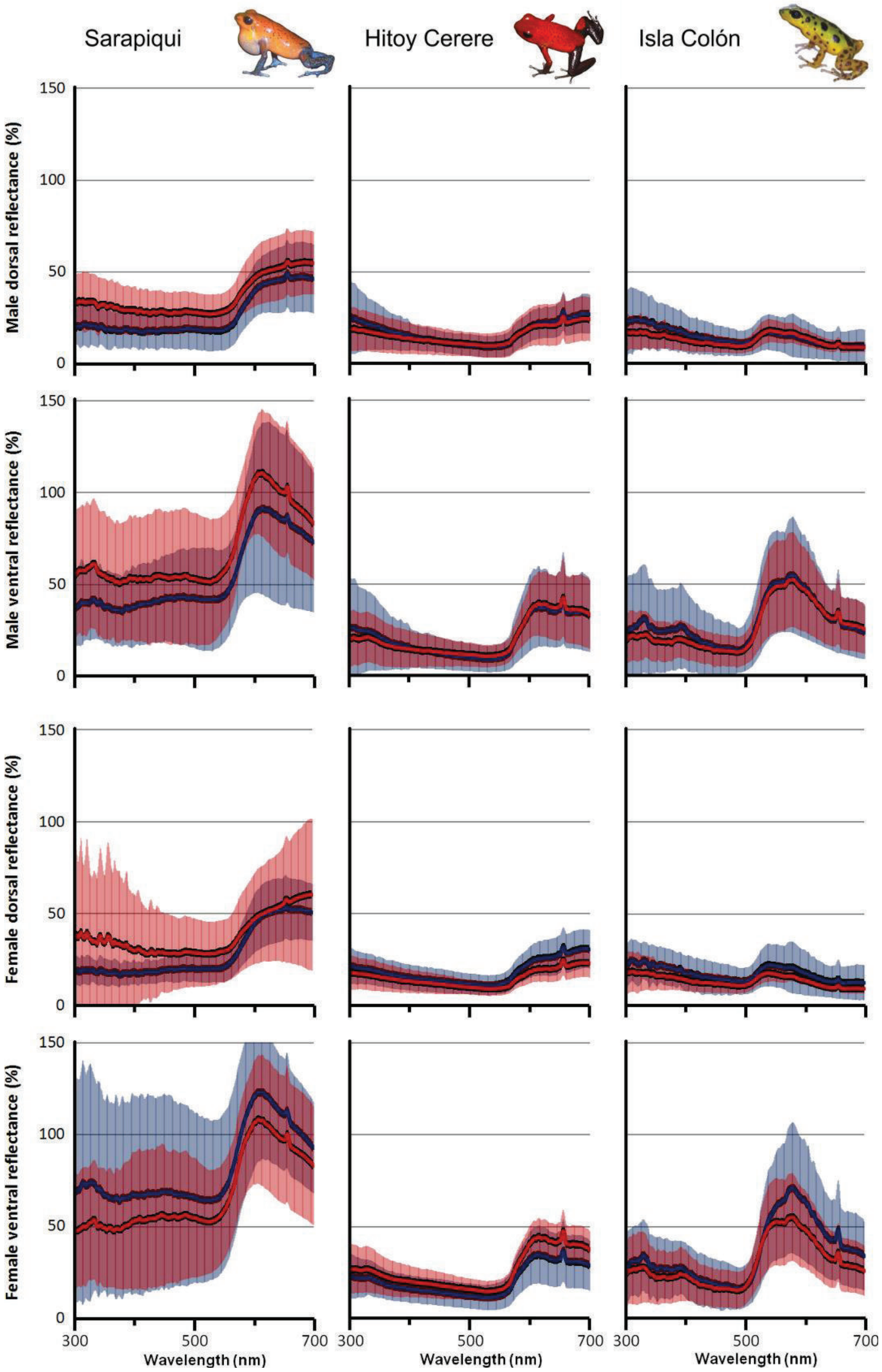
4.1 Supplementary material



Supplementary Material 1: Mean reflectance spectra (in % reflection relative to white standard) including standard deviation of mated (red line) and unmated (blue line) individuals of *Oophaga pumilio* from three study populations. Male and female, as well as ventral and dorsal reflectances are shown in separate graphs. Mean reflectance curves average spectra of 9 to 13 individuals (Sarapiqui males mated (dorsal and ventral): n = 9; for all Isla Colón spectra: n = 13, for all other spectra: n = 10). Reflectances over 100% emerge due to the brilliant surface of the frogs´ skin, while the used white standard has a dull surface. In order to verify accuracy of reflectance curves, all spectra were visually controlled for oversaturation.

5 Discussion

The influence of both natural and sexual selection on the evolution of color polytypism in the strawberry poison frog is likely and has been broadly investigated (Brown et al., 2010). However, questions remain about the mechanisms by which natural selection facilitated color divergence in *O. pumilio* (stated in (Richards-Zawacki et al., 2013)). As mentioned earlier, it is very likely that environmental conditions generally differ among geographically isolated populations (Hegna et al., 2012, Brown et al., 2010, Ruxton et al., 2004) and consequently, the direction and strength of natural selection might differ along the distribution range of a species. Furthermore, natural and sexual selection operate at the same time and might favor different outcomes of signal evolution. Unsurprisingly, the impact and direction of sexual selection might also differ among populations.

