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Ontogeny of Sexual Dichromatism in the Explosively Breeding Wood Frog

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***Abstract.*—Some species exhibit sex-specific color differences (sexual dichromatism) that are considered important for reproduction. The developmental timing of sexual dichromatism is largely unknown, particularly in amphibians, and is important for informing our basic understanding of sex-specific divergence in the physiology, anatomy, and ecology of species more broadly. Using a controlled laboratory environment, we explored the onset of sexual dichromatism in Wood Frogs (*Rana sylvatica*), a species in which adult females typically appear redder than males. We photographed juvenile Wood Frogs approximately every two months for one year and quantified coloration (hue, saturation, brightness). Our results show that Wood Frog hue becomes sexually dichromatic before their first winter, with yellower hues in males and redder hues in females. Hue differences persist throughout the following spring and summer, with the exception of immediately after emergence from overwintering when both sexes are dramatically less bright. Male and female Wood Frogs typically do not join breeding aggregations until their first and second year, respectively; our results therefore indicate that sexual dichromatism is established soon after metamorphosis and substantially before first breeding attempts. We also noted seasonal fluctuations in hue and luminance, both of which increased during the first fall, decreased during winter, and increased again during the second spring and summer. These fluctuations suggest that color plays a role not only in sexual signaling but also in terrestrial camouflage. Future work should explore the ecological and evolutionary relevance of the early onset of sexual dichromatism and the function of seasonal variation in frog coloration.**

Key Words.—hue; *Lithobates sylvaticus*; luminance; *Rana sylvatica*; saturation; sexual development

Introduction

Sexual dichromatism, differences between male and female coloration, has been documented in < 5% of all frog species (Bell and Zamudio 2012). Two classes of sexual dichromatism are recognized; dynamic dichromatism and ontogenetic dichromatism (Bell and Zamudio 2012). In dynamic dichromatism, males experience an ephemeral color change during the breeding season. In species with ontogenetic dichromatism, the sexes diverge permanently in color patterns during development. Ontogenetic dichromatism is far more prevalent in frogs and, while Bell and Zamudio (2012) note that color differences often develop at the onset of sexual maturation, the actual ontogeny of sexual dichromatism remains poorly studied in amphibians.

We are increasingly realizing that larval amphibians show sex-specific differences in response to environmental conditions. The wide-ranging North American Wood Frog (*Rana sylvatica* = *Lithobates sylvaticus*) has become a model for understanding such sex differences. In addition to exhibiting substantial behavioral and physiological differences during the breeding season (Swierk et al. 2014), males and females also differ in larval duration under food limitation (Warne and Crespi 2015) and in response to a hormonal chemical mixture exuded by plant roots (Lambert 2015). Sexual dimorphism at metamorphosis also emerges in response to the leaf litter type of natal ponds (Lambert et al. 2016). While these studies show that the sexes differ in developmental rates and sizes at or prior to metamorphosis, it is unclear the extent to which the sexes continue to morphologically differentiate during post-metamorphic development. However, given the strong sex differences observed during larval ontogeny, it might be expected that sexually dimorphic traits, like color, might continue to deviate before maturity.

Wood Frogs provide an excellent case study of the ontogeny of sexual dichromatism in amphibians. As

