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„Ontogenetic change of signal brightness in the foot-flagging frog species *Staurois guttatus* and *S. parvus*“

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INTRODUCTION

Conspicuous colors, patterns and patches can be observed in numerous animal taxa (Bradbury and Vehrencamp 2011). Ontogenetic change of coloration is generally unidirectional and usually occurs at the onset of sexual maturation of an individual (Hoffman and Blouin 2000; Wente and Phillips 2003; Galan 2008). Hormonal changes are suggested to affect ontogenetic variations in coloration (Richards 1982; Hayes and Menendez 1999; Hoffman and Blouin 2000). In Bocage's wall lizard (*Podarcis bocagei*) the onset of maturity can implement an abrupt, non-reversible change of dorsal and ventral coloration in males (Galan 2008). The bright yellow, orange or blue dewlap of male tree lizards (*Urosaurus ornatus*) are reliable status signals that can develop prior to sexual maturity (Thompson and Moore 1991).

Conspicuous and bright color displays were reported to influence female mate choice (Andersson 1982; Milinski and Bakker 1990; Hill 1991) and male-male competition (Krebs and Davies 1993; Hebets and Uetz 2000; Cummings et al. 2008). Hence, visual signals play a prominent role in sexual selection in several animal species. Visual displays are most conspicuous when the contrast of brightness, color, pattern and movement is enhanced relative to the background (Endler 1992; Hödl and Amézquita 2001; Bradbury and Vehrencamp 2011). Environmental conditions such as ambient light incidence or the structuring of habitats as the presence or absence of vegetation have a strong impact on the conspicuousness, thus on the transmission and detectability of a visual signal (Endler 1992; Bradbury and Vehrencamp 2011). To avoid predator detection, colorful signals of short duration, simple form and/or signaling during favorable light periods maximize intraspecific communication (Endler 1991; Harper 1991; Endler 1992; Sicsú et al. 2013). Males of the blue-black grassquit (*Volatinia jacarina*) adjust their timing of their acoustic and plumage displays to sunlight incidence to maximize detectability and minimize energetic costs (Sicsú et al. 2013).

Although anuran signals are primarily associated with acoustic cues for intraspecific communication (Gerhardt and Huber 2002), several species also use visual or multimodal displays for communication (Hödl and Amézquita 2001; Narins et al. 2003; Rosenthal et al. 2004; Hirschmann and Hödl 2006; Grafe and Wanger 2007; Preininger et al. 2009). Detection and discrimination of a sender is enhanced by conspicuous visual display like an inflated vocal sac in anurans (Narins et al. 2003; Rosenthal et al. 2004; Hirschmann and Hödl 2006; Taylor et al. 2011).

Many anuran species exhibit striking body patterns and colorations. Variations among hue and patterns are represented within species and/or across sexes (reviewed in Hoffman and Blouin 2000; Bell and Zamudio 2012). Numerous anuran species undergo distinct changes in body coloration from juvenile to the adult morph (Hoffman and Blouin 2000). Transformation from a green to a brown body coloration seems to be a common transition in anurans (Duellman and Ruiz-C 1986; Hoffman and Blouin 2000). However, some anuran species show a striking reversible dichromatism during the mating season (Bell and Zamudio 2012). In moor frogs (*Rana arvalis*) for example, the dynamic nuptial blue coloration in males is suggested as visual signal to promote mate recognition (Ries et al. 2008; Sztatecsny et al. 2010; Sztatecsny et al. 2012). Reports of visual displays and color signals in anurans have rapidly increased during the last decade (Hödl and Amézquita 2001; Bell and Zamudio 2012). Investigations of ontogenetic and dynamic color variations broaden our understanding of visual and multimodal communication systems.

The Bornean foot-flagging frogs *Staurois parvus* and *S. guttatus* are excellent model organisms to analyze differences in color change of foot webbings and body coloration. Investigations of the closely related species allow for a more general prediction regarding signal design. The diurnal and predominantly stream-dwelling species exhibit foot-flagging behavior in which legs are extended, rotated and brightly colored foot-webbings are displayed for a short period during male-male agonistic interactions (Grafe and Wanger 2007; Grafe et al. 2012). The interdigital webbings of adult individuals of *S. parvus* and *S. guttatus* are colored bright white and bluish respectively and pose a strong contrast to the dark coloration of their bodies. Males of *S. parvus* perch on black shale rocks close to the waterline, whereas males of *S. guttatus* display on branches and vegetation along waterfalls (Grafe and Wanger 2007; Grafe et al. 2012) and are well camouflaged in their respective habitat. Foot-flagging behavior occurs in male and female individuals of both species (Grafe and Wanger 2007; Preininger et al. 2012). The visual display can already be observed in juveniles of *S. parvus*, yet foot webbings at this life stage are still colored transparently grey. However, only few observations of juvenile individuals could be made in the natural habitat and close investigations on the behavior of foot-flagging frogs at this life stage are lacking. Due to the worldwide first breeding success of *Staurois spp*. at the Vienna Zoo (Preininger et al. 2012) it was possible to report visual signaling behavior in juveniles. Breeding at the Zoo provided a unique opportunity to investigate the differences in web and body coloration at different age classes starting immediately after metamorphosis.