Given that signal evolution is clearly affected by several evolutionary forces simultaneously, investigations of the importance of a single evolutionary force for trait divergence may give interesting insights, yet occasionally yield equivocal conclusions. Here, I present a project providing evidence that as well interspecific as intraspecific forces may affect signal divergence in a polytypic frog species. Both sexual and natural selection pressure vary considerably among geographically separated populations which supports the hypothesis that these evolutionary forces concurrently influence color divergence in this species.

5.1 The effect of predator selection on the evolution of aposematic coloration

In this part of the project I combined two methods aiming to verify how predation pressure as a mean of natural selection might have affected phenotypic trait divergence in the model species. First, I measured the level of predation risk in relation to coloration and second, I calculated visual models in order to evaluate how detectability of certain colors of living (or clay model) frogs might affect predation risk. Combining these two approaches I addressed both stages of a predation event, detection of prey (via visual modeling) and the decision made by the predator of whether to attack or not (evaluated through clay model experiments).

Visual modeling for three putative predators (avian, snake and crab) and a conspecific observer demonstrated that the values calculated for color and brightness contrasts were similar for the tetrachromatic avian compared to the trichromatic conspecific visual model. Furthermore, values for dichromatic crabs and trichromatic snake observers were alike. Generally, the concept of aposematism is described as the demonstration of unprofitability of prey (Guilford, 1990) through conspicuous signals such as color and/or brightness contrast (Ruxton et al., 2004). Prudic demonstrated with dichromatic Mantis predators that brightness contrast alone may suffice as an aposematic signal (Prudic et al., 2007). In *O. pumilio*, a trade-off between brightness and color contrast might exist (Crothers and Cummings, 2013) and certain extreme values for a single conspicuousness measurement might be masked by the calculation of an overall conspicuousness measurement only. Therefore, I consider the discussion of brightness and color contrast separately as worthwhile, instead of focusing on the results of the overall conspicuousness measurement.

Strawberry poison frogs from different populations vary in their coloration and accordingly, in their conspicuousness. Visual models showed that color contrast, brightness contrast and also overall conspicuousness differ strongly among populations. Interestingly, even the conspicuousness of red populations, i.e. Sarapiquí, Hitoy Cerere and Isla Solarte, strongly deviated from each other. Conspicuousness can be generated through different means, based on differences in brightness or hue (Cummings et al., 2008, Prudic et al., 2007, Cummings, 2007). Red frogs from Isla Solarte and Sarapiquí seem to follow different strategies to enhance their dorsal conspicuousness: the highest values for color contrast were calculated for frogs from Isla Solarte, while frogs from Sarapiquí yielded the highest brightness contrasts for all observers, respectively. Interestingly, frogs from Hitoy Cerere are relatively inconspicuous with low color and brightness contrasts though belonging to the monomorphic red Northern genetic group as the Sarapiquí population. In their study about sexual dichromatism in the Solarte population, Crothers and Cummings suggest that a trade-off exists between color and brightness contrast, which constrains the evolution of these features (Crothers and Cummings, 2013). Solarte represents the most conspicuous and most toxic population in this study. Additionally, frogs from this population show bold behavior which might furthermore enhance their detectability and strengthen their aposematic anti-predator strategy, as proposed by Pröhl and Ostrowski (Pröhl and Ostrowski, 2011). Usually, brightness and color information are used for different tasks. Concerning predation risk, brightness contrast is described to play a pronounced role in detection of objects at larger distances (Osorio et al., 1999, Ruxton et al., 2004, Crothers and Cummings, 2013) and might hypothetically also increase male conspicuousness for females approaching from a distance. Coloration on the other hand might be taken into account during close-range assessment of objects by predators (or mates). However these general assumptions might not be valid for all predator–prey combinations and clearly depend on the visual capacities of the respective predators. It is worthwhile to keep in mind that different features of conspicuousness might vary in their relevance depending on the ecological context (e.g. for predator deterrence or during mate acquisition) (Cummings et al., 2008).

Crothers and Cummings (Crothers and Cummings, 2013) applied visual modeling to evaluate whether sexual dimorphism in the Solarte population might be the result of predation pressure. They demonstrated that birds, which are thought to be major predators of *O. pumilio* (Hegna et al., 2011, Saporito et al., 2007a, Saporito et al., 2007c, Maan and Cummings, 2012), are hardly able to assess brightness differences among males within this population, while on the other hand crab, snake and conspecifics are able to discriminate within-population color differences. Based on this finding, they challenge the notion that predator selection might affect color evolution in this population (Crothers and Cummings, 2013). In my project I investigated how detectability for certain observers differs in relation to color differences among populations, instead of the analysis of differences among individuals within a population. The results showed that the relative conspicuousness - and therefore the ability to discriminate among colors of frogs from different populations - is very similar for an avian observer compared to a conspecific one (see Fig. 1, (Dreher et al., 2015)).