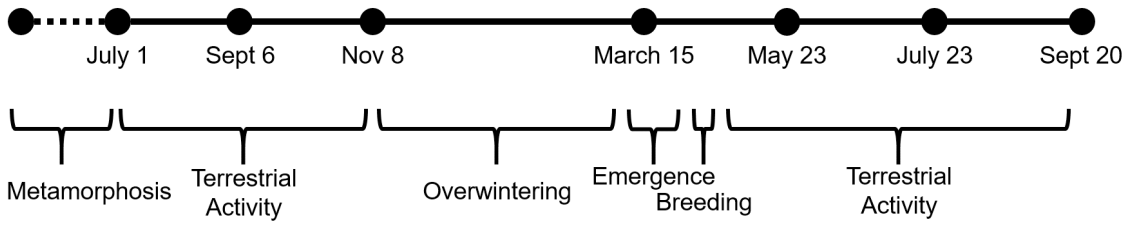

Figure 1. Conceptual schematic of metamorphosis and post-metamorphic ontogeny in Wood Frogs (*Rana sylvatica*). Points along the developmental timeline represent dates for each photograph period. After metamorphosis and after breeding, Wood Frogs are terrestrially active during summer and fall (May - October). Wood frog hibernation occurs during winter (November - March) and breeding activity typically occurs over a short interval (several days) in early spring (March or April). The time line is generalized and actual phenology can vary substantially across space and time.

breeding adults, males are tan or dark brown whereas females tend to be redder in color (Banta 1914). Experiments conducted over a century ago show that males readily amplex red females but ambivalently approach darker females (Banta 1914). This result suggests that males may use color to distinguish between other males and females during reproduction. Indeed, emerging evidence indicates that anurans may be able to discriminate discrete color (hue) and intensity (luminance) differences of mates, even under low light scenarios (Hailman and Jaeger 1974; Yovanovich et al. 2017). We note that color may be one characteristic, in addition to body size and chemical cues, that males use for sex identification (Banta 1914; Berven 1981). Being able to readily distinguish the sexes is likely important for Wood Frog males, as the breeding season may last only 1–3 d in early spring (March and April) and high male-male competition often results in only a small percentage of males succeeding in mating (Howard 1980; Berven 1981).

Here, we explore the ontogenetic onset of sexual dichromatism in post-metamorphic Wood Frogs. We reared Wood Frog larvae under common laboratory conditions and photographed post-metamorphic animals at regular intervals for over a year. Our goal was to track if, and when, color differences appear between the sexes at various post-metamorphic developmental and seasonal time points (Fig. 1).

Materials and Methods

Field collection and animal care.—We collected 41 egg masses from Wood Frogs in Pennsylvania State Game Lands #176 (Scotia Barrens; 40.7789°N; 78.0071°W), USA, in March 2012. To obtain egg masses, we captured adult Wood Frogs using drift fences and pitfall traps as they migrated to vernal (temporary) ponds to breed. At the edge of natural breeding ponds, we placed frogs in mixed-sex groups in circular wading pool arenas (115 × 33 cm, diameter × height) that we filled with pond water and natural oviposition substrates, as part of a separate study (Swierk et al. 2014). We left arenas undisturbed until egg masses were produced approximately 12–36 h later.

At Pennsylvania State University, we placed each mass on a mesh sling in an individual 18 L tank containing dechlorinated, aged water. Larvae began hatching 4–7 d later. After hatching, we retained 50 larvae per egg mass in clutch-specific 18 L tanks, and we released the remaining larvae back into their pond of collection. Approximately once weekly we performed partial water changes and monitored larval health daily. Larvae ate a 3:1 ground mixture of alfalfa pellets (Kaytee Products, Inc., Chilton, Wisconsin, USA) and TetraFin Goldfish Flakes (Spectrum Brands, Inc., Blacksburg, Virginia, USA) *ad libitum* three times per week until forelimb emergence. When forelimbs emerged (Gosner stage 42; Gosner 1960), we transferred each froglet to an individual plastic container (11.4 × 16.5 × 7.6 cm; width × length × height) lined with moistened bleach-free paper towels, containing a shelter and a petri dish of water, and covered with a secure and ventilated lid.