In the course of this study we investigated the variation of color parameters of foot-webbings and body coloration of different age classes in *S. parvus* and *S. guttatus* by the means of spectrophotometry. Additionally we compared previous color measurements of an adult wild cohort from Brunei and discuss color variation in relation to diet, signaling behavior and habitat.

METHODS

STUDY SITE AND SPECIES

The study was conducted in a bio-secure container facility of the Vienna Zoo from November 2012 to February 2013. The Vienna Zoo has successfully established a breeding program for the species *S. guttatus* und *S. parvus* from individuals collected in the Ulu Temburong National Park, Brunei Darussalam in Borneo in 2010. Several generations of juveniles are housed in separate aqua terraria equipped with plants, tree branches and a water pump. Juveniles of both study species were supplied with *Drosophila sp*. and *Collembola*. Tadpoles were fed with algae tablets, fish food flakes and fish filet (for details see Preininger et al. 2012).

In nature, *S. parvus* and *S. guttatus* live in sympatry along fast-flowing streams. Males perch on rocks and branches along forest streams and display conspicuous visual signals during male-male agonistic encounters (Grafe and Wanger 2007; Grafe et al. 2012). Foot-web coloration of adult individuals of *S. parvus* appears bright white and light blue in *S. guttatus* and poses a contrast to the darker body coloration in both species. We observed foot-flagging displays in freshly metamorphosed juveniles. Foot-webbing coloration at early life stages appears translucent grey.

FROG SAMPLING AND REFLECTANCE MEASUREMENTS

At the beginning of the study we separated 30 recently metamorphosed individuals of each study species from their breeding terraria and housed them in smaller enclosures (50 x 60 x 70 cm). Older age classes were obtained from previously separated individuals since the species were first bred successfully in October 2011 (*S. parvus*) and March 2012 (*S. guttatus*). We performed monthly reflection measurements of recently metamorphosed juveniles and additional measurements of individuals of older age classes to obtain reflectance data over a period of 12 months for *S. parvus* and 7 months for *S. guttatus*. The classification of age resulted from the period of metamorphosis, which was taken as reference point.

Reflectance spectra of foot webbings and backs of juveniles of both study species were obtained with a spectrometer (Ocean Optics JAZ) between 300 and 700 nm, the spectral range of UV and visible light (Zuk and Decruyenaere 1994; Cuthill et al. 1999; Grill 2000; Ries et al. 2008). The spectrometer had an integrated pulsed xenon light source (Jaz-PX) with a spectral response from 190-1100 nm. The reflectance data were collected from 300-700 nm and expressed in per cent relative to a white standard (WS-1 Diffuse Reflectance Standard, Ocean Optics). We used a custom made probe holder keeping the reflection probe at a distance of 5 mm and an angle of 45° to the frog's skin surface in order to reduce specular glance. The probe holder touched the frog skin preventing stray light from entering. Three obtained measurements from one individual were averaged respectively.

All reflection measurements were taken on hand-held, non-anesthetized frogs immediately after taking them out of the terraria to shorten handling time and disturbance. We took reflectance scans on two body parts: the dorsal skin on the frog's back as a proxy of the frog's general body coloration and the foot webbing of the right hind foot. As foot webbings at early life stages appeared translucent, black rubber gloves were worn to avoid light reflection from the human skin. We measured snout-vent-length (SVL) of each individual to the nearest of 0.1 mm with sliding calipers and body mass to the nearest of 0.1 g with electronic miniscale.

Additionally we compared our measurements with previous data from the adult wild cohort collected in January to March 2010 in the Ulu Temburong National Park, Brunei Darussalam (Preininger et al. 2013). Reflectance measurements of the wild cohort was conducted in the same manner as measurements carried out in the Zoo.

Given that no correct age classification could be obtained from the Brunei cohort, only basic comparisons were conducted and visualized.

SPECTRAL DATA AND STATISTICAL ANALYSIS

For each frog, three parameters of coloration were extracted from the reflectance spectra using Avicol software v6 (Gomez 2006): brightness, hue and UV Blue chroma. Brightness corresponds to the total reflectance, calculated as the surface area under the spectral curve. For the parameter hue we considered the most rapid change of a spectrum, the wavelength of the maximum slope (Endler 1990). UV Blue chroma corresponds to color saturation, calculated as the proportion of the total reflectance located between 300 and 450 nm: (R300 nm – R450 nm) / (R300 nm – R700 nm).