Depending on their sensory capacities, different elements of a warning signal may be relevant to different predators. For example, some predators may have inherent avoidance of conspicuous aposematic coloration, while others may pay attention to internal contrasting patterns (Bowdish and Bultman, 1993, Aronsson and Gamberale-Stille, 2012). In experiments with the firebug (*Pyrrhocoris apterus*), the white admiral butterfly (*Limenitis camilla*) and *O. pumilio* coloration in itself seems to be a better predictor of predation risk than internal contrast between red and dark patterns (Exnerova et al., 2006, Hegna et al., 2011, Stobbe and Schaefer, 2008). Gamberale-Stille and Guilford (Gamberale-Stille and Guilford, 2003) tested, whether chicks responded to coloration or internal contrast to discriminate between palatable and unpalatable food sources, and did not find evidence for the use of contrast in these tasks. Hence, they concluded that birds might generally use color cues and neglect information of contrast in avoidance learning. This conclusion was corroborated by further experiments demonstrating that chicks in control experiments did not avoid a pattern (black spots or stripes) they had been trained to connect to unpalatable food items, but rather responded to the color content of the learned aversion (Aronsson and Gamberale-Stille, 2008).

In their study, Hegna and his colleagues detected no effect of a dark spotting pattern on predation probability in *O. pumilio* (Hegna et al., 2011) which suggests that black contrasting patterns may not be of major importance for predation risk in this species. Therefore, in my experiments neither a dark spotting pattern was painted on the clay model frogs nor the black spotting pattern was included in the visual models. However, Hegna et al. investigated the effect of a spotting pattern consisting of small black spots as typically found in strawberry frog populations of the Northern genetic group to evaluate the effect of spotting pattern on predation risk (Hegna et al., 2011). The anti-predator effect of a spotting pattern with big dark spots, as present in some populations of the Southern genetic group (e.g. in my study populations Río Gloria and Isla Colón), possibly differs from a pattern with small spots in its importance and effectiveness for predator deterrence. A recent study of Preißler and Pröhl discovered that in one of my study populations (Hitoy), the overall risk of an attack covaries with the size of a contrasting pattern (Preißler and Pröhl, 2017), which supports the hypothesis that black internal body patterns of some populations of *O. pumilio* might increase avoidance learning and/or recognition of toxic prey and might thus play a role in aposematic signaling.

Using clay model frogs of four different colors I measured the intensity of predation pressure and assessed how the probability of an attack relates to the local vs. nonlocal coloration of frogs in the respective population. I furthermore addressed the question how the composition of predator communities differs among populations. As expected, predation pressure strongly varies among populations. Contrary to the general expectation of predation pressure to be lower on islands compared to the mainland (Meiri et al., 2005, Williamson, 1981), I measured higher predation rates at the two island populations compared to the four mainland populations. Hegna and colleagues conducted a similar study using clay model experiments to measure predation pressure at the Bocas del Toro Archipelago (Hegna et al., 2012). They compared predation rates present at the island of Colón to those measured at one population on the Costa Rican mainland. Based on the results, they suggested that low predation pressure at island populations of *O. pumilio* might have facilitated the evolution of color polytypism in the area of the Bocas del Toro Archipelago (Hegna et al., 2012). In my project, I evaluated predation pressure in six populations, including two island and two mainland populations of the Bocas del Toro Archipelago and two mainland populations of Costa Rica. Unlike Hegnas investigation, my results provide evidence for a higher overall predation risk on the islands compared to mainland populations and therefore oppose the hypothesis that low predation pressure facilitates color divergence at the Archipelago.

In the present study, the majority of attacks on clay models were assigned to bird predators, supporting the idea of birds to represent the major predator taxa for dendrobatid frogs (Hegna et al., 2011, Maan and Cummings, 2012, Stuart et al., 2012, Saporito et al., 2007c). *Oophaga granulifera* is a sister species of *O. pumilio* and inhabits the pacific slope of southern Costa Rica and northern Panama (Wang, 2011). Along their geographic distribution, populations of *O. granulifera* have diverged in coloration including green and red populations as well as intermediate populations (Brusa et al., 2013). Interestingly, in *O. granulifera* toxicity is inversely related to conspicuousness, with cryptic green populations being more toxic than conspicuous red ones (Wang, 2011). Similar to my study, Willink and her colleagues linked the results of visual modeling to those of clay model frog experiments in their investigation, but additionally monitored the clay model frogs with cameras to identify the predators causing damages on clay model frogs. They discovered that predation pressure in the study species was exerted by three predator taxa: birds, crabs and lizards. Here, attack probability followed a specific pattern, with birds consistently avoiding the attack of models which resemble the local color morph, while lizards mostly preyed upon red model frogs in all populations (Willink et al., 2014). My here presented study of predation pressure in *O. pumilio* did not provide such a distinct pattern. Predation events caused by predator taxa other than birds were rare and avian attacks did not follow a specific pattern regarding the local coloration of the frogs, i.e. clay models of local coloration were neither better nor worse protected than nonlocal colored ones in all populations. This result is interesting, as in previous studies in *O. pumilio* and for several other dendrobatid frog species, probability of an attack frequently varied as a function of the origin of the presented color morphs. In the study of Hegna et al. on *O. pumilio*, the probability of an attack at the island population of Colón was higher on local color morphs than on red aposematic or brown cryptic controls (Hegna et al., 2012). However, at the neighbor island of Bastimentos two aposematic colors, red and yellow, were highly protected, both in a polymorphic population where frogs of both color occur and in a monomorphic red population (Richards-Zawacki et al., 2013). *Ranitomeya imitator* and *Dendrobates tinctorius* also show geographical variation in coloration, and predator selection acts in a purifying way against novel phenotypes in both species (Chouteau and Angers, 2012, Noonan and Comeault, 2009).