Beginning on 1 July 2012, we re-housed a subset of froglets (n = 120) in the laboratory for the remainder of our monitoring period. This subset included the frogs that metamorphosed in the peak metamorphosing time window (about 20 May to 15 June); later metamorphosing frogs were not included. We housed these frogs in groups of four in larger, mesh-covered enclosures (40.1 × 26.2 × 17.8 cm; W × L × H), which also contained moistened bleach-free paper towels, a shelter, and a petri dish of water. Froglets ate Common Fruit Flies (*Drosophila melanogaster*) and House Crickets (*Acheta domesticus*), dusted with calcium (Rep-Cal Research Labs, Los Gatos, California, USA) and multi-vitamin powder (Sticky Tongue Farms, Sun City, California, USA), ad libitum. We maintained the animal facility on a 12:12 h light:dark cycle at 20° C, and froglets had access to a low-heat lamp and UV light (Exo-Terra Repti-Glo 10.0 Compact Fluorescent Terrarium Bulb 13W, Rolf C. Hagen, Inc., Mansfield, Massachusetts, USA).

Between November 2012 and March 2013, we overwintered all frogs in a 4° C laboratory refrigerator with their housing groups in moistened plastic storage containers (30 cm × 15 cm × 10 cm; W × L × H) with lids that allowed air circulation. We angled containers to approximately 10° and filled each with approximately 1 cm dechlorinated water, so that about half the container was wet and half was dry. In preparation for hibernation, we slowly acclimated frogs to their overwintering conditions in an incubator that we set to decrease temperature at a rate of 0.16° C per hour or about 3.8° C per day until 4° C was reached (Costanzo et al. 1991), at which point we placed frogs in the laboratory refrigerator for the duration of hibernation. For the first week of hibernation, we changed water daily and after the first week we changed water weekly; we chilled replacement water to match the temperature of the frogs in hibernation (4° C). We did not feed frogs during this period, as their metabolic rate is too low during hibernation for digestion. In March (typical Wood Frog emergence date in Pennsylvania; unpubl. data), we warmed frogs at a rate of 3.8° C per day for about 4 d in the incubator to prevent organ damage (Layne and First 1991). We then returned frogs to their previously described housing and husbandry conditions.

Photography.—As part of a separate study (Lindsey Swierk and Tracy Langkilde, unpubl. data), we monitored growth and mortality of the lab-reared larvae and, later, the metamorphosed froglets, from March 2012 to September 2013. Once froglets were group-housed, we toe-clipped each for identification purposes and photographed froglets approximately every 2 mo except during hibernation: 1 July 2012, 6 September 2012, 8 November 2012, 15 March 2013, 23 May 2013, 23 July 2013, and 20 September 2013. We took all photographs with a tripod-mounted Cyber-shot DSC-H7B digital camera (Sony Corporation, Tokyo, Japan). This camera records images as JPG files rather than TIFF or RAW files, and JPG image compression can be associated with lost data (Stevens et al. 2007). To minimize this problem, we used the lowest compression setting (fine) on this camera, a solution that other studies of animal coloration have successfully employed (Langkilde and Boronow 2012). All seven photography sessions employed the same lighting conditions (overhead fluorescent lights) and camera settings: effective pixel count of 8.1 megapixels, optical zoom (1.5), macro mode, shutter speed 1/30 s, F = 3.2, and manual white balance. No auxiliary source of light was used. We photographed each frog against a white background and next to a ruler for scale.

We chose to use digital photography to study coloration as opposed to using a spectrophotometer. Unlike spectrophotometry, digital photography permitted us to rapidly measure large regions of color with only minimally manipulating the subject, thus reducing any chance of stress-induced color change (unknown in this species, but common in other taxa; e.g., Boyer and Swierk 2017). In addition, Wood Frog dorsa are usually mottled or patterned, making spectrophotometry a difficult technique to use successfully on this species. Digital photography is a powerful tool, but, due to the inability of most cameras to detect ultraviolet colors, must be used with caution (for recent reviews, see Troscianko and Stevens 2015; White et al. 2015). We therefore acknowledge that our findings solely quantify color variation in the human-visible spectrum.