To test for ontogenetic differences in color parameters we compared the first measurements (age cohort 1 month) with the last measured age class (*S. guttatus*: 7 month; *S. parvus*: 12 month) using Mann-Whitney U tests. As we found the most significant differences in the achromatic component of the coloration, brightness was chosen as variable for further statistical analyses to characterize coloration.

Total brightness of different age classes, body size and body weight were compared, using Linear Mixed Models (LMMs). The LMM allows repeated measurements of the same individual to be fitted in the model as random variables, thus controlling for measurements of the same individuals in differing age classes. The statistical assumptions for LMM analysis were met (Kolmogorov-Smirnov test) and non-normal data were square-root transformed to meet the criteria. To test if foot and back brightness are dependent on age, body size or body mass of *S. parvus* and *S. guttatus*, six models were run respectively, entering square-root transformed brightness values of foot or back of the respective species as dependent variable, with either age, body size or body weight as predictor variable. The identity of individuals was entered as random variable.

To test for differences of body size and body weight between individuals of an adult wild cohort and the oldest measured age class of the Zoo cohort (*S. guttatus* = 7 month, *S. parvus* = 12 month) we used Mann-Whitney U tests. All analyses were run using SPSS version 19 (SPSS Inc., Chicago, IL, USA).

RESULTS

Foot coloration of *S. guttatus* and *S. parvus* changed during ontogeny (Fig. 1). Measurements of color variables at an early life stage (1 month in both species) compared to 7 months later in *S. guttatus* and 12 months later in *S. parvus* show that foot colorations differ in intensity or brightness (Tab. 1; Fig. 2A and 2B). We found no differences in hue or UV Blue chroma for foot colorations (Tab. 1). The body coloration of both species was green after metamorphosis and changed to a light brown dorsal coloration in *S. guttatus* and overall dark grey coloration in *S. parvus* (Fig. 2C and 2D).

In *S. guttatus* the total brightness of foot-webbings and back coloration increases with age (Foot: LMM pairwise comparison: ß = 5.160; S.E. = 0.469; t = 10.996; $P \leq 0.001$; Back: LMM pairwise comparison: ß = 1.165; S.E. = 0.327; t = 3.566; $P \leq 0.001$) (Fig. 3A).

In *S. parvus*, webbing brightness increases with age (LMM pairwise comparison: ß = 4.883; S.E. = 0.332; t = 14.696; $P \leq 0.001$), whereas no differences in back brightness between age classes were found (LMM pairwise comparison: ß = -0.227; S.E. = 0.154; t = -1.477, P = 0.141) (Fig. 3B). Additionally in both species foot brightness increased with SVL (LMM pairwise comparison: *S. guttatus*: ß = 114.33; S.E. = 11.50; t = 9.943; $P \leq 0.001$; *S. parvus*: ß = 190.44; S.E. = 14.40; t = 13.23; P ≤

0.001) (Fig. 4) and weight (LMM pairwise comparison: *S. guttatus*: ß = 32.29; S.E. = 3.90; t = 8.27; P ≤ 0.001; *S. parvus*: ß = 59.87; S.E. = 4.84; t = 12.36; P ≤ 0.001).

The mean reflectance of foot webbings of adult individuals of a wild cohort (26.6% ± 1.7; n = 13) compared to the oldest individuals of *S. guttatus* (7 month: 9.5% ± 0.8; n = 14) measured in the Zoo was three times higher (Fig. 5A). Body size (Z = 0.000; P ≤ 0.001) and body mass (Z = 0.000; P ≤ 0.001) of the 7-month-old age class and the wild cohort differed significantly.

The foot webbings of an adult cohort of *S. parvus* (31.3% ± 1.9; n = 13) reflected twice as much light than of 12-month-old individuals (14.9 ± 2.0; n = 20) of the study cohort (Fig. 5B). No differences in body size (Z = 119.5; P = 0.153) and body mass (Z = 90; P = 0.961) were found among the wild and the captive individuals of *S. parvus*.

DISCUSSION

Our results show that the coloration of foot webbings of *Staurois guttatus* and *S. parvus* changes with age. We found that brightness is the parameter which most impacts the change of coloration of foot webbings of both study species.

The interdigital webbings of recently metamorphosed frogs are colored translucent grey. The body coloration of both species at early life stages is bright green. Juveniles were observed in mossy areas close to the stream (Wampula pers. comm.). Considering that individuals already display foot-flagging signals at early life stages, we suggest that the green body coloration and inconspicuous web coloration could camouflage juveniles and reduce predation risk in mossy habitats. Likewise, the tropical python *Morelia viridis* undergoes an ontogenetic change from a yellow or red morph to a green phenotype to camouflage in different habitats used by juveniles and adults (Wilson et al. 2007).