Although the independence of attack risk and origin of the model frog coloration in my study was unexpected, there are several possible explanations for this result. First, despite the recent formation of the Bocas del Toro Archipelago in the last 10,000 years predator communities might have changed since the initiation of color divergence among populations of *O. pumilio* (Richards-Zawacki et al., 2013), and the selective advantage of certain colors during the past might no longer be present. Second, further aspects of an anti-predator strategy, which might affect probability of an attack on live frogs, e.g. behavior, activity patterns, smell, toxicity and internal body patterns, were neglected in my study as in previous research. Last, but maybe most importantly, color divergence might not be shaped exclusively by predation pressure, but be affected by sexual selection and stochastic factors at the same time. Different evolutionary forces might favor different expressions of a trait, as shown in the wood tiger moth (Nokelainen et al., 2012), and the impact of one force might be overriden by another one. Interactions among these forces might complicate a correct interpretation of results in studies investigating evolutionary drivers of trait divergence.

Although predation rate on specific colors was independent of the local coloration, it differed among the four colors of clay model frogs which were presented. While red clay models suffered intermediate predation risk, blue frog models were significantly more prone to be attacked by avian predators than green and yellow ones. Visual modeling showed that yellow frogs possess high conspicuousness to the avian visual system, which might lead to low predation rates through avoidance of aposematic prey. Green model frogs on the other hand, might be protected against bird attacks through low conspicuousness to avian predators. Recalling that a predation event comprises two elements, namely detection of prey items and the decision of the predator on whether to carry out an attack, yellow and green frogs might be protected against avian attacks through differential mechanisms. Attacks on green frogs (with low conspicuousness to the avian visual system) might be low based on difficulties in detection of prey items. Contrary, yellow clay frogs might be spotted easily, but based on the aposematic signal content of coloration, an attack might be impeded during the second stage of the predation event.

Imprints in clay models revealed that birds are very likely to be the main predators of *O. pumilio*, as damages in clay models were mainly assorted to the categories of bird marks (with u- and v-shaped imprints) or potential bird marks (with holes and scratches potentially caused by birds through manipulation of the models). Predation events assorted to other categories were scarce and therefore summarized to the category of non-bird predation (see Fig. 2, (Dreher et al., 2015)). Therefore, the results did not allow for an analysis of differences in predator communities among populations. Calculated conspicuousness values for an avian observer were very similar to those for a conspecific *O. pumilio* observer. Hence, I conclude that the evolution of bright coloration, which is favored by sexual selection, might cause easy detection by birds as the major predator class. As coloration in several populations in this species is selected as part of an aposematic defense strategy and serves as an honest indicator of toxicity (Maan and Cummings, 2012), easy detection at larger distances might enhance the aposematic signal function and favor predator avoidance learning (Endler and Mappes, 2004). Interestingly, predation pressure exerted by birds as the main predator covaried with the overall conspicuousness of local frogs. Hence, contrary to the hypothesis that highly conspicuous as well as very cryptic color morphs might have evolved under high predation pressure, general probability of an attack was low in cryptic populations of strawberry poison frogs (see Fig. 4, (Dreher et al., 2015)). To complement this study I furthermore interpreted differences in predation risk among color morphs with respect to the conspicuousness of clay colors to the visual system of different predators. In agreement with the traditional hypothesis (Hegna et al., 2012, Maan and Cummings, 2012), I conclude from the results of this study that divergence in coloration among different populations of strawberry poison frogs may potentially be affected by predation pressure.

5.2 Multiple sexual signals: calls over colors for mate attraction

As described earlier, sexual signals are important means for intersexual communication. Prezygotic isolation based on strong preferences for local signals might play a pivotal role during speciation. Here, I focused on call and color diversity within one dendrobatid species to evaluate the importance of sexual signals of different sensory channels for mate selection and thus for reproductive isolation among phenotypically diverged populations. The present study incorporates several understudied aspects of prezygotic isolation between diverging populations and investigates the importance and interaction of multiple sexual signals under field conditions in several populations. I tested preference functions of female frogs using a combination of local and nonlocal advertisement calls and color patterns in multiple populations in two genetic lineages and across the species distribution of *O. pumilio*. By observing courting frog couples until courtship behavior was undoubtedly identified, I assured that females were receptive (an issue neglected in other studies) and their choices were related to mate selection preferences. In total I conducted five types of experiments and tested 452 females in their natural home ranges, which span around ~ 20 – 100 m² of tropical primary or secondary forest (Pröhl and Berke, 2001, Meuche et al., 2011, Meuche and Pröhl, 2011).