Digital measurements.—We quantified dorsal color and SUL of each frog over 15 mo using Adobe Photoshop (version CS6, Adobe Systems Inc., San Jose, California, USA). To obtain SUL, we measured the length between the snout and urostyle of each frog after setting the scale of the photo using the ruler (Browne, R. 2014. Measuring amphibian morphometrics using ImageJ. Amphibian and Reptile Conservation Protocols 2. Available from http://www.redlist-arc.org/Protocols.html [Accessed 14 January 2016]). Prior to obtaining color measurements from photos, we standardized the color of each photo to the background standard (Calisi and Hews 2007) using the curves adjustment function in Adobe Photoshop to eliminate any differences in ambient lighting (Ng et al. 2013). We drew an oval over the dorsum of each frog, beginning at the midpoint of a line spanning the posterior corner of the eyes, width spanning the distance between the dorsolateral dermal plicae, and typically ending at the sacral hump. We then used the average blur function to obtain the average dorsal color (Langkilde and Boronow 2012). We used the color picker tool to obtain the hue, saturation, and brightness values of the averaged area. Following Rudh et al. (2007), hue represents the color reflected off an object as defined by its placement on the color wheel from 0° to 360°. Roughly, hue values closer to 0° are more red, those near 60° are more yellow, near 120° are green, near 180° are turquoise, near 240° are blue, 300° are purple, and towards 360° approaches red again. Saturation (i.e., chroma) refers to the percentage of grey relative to hue (color) in a given unit of measurement. Saturation values closer to 0 are entirely grey whereas higher values indicate more saturated color. Luminance is a measure of brightness, in which lower values indicate 0% white (100% black) and higher values indicating 100% white.

Sex identification.—In September 2013, immediately following the final photo session, we euthanized all frogs. We determined sex by dissection and identification of testes or ovaries (Lambert 2015; Warne and Crespi 2015).