Until the age of 12 months the body coloration of *S. parvus* changes to dark grey and showed no increase in brightness. The dorsal color of *S. guttatus* remains green with brown spots and an increase in brightness was measured in the first seven months.

Directional ontogenetic color change of body coloration was observed in at least 39 species (Hoffman and Blouin 2000). In most species hormonal changes were suggested to control age-related color variations (Richards 1982; Hayes and Menendez 1999; Hoffman and Blouin 2000). We suggest that the ontogenetic increase in foot-webbing brightness enhances signal conspicuousness with sexual maturity and most likely reflects androgen levels. During mating season, males of both species signal the readiness to defend perching sites via visual displays. The foot-flagging displays were suggested to signal the motivation to defend a signaling site especially in agonistic male-male interaction (Preininger et al. 2013). We suggest the increase in signal brightness of foot webbings from juvenile to adult leading to an enhanced visual signal-to-noise ratio to the ambient habitat and body coloration. A comparison between juvenile and adult individuals suggests the following visibility-enhancing strategies in both of the investigated species: maximizing contrast to the overall body coloration, maximizing contrast to the environmental background by increasing the brightness of foot webbings, and a movement contrast through the dynamic foot flagging display (sensu Endler 1992; Bradbury and Vehrencamp 2011).

The enhanced web brightness of the oldest individuals measured in the Zoo and the wild cohort could derive from differences of diet or food availability of the respective cohort. In some anurans, the nutritional supply with carotenoids seems to have a strong impact on development and growth, but also on reproductive success and coloration of adult individuals (Ogilvy et al. 2012). For instance the coloration of the red ventral patch of *Bombina orientalis* is strictly dependent on the supply of pigment substances, a lack of these carotenes under rearing conditions leads to a yellowish coloration (Steinicke 1976; Frost and Robinson 1984). Likewise, the Japanese newt *Cynops pyrrhogaster* shows yellow ventral skin when lab-reared and undersupplied with pigment substances (Matsui et al. 2002). *Staurois parvus* and *S. guttatus* were fed similar to what was observed in the adult wild cohort, however observations of food intake were rare (Preininger pers. communication) and there are no reports available what tadpoles feed on in their natural stream habitat.

Another explanation for the increase in intensity might be, that foot-webbing brightness could signal male age and/or mating activity, thus again indicate the hormonal status of an individual. Since brightness not only increased with age, but also with body size and weight in this study, we suggest that the latter could be a byproduct of the simultaneous ontogenetic changes. The comparisons of body size and weight between 12-month-old individuals of *S. parvus* and the wild cohort showed no differences, whereas foot brightness doubled. However we have no record of actual age of the wild cohort, therefore the proposed age and brightness dependency of individuals older than 12 months remains speculative. Nevertheless mating activity could have influenced web coloration of the wild cohort. All observed male individuals were emitting advertisement calls and engaged in agonistic interactions (Preininger, pers. comm.). In several amphibians coloration and traits are emphasized during mating. Males of the spot-tailed warty newt (*Paramesotriton caudopunctatus*) for example display fanning movements with their tail which exhibits a bright spot on the tip during the mating season (Sparreboom 1983). Male individuals of *Rana arvalis* display conspicuous dynamic nuptial colorations during the breeding period (Sztatecsny et al. 2012). The Australian stony creek frogs (*Litoria wilcoxii*) show a striking example for hormone-driven dynamic color change. Males react with reversible color change from brown to bright yellow during amplexus due to stress hormones (Kindermann et al. 2013).

Future investigations on age-related color change in *Staurois spp*. should clarify if foot-webbing brightness further enhances with increasing age, or differs between individuals held in a large breeding arena with regular mating activity and smaller terraria where no breeding takes place. Additionally, foot flagging was already observed in juvenile frogs in short range interaction during feeding (Preininger et al. 2012), which suggests that the display is used as spacing mechanism already in early life stages. Further studies of juveniles should investigate if foot-flagging frequency is dependent on population density e.g. during feeding.

Investigating signal behavior and design of foot-flagging displays in juveniles in comparison to adults will help to explain the function and development of visual signals and multimodal communication.

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APPENDIX

Table 1. Median values ± standard error of color variables of foot and back measurements in *Staurois guttatus* and *S. parvus*.