The experiments yielded several interesting results: First, contrary to my expectation, presentation of model frogs did not affect female choice in a significant way, neither were preferences for certain calls reinforced through presentation of matching models, nor were they modified by a combination with non-matching models. Nevertheless, the results of one experiment (experiment V) indicate that instead of the expected preference for the local model, there are tendencies to select the brighter models, which resemble the local yellow frog coloration in Río Gloria, but the nonlocal red frog model in Tierra Oscura. Second, regarding advertisement calls, female strawberry poison frogs from the Southern genetic group discriminated strongly against the Northern genetic group call, but not against the group call of their own genetic group (experiment I and II, as well as III and IV). Females from the Northern genetic group on the other hand were indifferent about presented calls with one exception. Females from Hitoy Cerere preferred the local call over the nonlocal group call (experiment II), but only when presented without models (please compare to experiment III and IV). These results are surprising because of two reasons: First, compared to the Southern group call the Northern group call has a lower dominant frequency, which is supposed to be attractive to anuran amphibians, as it indicates a large body size of males (Ryan, 1980). Second, while color variety is located in the Southern genetic group, advertisement calls of populations show little divergence in this area. Conversely, in the monomorphic Northern genetic group call variation among populations is high (Pröhl et al., 2007), wherefore I expected females from this populations to strongly discriminate against nonlocal calls. On the other hand, divergence in advertisement calls among populations of the Northern genetic lineage might have ultimately been facilitated by females being unselective regarding acoustic sexual signals and allow for divergence in this trait. To summarize, the results demonstrate that Panamanian frogs from the Southern genetic group show pronounced preferences for local calls, while Costa Rican females even accept the advertisement call sequence from the South with a low dominant-frequency. This result coincides with the study of Wang and Summers, who analyzed microsatellite loci and discovered that gene flow among Costa Rican populations is high, while gene flow among populations of the Southern genetic group is negligible (Wang and Summers, 2010). Prezygotic isolation in strawberry poison frogs based on differences in advertisement calls is therefore most likely to play a role for signal divergence in areas, where female frogs from the Southern genetic group with strong preferences for local calls encounter males with Northern call attributes, i.e. in populations along the borderline between the two genetic groups. Still, gene flow has been found between the two genetic groups (Hauswaldt et al., 2011), i.e. prezygotic isolation is not complete and parapatric mechanisms might be involved in trait divergence of *O. pumilio*.

The results of bimodal playback-experiment to test females for their preference for local versus nonlocal male calls and colors demonstrated that unexpectedly, mate selection in strawberry poison frogs is not affected by these signals in a multiplicative way. Rather, advertisement calls override coloration in its importance for mate selection. Under natural mate choice situations, females are attracted by males over longer distances. Advertisement calls of male strawberry poison frogs transmit several meters across their habitat. Hence, receptive females searching for mates most likely perceive advertisement calls of several males at the same time. However, sight of calling males might be handicapped by obstacles in the habitat, such as plants, trunks, branches or stones. Therefore, as expected, advertisement calls were sufficient to induce a positive phonotaxis of receptive females during playback experiments. I suggest that strawberry poison frogs might use acoustic and visual cues in a sequential order with advertisement calls attracting potential mates and facilitating selection at a distance. Visual cues might be important as a close-range cue at a later stage of courtship, hence, potential preferences might not have been detected during my experiments. None the less the results point to a higher importance of acoustic cues for mate choice in strawberry poison frogs, because a failure in long-distance attraction will impede the stage of close-range assessment.

Pairwise comparisons show that sexual preferences of female strawberry poison frogs do vary not only between genetic groups, but also among populations, and are dependent on the alternative choice provided. These results are consistent with the results of other studies on sexual communication (Maan and Cummings, 2008, Ryan et al., 2003). The strength of sexual preferences is variable among populations of *O. pumilio*, which highlights the importance to consider an evaluation of multiple wild populations in studies of behavioral isolation. The results proved that strong sexual selection on advertisement calls of males is present in the Southern genetic group, while sexual selection on coloration was far less pronounced. The experiments did not reveal a preference of females to choose frog models of their local coloration. Females did not show preferences indicating a rejection of calls from nonlocal populations within their local genetic group neither in the Northern genetic group, with rather non-discriminating females, nor in the Southern genetic group, with females being highly selective against the Northern group call. This might indicate that prezygotic isolation among populations within a genetic group might not be complete. If mate choice was based on the more prominent cue of acoustic signals solely, secondary contact among populations (within a group) would be followed by intense gene flow.