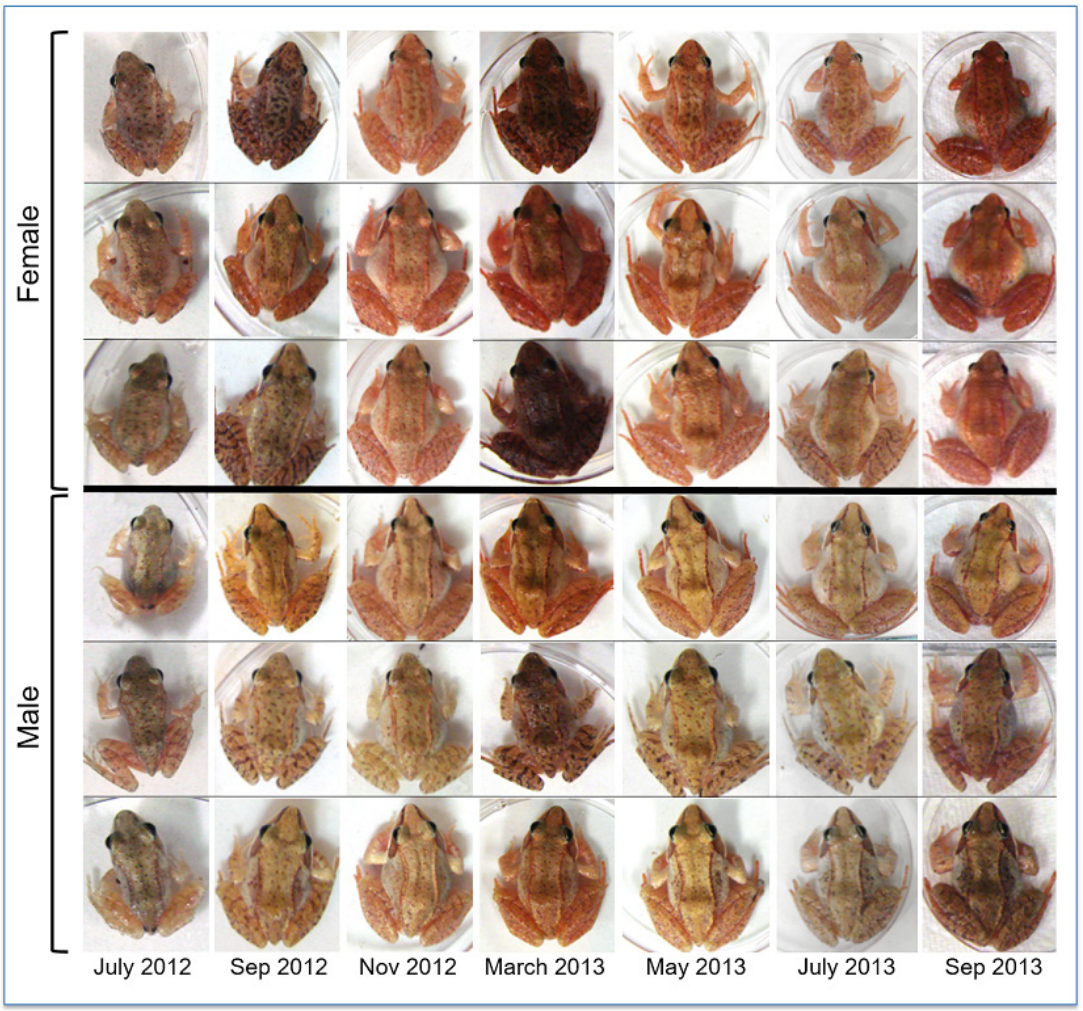

Figure 2. Representative color variation within and between female and male Wood Frogs (*Rana sylvatica*) and across all seven developmental time periods. Each row represents an individual frog across ontogeny and each column represents a given developmental time period. (Photographed by Lindsey Swierk).

Statistical analyses.—We examined color at time points that represented key developmental periods throughout the first year of post-metamorphic development (Fig. 1). As such, our modeling was aimed at understanding whether color varied over time and whether sexual dichromatism occurred at each discrete time step. We considered all differences significant at $P < 0.05$.

We used linear mixed effect models (LMM) to test whether each color variable (hue, saturation, and brightness) differed across ontogeny and sex. To do this, we used the R package *lme4* (Bates et al. 2015) and treated the developmental period and sex as fixed effects and each frog as a random effect to account for repeated measures across time. We used the r.squaredGLMM function in the R package *MuMIn* to calculate r^2 values for mixed effects models and used the glht function in the R package *multcomp* to perform Tukey's HSD tests for multiple comparisons among each developmental period. The r.squaredGLMM function provides two r^2 values: a conditional r^2 (cr^2) for the full model including both fixed and random effects, and a marginal r^2 (mr^2) to express the variance explained by only the fixed effects after accounting for random effects.

We started with a model incorporating an interaction between sex and developmental period, using likelihood ratio (LR) tests with the function Anova to test whether the interaction was significant. If non-significant, we removed the interaction from the model and subsequently tested whether the two fixed effects were significant using LR tests. If a hue, saturation, or brightness showed significant variation between the sexes and across time, we tested whether that color variable predicted sex using separate binomial generalized linear models (GLM) for each time point.

Results

Post-metamorphic Wood Frogs showed distinct color patterns by sex across ontogeny (Fig. 2). For hue,

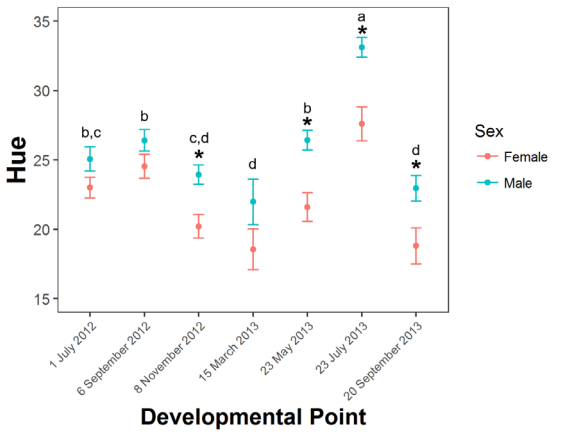

Figure 3. Wood frog (*Rana sylvatica*) hue differs across ontogeny and by sex. Higher hue values indicate frogs that are more yellow whereas lower hue values indicate frogs that are more red. Asterisks indicate developmental points where males have higher hue values than females. Letters indicate Tukey's groupings for each time point, controlling for sex. Hue tends to decline towards the frogs' first winter, immediately increasing during spring and summer before decreasing again before the second fall. This ontogenetic pattern is the same for both sexes. Points are mean ± 1 SE. Dates correspond to those in Fig. 1.