Species Age (month)	Total brightness (300-700 nm)	*Z* *P*	Hue (nm)	*Z* *P*	UV blue chroma (300-450 total^{-1})	*Z* *P*
Staurois guttatus						
Foot						
1 (*n* = 20)	913 ± 76	400	414 ± 9.2	163	0.21 ± 0.02	204
7 (*n* = 20)	3353 ± 300	**<0.001**	411 ± 4.6	0.314	0.23 ± 0.01	0.914
Back						
1 (*n* = 20)	401 ± 53	359	513 ± 0.7	146	0.00 ± 0.02	397
7 (*n* = 20)	854 ± 74	**<0.001**	504.5 ± 31.4	0.143	0.25 ± 0.01	**<0.001**
Staurois parvus						
Foot						
1 (*n* = 20)	580 ± 96	280	414 ± 15.8	91	0.20 ± 0.03	189
12 (*n* = 14)	6227 ± 794	**<0.001**	331.5 ± 10.6	0.086	0.26 ± 0.01	0.086
Back						
1 (*n* = 20)	350 ± 4	107	513 ± 10.1	110	0.00 ± 0.01	187
12 (*n* = 14)	88 ± 74	0.248	494.5 ± 26.6	0.292	0.03 ± 0.04	0.065

Z, Mann-Whitney U-statistic for comparison between age classes; P, significance level; n, sample size; figures in bold indicate significant P-values for α = 0.05.

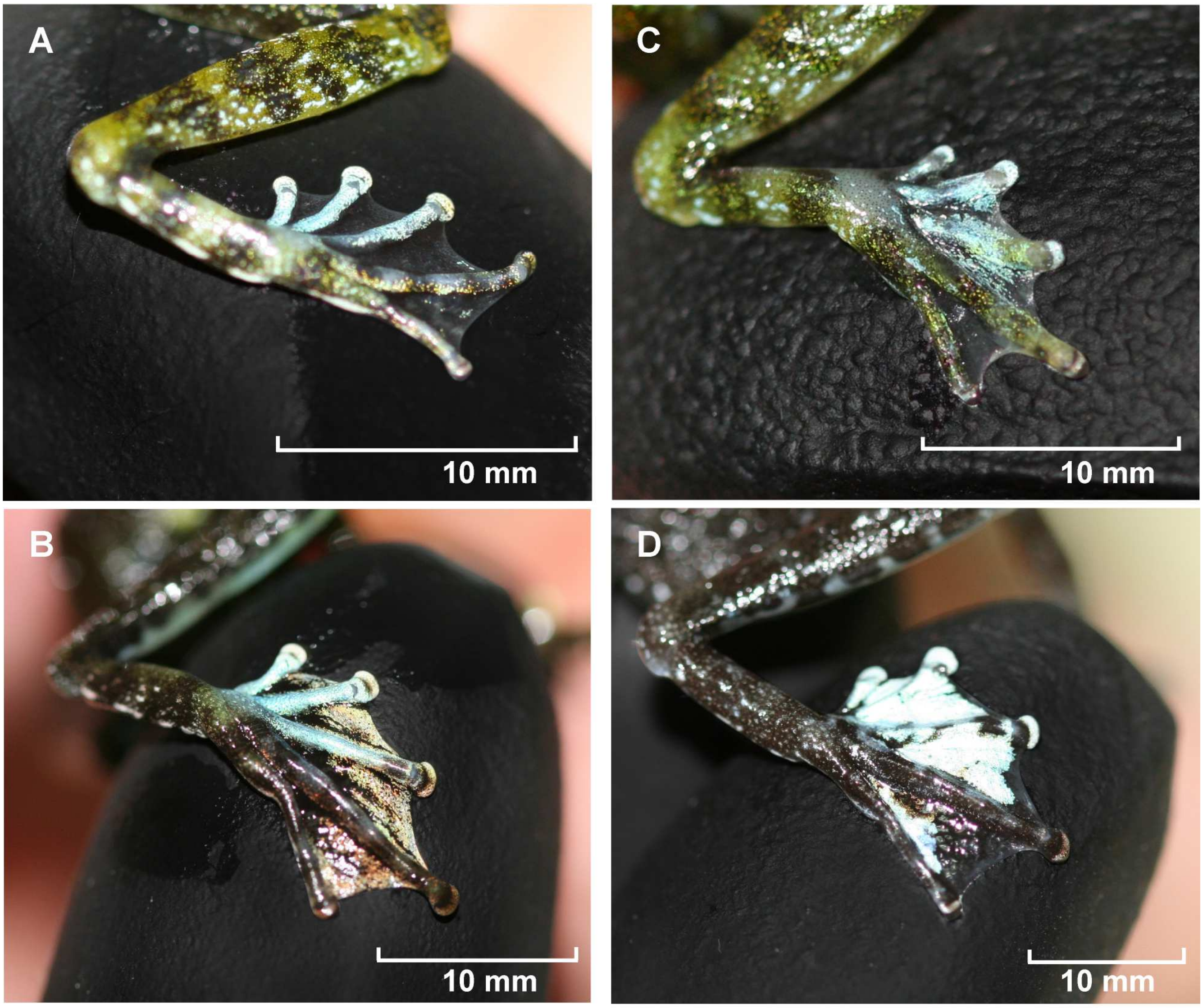

Figure 1. Foot-webbings of *Staurois guttatus* (A,B) and *Staurois parvus* (C,D) at an age of 1 month (A,C), 7 months (B) and 12 months (D) respectively.