5.3 Are brighter animals more sexy? – Mate attraction in strawberry poison frogs

In the last part of the presented project I compared the brightness and coloration of strawberry poison frogs that were engaged in courtship to those not engaged in courtship. Using visual models for a conspecific *O. pumilio* observer, I evaluated, whether relatively bright animals actually have a reproductive advantage over duller individuals of the same population. Female preferences for brighter males has been found in 3 of 4 studied populations of strawberry poison frogs during laboratory experiments (Maan and Cummings, 2009), however, evidence based on field data was not available prior to this study.

As described earlier, sexual selection is thought to play a substantial role for intraspecific color divergence in the study species (Maan and Cummings, 2009, Richards-Zawacki et al., 2012). Coloration and brightness seem to be important for both intersexual interactions (during mate selection) and for intrasexual interactions during male-male competition (Crothers et al., 2011, Crothers and Cummings, 2015, Crothers et al., 2016, Galeano and Harms, 2016). The existence of sexual dimorphism in a certain trait, as described for brightness in the Isla Solarte Population (Maan and Cummings, 2009), is no evidence for the impact of sexual selection on this trait (Butlin et al., 2012). Yet, in the same population, females did show a preference for brighter males under laboratory conditions, and aggressiveness of males was predicted by their own and the rivals´ brightness (Crothers et al., 2011, Crothers and Cummings, 2015). Taken together, the impact of sexual selection on divergence in brightness in the population of Isla Solarte is highly probable. According to Crothers et al. (Crothers and Cummings, 2013, Maan and Cummings, 2009), brightness and coloration are interdependent, hence, sexual selection causing directional selection toward elevated brightness might facilitate changes in coloration at the same time.

Toxicity in strawberry poison frogs varies strongly among populations and covaries positively with conspicuousness, i.e. conspicuousness indicates toxicity of the frogs (Maan and

Cummings, 2012). One interesting study of Cummings and Crothers (Cummings and Crothers, 2013) integrated information of studies of sexual selection and natural selection. They argue that populations with high toxicity levels are sufficiently protected from predation, i.e. natural selection pressure is low, which allows coloration to be shaped by sexual selection. On the other hand, crypsis as an anti-predator strategy might be favored by natural selection in populations with relatively low toxicity levels. The measurements presented in my project demonstrated that dorsal reflectance of strawberry poison frogs from Sarapiquí is high compared to Hitoy and Colón and that a detectable difference between mated and unmated animals was present exclusively in the Sarapiquí population. Here, mated strawberry poison frogs possessed significantly higher brightness compared to unmated animals and color contrasts were significantly lower for mated compared to unmated animals. According to the hypothesis of Crothers and Cummings (Crothers and Cummings, 2013) and due to low attack rates measured in Sarapiquí (Dreher et al., 2015), I suggest that natural selection pressure in Sarapiquí may be sufficiently low to allow the evolution of coloration and brightness in this population to be driven by sexual selection. Another possible explanation for low predation rates in Sarapiquí is that natural selection by predators has favored very conspicuous warning signals and predator avoidance learning in this population. Although toxicity of the Sarapiquí population has never been assessed, one geographically close population (La Selva), which might be similarly toxic, appeared to contain relatively low toxicity levels (Mina et al., 2015), which is contrary to my expectation. In agreement with the expectation, cryptic strawberry poison frogs from Colón (Pröhl and Ostrowski, 2011) possess very low toxicity levels (Maan and Cummings, 2012) and mated and unmated animals did not differ for any measurement of conspicuousness.

Several animal taxa use changes in coloration as a mean for intersexual communication during courtship or breeding seasons (Sztatecsny et al., 2012, Hutter et al., 2012, Hanlon, 2007, Stuart-Fox and Moussalli, 2008). Chameleons and cephalopods are an impressive example for instant changes in color and pattern displaying their mood and intentions (Hanlon, 2007, Stuart-Fox and Moussalli, 2008) and male moor frogs change their coloration for several days during breeding season (Sztatecsny et al., 2012). I cannot completely exclude the possibility that measured differences between mated and unmated animals might be caused by a temporary variance in coloration during courtship. However, long-term changes lasting for more than the actual courtship are not probable, as male strawberry poison frogs can't anticipate whether a receptive female will mate with them at the respective day. Short-term changes on the other hand, will probably have disappeared at the moment of measuring, because spectrometric measurements were taken several hours after collection of frogs in their habitat. Therefore, I consider the measured differences between mated and unmated frogs to be based on actual differences in coloration of the animals.

This is the first study to analyze color and brightness of naturally occurring couples, with mates selected under undisturbed conditions. The results provide evidence that brightness and coloration of strawberry poison frogs does predict reproductive success in at least some populations, which demonstrates that sexual selection acts of the evolution of these traits.

6 Conclusions

The investigation of the effect of natural selection on phenotypic divergence in *O. pumilio* yielded several interesting results. The experiments provided evidence that predation pressure varies strongly among populations and that the origin of frog models – local or nonlocal – did not predict attack probability. Furthermore, birds were confirmed to be main predators across populations, and avian attack rates covaried with overall conspicuousness and brightness contrast of local frogs. Hence, in populations where predation pressure exerted by birds is high, local frogs exhibited high brightness contrast and overall conspicuousness, which might suggest the involvement of natural selection in the evolution of color divergence among populations of *O. pumilio*.