the interaction of sex by developmental time was not significant (P = 0.318) but both sex (P < 0.001) and developmental time (P < 0.001) were significant. The LMM incorporating sex and developmental period (cr^2 = 0.51, mr^2= 0.28, intercept estimate = 22.4818° ± 0.7449) indicated that males generally had higher hue values than females (estimate = 3.1537° ± 0.8558) controlling across time points (Fig. 3). Both sexes generally showed decreasing hue values towards the onset of winter, increasing again in spring, and decreasing again during their second summer (Fig. 3, all Tukey's pairing P < 0.05). Binomial GLMs indicated that males had higher hue values at time points 3, 5, 6, and 7 (all P < 0.05).

There was no significant interaction or effect of sex on saturation (both P > 0.05) or luminance (both P > 0.05), but saturation and luminance did vary across developmental time (saturation: P < 0.001, cr^2 = 0.64, mr^2 = 0.39; luminance: P < 0.0001, cr^2 = 0.64, mr^2 = 0.44). Tukey's HSD identified strong seasonal fluctuations in saturation (Fig. 4, all groupings P < 0.05) and luminance (Fig. 4, all groupings P < 0.05). Frequency distributions for hue, saturation, and luminance values between sexes and among developmental time generally exhibit unimodal, normal distributions (Fig. 5). Sample sizes, average snout-urostyle length (SUL), and mass at varied among time points but were similar between sexes (Table 1).

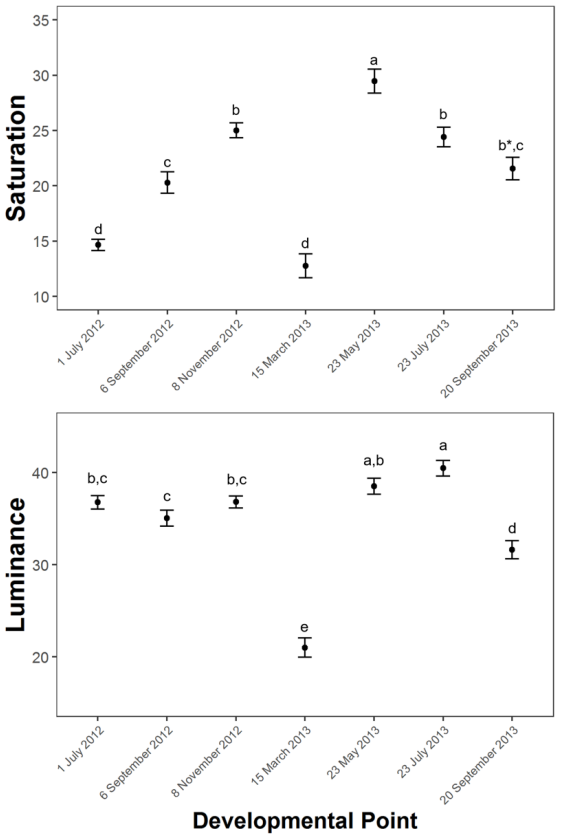

Figure 4. Wood Frog (*Rana sylvatica*) color saturation (top) increases during the first summer, decreasing during fall and into their first winter, and peaking again during their second spring and summer. Luminance (bottom) remains relatively constant and high after metamorphosis, reaching a low for the winter, and peaking in spring again. This pattern is indicative of the stark light-to-dark transition at the onset of winter and similarly stark dark-to-light transition after winter. Letters indicate Tukey's groupings for each time point. The sexes did not differ significantly in saturation or luminance. Points are mean ± 1 SE. Dates correspond to those in Fig. 1. The asterisk here indicates that saturation is marginally higher on 23 July 2013 compared to 20 September 2013 (P = 0.057).