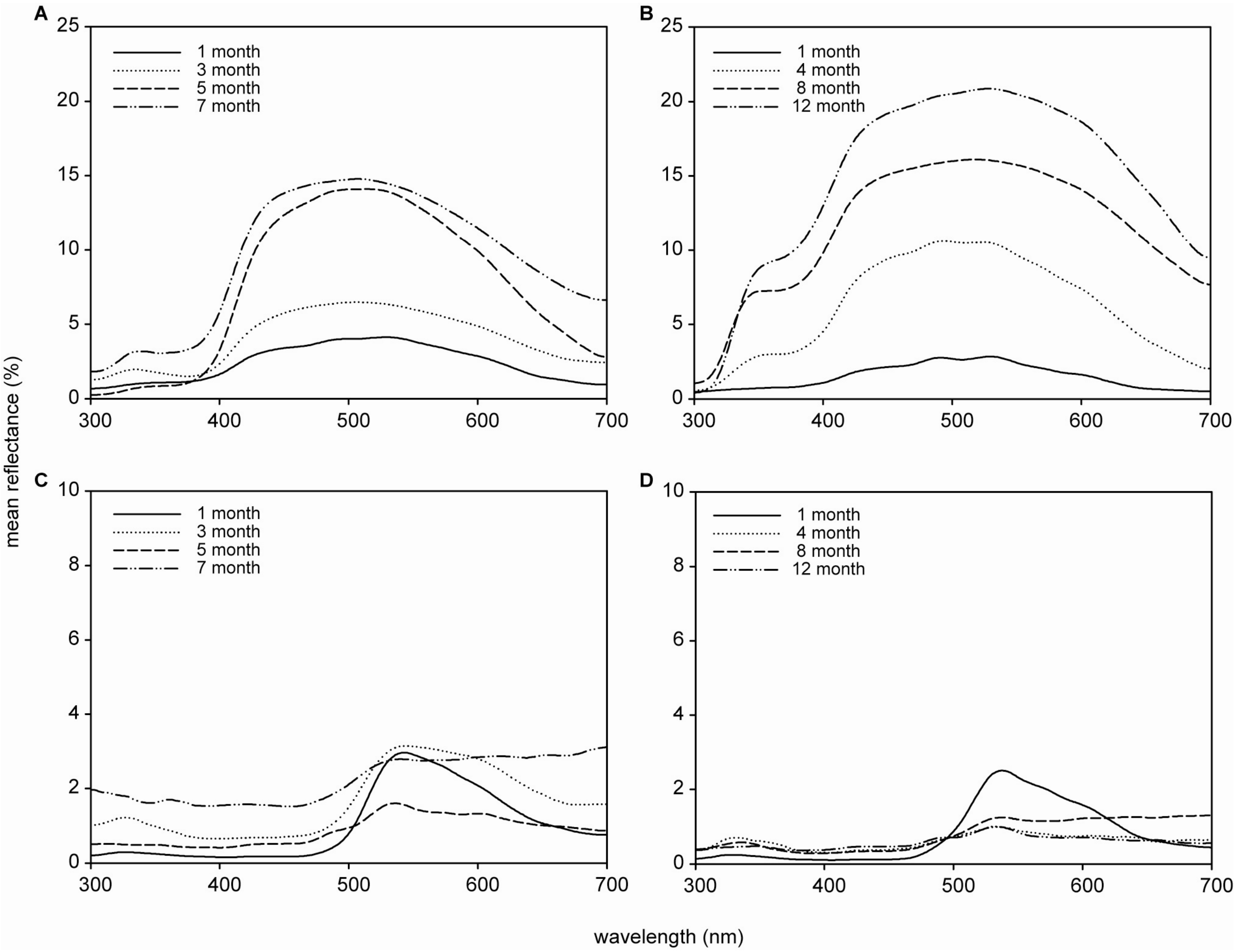

Figure 2. Mean reflectance of *Staurois guttatus* (A, C) and *S. parvus* (B, D) at different age classes. A and B show foot reflectance; C and D back reflectance (note different scales of y-axis). *Staurois guttatus*: (*n* = 20) for all age classes; *S. parvus*: 1 month (*n* = 20), 4 month (*n* = 12), 8 month (*n* = 19), 12 month (*n* = 14).