The results of mate choice experiments clearly demonstrated that multiple signal modalities – including aposematic coloration and acoustic mating signals - differ in their importance for sexual selection. Advertisement calls clearly outpaced visual signals during mate selection and females from the southern populations strongly preferred local acoustic signals over nonlocal ones. This result implies a role of mate choice for acoustic divergence between the two genetic groups. Coloration on the other hand seems to be of minor importance, and only two of six populations showed a tendency to select the brighter - but not necessarily the local - color morph, which does provide marginal support for the hypothesis of sexual selection to affect divergence in visual signals. Preferences strongly varied among populations and between the genetic groups, which emphasizes the importance to survey an array of populations in studies of behavioral isolation.

The mechanisms which facilitate and maintain color divergence among populations in aposematic organisms are a popular research topic, however, divergence in coloration within populations has received much less attention. In one of three study populations, frogs which were found engaged in courtship, were significantly brighter than unmated animals. This indicates that within-population variation in coloration affects reproductive success in this population and thus provides evidence of directional sexual selection towards brighter mates. The mechanisms involved in this process might facilitate shifts in hue as well and thus be involved in the intense diversification of coloration among population in this species.

I conclude that the rapid phenotypic divergence among populations of the strawberry poison frog has been affected by both natural selection and sexual selection on several signals of different sensory modalities, including both visual and acoustic properties. Possibly, the importance and/or direction of these evolutionary forces might have changed since the initiation of divergence. I suggest that the effect of both factors likely varies among populations due to local ecological differences (e.g. in predator communities or availability of toxic food items), for which reason the results of all parts of this study differed among geographically separated populations. Even though the outcome of this project clearly indicates that both natural and sexual selection affect and interact during ongoing phenotypic divergence, it also lights the way for further research aiming to integrate studies on different evolutionary drivers of divergence.

7 Limitations of the project

The presented study investigated the relevance of multimodal signals for prezygotic behavioral isolation and the importance of predator selection based on coloration in different populations. This study advances our understanding of the evolution of phenotypic diversity among populations of a species. Nevertheless, there are some limitations in the project which remain to be mentioned.

In the multimodal mate choice experiments I used models of strawberry poison frogs as an alternative to living specimens. The model frogs were manufactured to resemble living frogs as much as possible. However, the models were stationary during experiments, and I cannot exclude that movement, including vocal sac inflation, might be a sexual signal female strawberry poison frogs employ during mate selection. Vocal sac inflation has been found to be elementary for the induction of aggressive behavior in another dendrobatid frog species (Narins et al., 2003), and may also serve as an important sexual signal to attract females. The use of living specimens would have entailed other restrictions, including a potential influence of male frog behavior on female choice, as well as possible interferences of broadcasted calls with emitted calls by object males. Therefore, I decided to use non-living model frogs.

In addition, the results indicated that coloration might be applied as a short-distance cue during mate choice. Hence, the effect of frog coloration might have been underestimated applying the described methodology, as the experiment ended as soon as the female had approached one loudspeaker to less than 20 cm distance. The applied method failed to detect potential rejection of certain color morphs during close-range interactions.

Furthermore, male acoustic signals were broadcasted via loudspeakers during playback-experiments. The used sound system (Apple ipod®, amplifier, loudspeaker) was controlled for unaltered reproduction of the sound frequency band the frogs are believed to be sensitive to. However, no detailed knowledge about audiometric sensibilities of the auditory system of *O. pumilio* is available, therefore, I cannot exclude that broadcasted calls might have sounded somewhat unnatural to female frogs.

The identification of the actual predators of a species is essential for the evaluation of the importance of natural selection for signal divergence (Endler and Mappes, 2004, Mappes et al., 2005). However, as Maan and Cummings stated ”*documenting predation in the wild is notoriously difficult, even more so for aposematic prey with extremely low attack rates*” (Maan and Cummings, 2012). Despite this difficulty, I evaluated predation pressure in the polytypic aposematic frog species *O. pumilio* and interpreted attack probabilities under consideration of the conspicuousness of different prey items to birds as the major predator class of birds. There are several restrictions to this analysis. First, I do not have information about the entire predator community of *O. pumilio*, as only anecdotal observations of predation events on *O. pumilio* are available and with the experimental setup used in my project I did not detect substantial predation by further predator classes but birds. Second, the actual attack probability on living frogs might deviate from the one on clay model frogs, because beside coloration, predators might consider further cues, such as gloss, movement and odor. Third, while interpreting the results of attack probability on different prey colors with respect to the local coloration of toxic frogs, I did not take into account whether local predators are experienced with further certainly colored toxic prey species. Fourth, the visual models calculated are based on the microspectrophotometric analyses of few individual animals for each visual model. The crab model uses the visual sensibility of the European crab, and the conspecific *O.pumilio* visual model is based on one individual frog. Clearly, visual models based on the average visual properties of several animals might lead to more reliable estimations. Finally, no data outrules the possibility that differently colored frog populations might not only differ in the pigments coloring their skin, but may also deviate in the composition and quantity of light-absorbing pigments in their retinae.