Discussion

Here, we show that sexual dichromatism in Wood Frogs is absent immediately post-metamorphosis, develops prior to the first winter, is not present immediately after overwintering, but re-emerges and persists through the second spring and summer. Both sexes show similar seasonal cycles in hue levels but males maintain higher hue than females for four of the seven time points measured here. Unlike some other sexually dichromatic anurans that change color instantaneously or within minutes during breeding (Doucet and Mennill 2009; Kindermann et al. 2014),

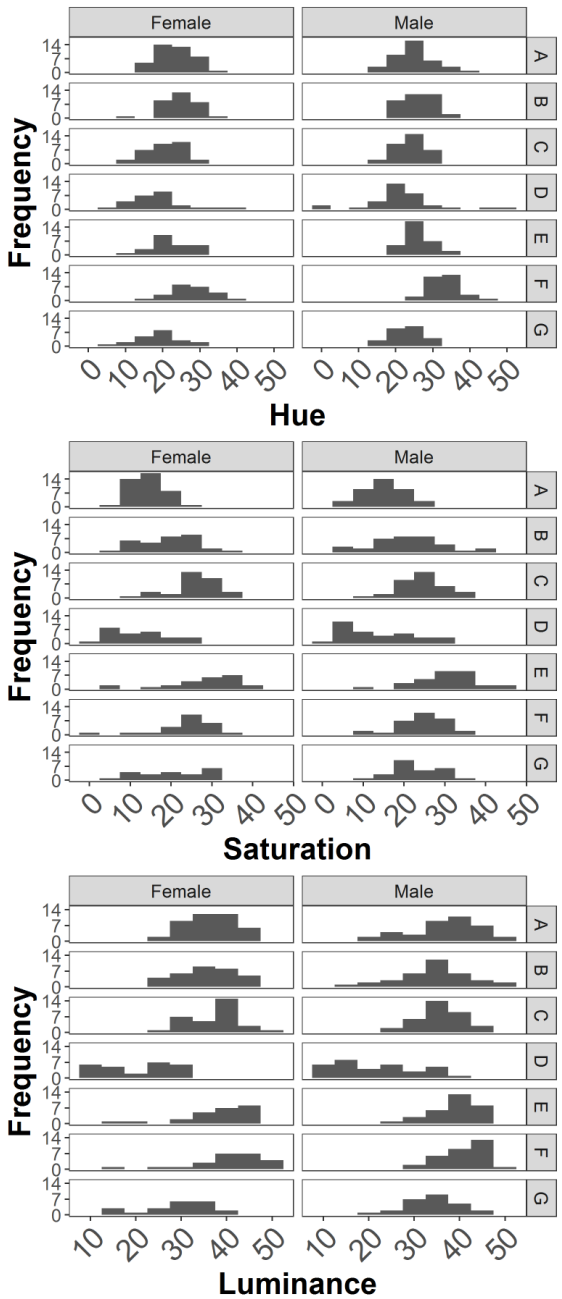

Figure 5. Frequency distributions for hue, saturation, and luminance at each time point (right axis) and for each sex of Wood Frogs (*Rana sylvatica*). Time points are A = 1 July 2012, B = 6 September 2012, C = 8 November 2012, D = 15 March 2013, E = 23 May 2013, F = 23 July 2013, and G = 20 September 2013.

color change in Wood Frogs develops over days or weeks and persists after the breeding season. In the range of hue values measured in these Wood Frogs (about 15 – 40°), males are more yellow than females, which are redder. We note that the more yellow hue of males presents as a darker tan or brown color rather than a vibrant yellow. Importantly, because we raised frogs under common garden conditions, we are able to show that these color differences between females and males are innate and not due to environmental effects (e.g., diet, temperature, background color). However, it is possible that the environment may also mediate color to some extent in a natural setting.