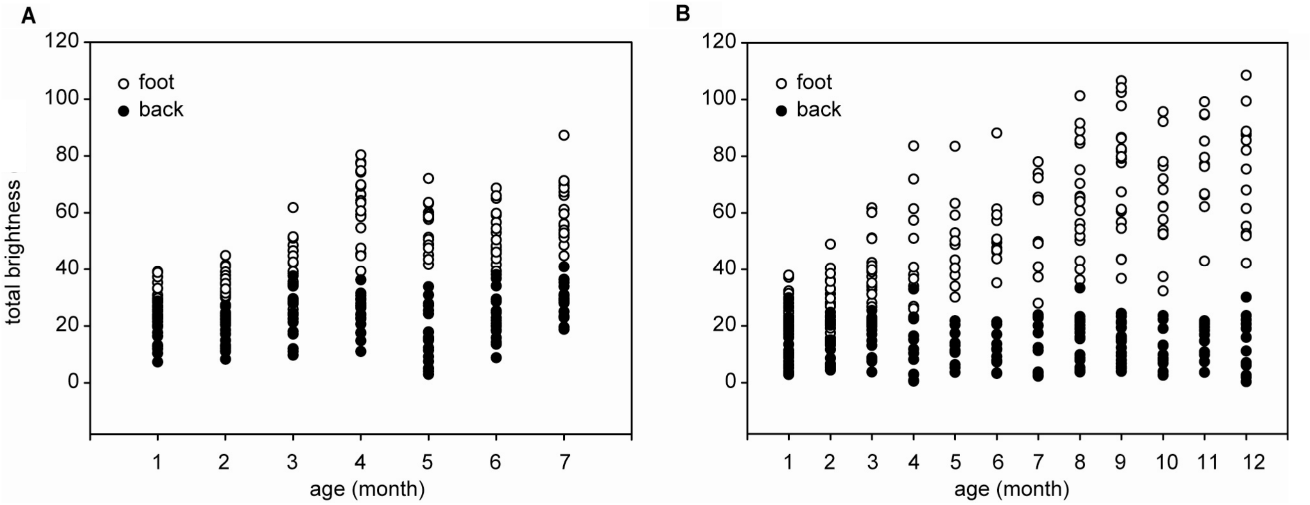

Figure 3. Scatterplots of foot and back brightness (square-root transformed values) of measured age classes of *Staurois guttatus* (A) and *S. parvus* (B). Plots show original data of each individual (not estimates of the LMMs) for illustration and do not correspond directly with the statistical results.

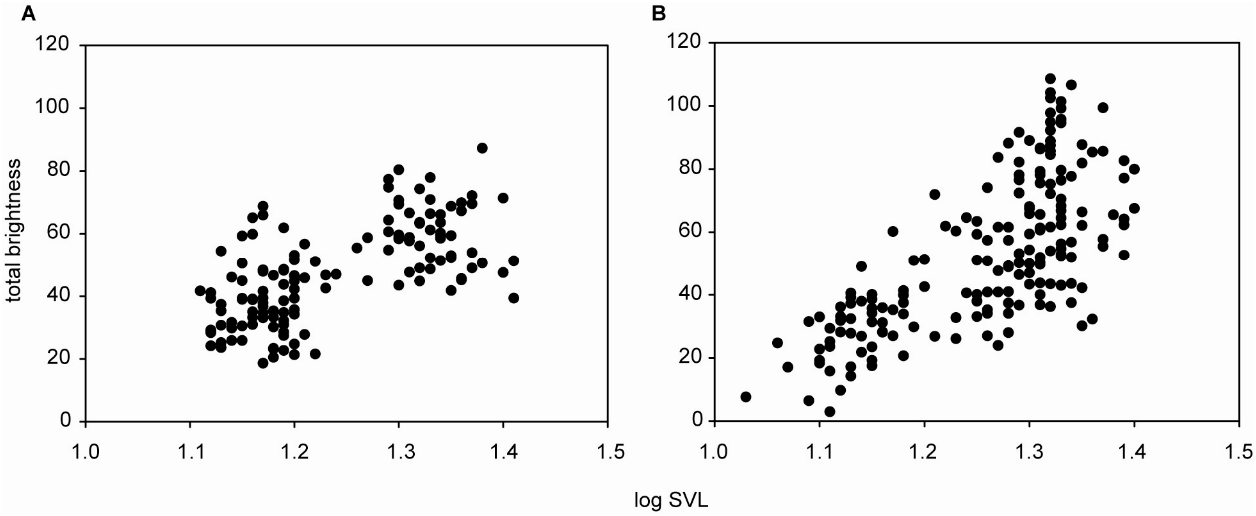

Figure 4. Scatterplots of foot brightness (square-root transformed values) and log snout-vent length (SVL) of *Staurois guttatus* (A) and *S. parvus* (B). Plots show original data of each individual (not estimates of the LMMs) for illustration and do not correspond directly with the statistical results.