8 Future Projects

The presented project gives interesting insights about the impact of natural and sexual selection on phenotypic divergence in neotropical frogs. Likewise, it offers prospects for future work.

For a more significant interpretation of the importance of predator selection on color divergence in *O. pumilio*, we need to know about the actual predators of these frogs, including a comparison about geographic differences concerning this issue. Furthermore, we should pay attention to seasonal differences in predation pressure (Mappes et al., 2005). However less pronounced in the tropics, predation might differ seasonally, e.g. through bird migration or caused by shifts in predator diet through changes of availability of alternative food sources like fruit. I suggest to conduct long-term experiments using video traps in several populations along the distribution range. Using actual predators, the importance of additional features like spotting pattern, olfactory cues and movement on attack probability should be evaluated through laboratory and field-base experiments.

An important complement to studying predation pressure would be an evaluation of the availability of toxic prey items of frogs among populations. Toxicity levels need to be estimated on a broader base and compared among populations and different species of sympatric frogs or sister species (e.g. *O. granulifera*). Connecting the results of these approaches can allow to disentangle the interplay between conspicuousness, toxicity and behavior and give evidence about the importance of predation pressure as a mean of natural selection on phenotypic divergence in polytypic frog species.

The presented study of sexual selection concentrates on the relative importance of visual versus bioacoustics signals. These multimodal experiments including calls and colors shall also be conducted in contact zones between the two genetic groups of *O. pumilio* and might give interesting insight on the degree of prezygotic isolation between diverging lineages. Furthermore, the same type of experiments can be used to evaluate sexual preferences in contact areas between different color morphs.

Due to the higher importance of calls over colors as indicated by the results of my thesis, I recommend to adjust the methodology of multimodal experiments and use moving frogs during the experiments. In particular, movement of vocal sacs might be important for female attraction in strawberry poison frogs. Movement of males and vocal sacs might enhance female attention and therefore strengthen preferences for call types or color morphs.

I furthermore consider it promising to develop an experimental set-up with a two-stage two-choice playback experiment. I propose to use advertisement calls to facilitate long-distance mate attraction according to the experiments conducted in this project and an additional choice offered during close-range assessment of the potential mate, which might prevent an underestimation of the importance of coloration for mate selection.

Females from populations of the Southern genetic group showed strong preferences for local calls over nonlocal calls during my experiments. However, the nonlocal call was the average call of the other genetic lineage. Differences in call parameters among populations within the Southern genetic group are less pronounced. Whether Southern females also discriminate against nonlocal calls from other Southern populations, which might be located in closer distance, may help to uncover the role of call divergence during the rapid evolution of color morphs at the Bocas del Toro Archipelago.

A promising supplement to the evaluation of female preferences might be to investigate whether males also exert assortative mate preferences and hence contribute to ongoing signal divergence. Males of strawberry poison frogs may be selective about their mates because males provide parental care of the laid eggs moistening clutches each day until hatching. An approach to this question will be to evaluate whether males court nonlocal females with the same effort as local females.

Oophaga pumilio is only one exemplary study species showing divergence in several phenotypic traits. Similarly, other dendrobatid frog species show call and color diversity among populations, e.g. *O. granulifera* and *O. vincentei*. This offers the possibility to evaluate the mechanisms of signal divergence mediated through different sensory channels including visual and acoustic signals in order to identify the ultimate drivers of speciation in this taxonomic group.

Likewise, the difference between mated and unmated animals should be investigated on a broader scale. I recommend extending the studies on further populations of *O. pumilio* and to other polytypic species to investigate, whether directional sexual selection toward brighter individuals is a general trend in dendrobatids. A necessary extension to this study is an evaluation of toxicity levels in order to test, whether and how toxicity affects the evolution of phenotypic divergence.

In order to exclude a potential temporary change in coloration during courtship, I recommend to survey coloration of focal male and female animals during a longer time period, including different courtship situations as well as different seasons, climates and daytimes. This approach might also provide insights about potential phenotypic plasticity in coloration of individual *O. pumilio*.

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List of publications

Parts of this thesis have already been published or communicated.

Publications:

Dreher CE and Pröhl H (2014) Multiple sexual signals: calls over colors for mate attraction in an aposematic, color-diverse poison frog. *Front. Ecol. Evol.* **2**:22. doi: 10.3389/fevo.2014.00022

Dreher CE, Cummings ME, Pröhl H (2015) An Analysis of Predator Selection to Affect Aposematic Coloration in a Poison Frog Species. *PLoS ONE* 10(6): e0130571. https://doi.org/10.1371/journal.pone.0130571

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Oral presentations:

The Importance of calls and colors for mate choice in strawberry poison frogs (*Oophaga pumilio*); Corinna Dreher & Heike Pröhl; 17th Graduate Meeting of Evolutionary Biology of the DZG; 02. & 03. März 2012, Siegen, Germany.

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