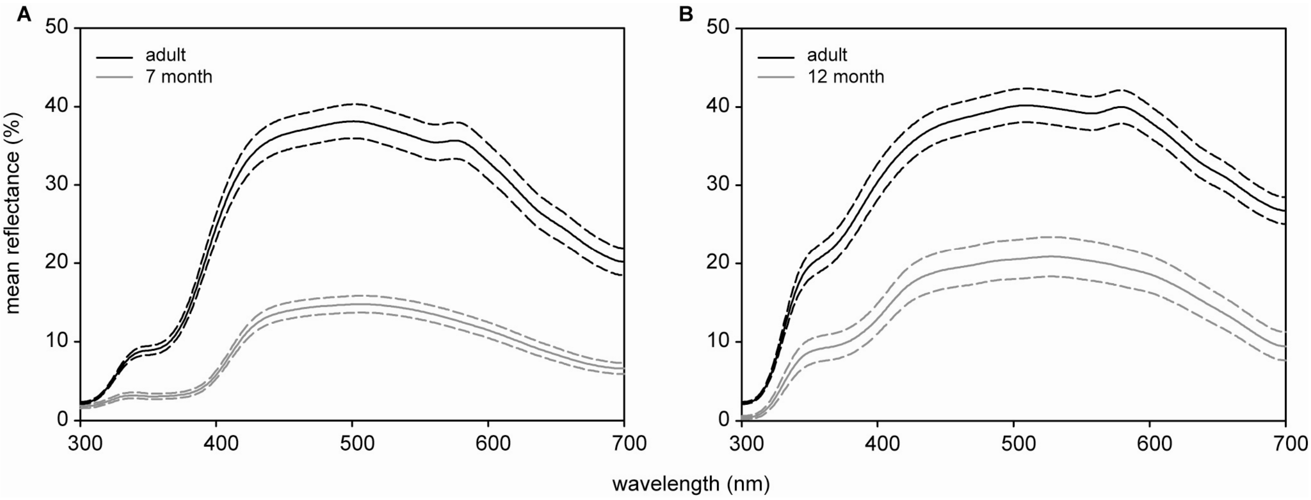

Figure 5. Mean reflectance of foot webbings of *Staurois guttatus* (A) and *S. parvus* (B) of the last age class measured in the Zoo and of adults measured in their natural habitat (Brunei) respectively. Dashed lines below and above solid lines indicate standard errors. *Staurois guttatus*: 7 month (*n* = 20), adult (*n* = 13); *S. parvus*: 12 month (*n* = 14), adult (*n* = 13).

ABSTRACT

Adult individuals of several anuran species exhibit conspicuous visual displays during intraspecific communication. While signal properties in adults have been subjects to an increasing number of studies, little is known about the variation of visual signals in juveniles and during ontogenetic changes. Foot-flagging signals of the Bornean frogs *Staurois guttatus* and *S. parvus* were observed in juveniles already a few days after metamorphosis. We investigated color parameters of foot-webbings and body coloration of individuals bred at the Vienna Zoo and their relation to age and body size using spectrophotometry. Our results show that the brightness of foot webbings of *S. guttatus* and *S. parvus* increased with age. Additionally we compared the results with measurements of adult individuals from Brunei and discuss possible differences related to diet and age as well as the habitat use of juveniles and adults. We suggest that the ontogenetic increase in foot-webbing brightness enhances visual conspicuousness and the signal-to-noise ratio of the visual signal with sexual maturity.

ZUSAMMENFASSUNG

Während intraspezifischer Kommunikation bedienen sich einige Anuren auffälliger optischer Signale. Im Gegensatz zu Signaleigenschaften bei Adulttieren, die mittlerweile gut dokumentiert sind, ist über die Variation und die ontogenetischen Veränderungen von optischen Signalen bei Jungtieren nur wenig bekannt. Bereits wenige Tage nach der Metamorphose wurde das Winken mit den Hinterbeinen bei den aus Borneo stammenden Froscharten *Staurois guttatus* und *S. parvus* beobachtet. Wir untersuchten die Farbparameter der Schwimmhäute und der Rückenfärbung in Abhängigkeit von Alter und Körpergröße, einer Nachzucht des Zoo Schönbrunns, mittels Spektrophotometrie. Unsere Ergebnisse zeigen, dass die Helligkeit der Schwimmhäute bei beiden Winkerfroscharten mit dem Alter ansteigt. Zusätzlich verglichen wir unsere Messresultate mit jenen einer Adultpopulation von Brunei und diskutieren mögliche Unterschiede hinsichtlich Ernährung, Alter und unterschiedlicher Habitatnutzung von Juvenilen und Adulten. Wir vermuten, dass der ontogenetische Anstieg der Helligkeit der Schwimmhäute die optische Auffälligkeit, und somit das Signal-Rausch-Verhältnis verbessert und mit dem Erreichen der Geschlechtsreife zusammenhängt.

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