Table 1. Sex, sample size (n), mean (SE) snout-urostyle length (SUL), and mean (SE) body mass of Wood Frogs (*Rana sylvatica*) at each date photographed.

Date	Sex	n	SUL (mm)	Mass (g)
1 July 2012	Female	41	14.9 (0.3)	0.32 (0.02)
	Male	38	14.8 (0.2)	0.30 (0.01)
6 September 2012	Female	32	21.3 (0.4)	0.99 (0.06)
	Male	35	22.0 (0.4)	1.05 (0.06)
8 November 2012	Female	32	28.2 (0.7)	2.51 (0.17)
	Male	34	29.8 (0.7)	2.99 (0.21)
15 March 2013	Female	26	27.8 (0.8)	2.15 (0.16)
	Male	33	29.1 (0.7)	2.45 (0.18)
23 May 2013	Female	24	32.0 (1.0)	3.07 (0.26)
	Male	31	33.3 (0.7)	3.24 (0.20)
23 July 2013	Female	24	36.5 (1.0)	5.00 (0.43)
	Male	31	37.1 (0.8)	5.05 (0.32)
20 September 2013	Female	21	38.4 (1.1)	6.90 (0.60)
	Male	26	38.6 (0.8)	6.32 (0.41)

Wood Frogs also showed seasonal variation in saturation and luminance, independent of sex. Saturation increases from metamorphosis through summer and into the first fall, is low while overwintering, peaks during the second spring, and slowly decreases again during the second summer and fall. Because higher saturation corresponds to more vibrant colors, this pattern might generally reflect the increasing need for crypsis during terrestrial movements and foraging. Alternatively, saturation is highest at time point 5, the time point that may most accurately reflect color differences during breeding. High saturation at this time point may indicate that dichromatism is most important during breeding activities but that maintaining saturated colors is costly and so color saturation declines after the breeding season. Luminance, or brightness, is generally high from metamorphosis to hibernation, drops dramatically during hibernation, is at its highest in the second spring and summer, and declines again during the second fall. The decrease in luminance during hibernation further supports the hypothesis that color is costly and likely unnecessary to maintain during winter stasis.

Interestingly, sexual dichromatism is absent at time 15 March (time point 4), immediately after overwintering, but is present both in the fall before overwintering and again in late spring after breeding typically occurs in

wild populations. Given that breeding Wood Frogs are known to be sexually dichromatic (Banta 1914; King and King 1991), this result suggests that the seasonal onset of dichromatism occurs in a relatively short window of time (days or weeks) in the spring when Wood Frogs emerge from overwintering but before they enter ponds to breed. This result also indicates that 4 d of increasing temperature following hibernation was insufficient to elicit the spring onset of sexual dichromatism. We note, however, that the variation in hue at this time point was substantial. For females, the coefficient of variation (CV) for hue on 15 March was 0.40 but ranged from 0.20–0.32 for all other time points. For males, CV was almost double or greater on 15 March (0.43) compared to other time points (range = 0.17–0.22). While the frequency distribution indicates a single mode for both sexes at this time point, there are many low and high values for each sex contributing to the high variation.

This variation perhaps suggests that individuals differ in the speed at which their sexual coloration is expressed after overwintering. However, we cannot discount the possibility that this variation is a by-product of the overall darker coloration at this time point and potential difficulties in discriminating subtle color differences when little light is reflected. Future work at a finer temporal resolution is needed to understand the time course of sexual dichromatism between overwintering and breeding. Currently, it is unclear which environmental conditions (e.g., light, temperature, food, etc.) influence color change after hibernation. Furthermore, it is known that mature female Wood Frogs exhibit color polymorphisms where some are redder and others tanner (Banta 1914). The extent to which this variation in mature females is genetic or due to differences in color changes after overwintering is currently unknown.

Color differences are typically believed to be a sexually selected signal of condition in species with prolonged breeding seasons, but this may be less likely in species such as Wood Frogs with abbreviated breeding seasons (*sensu* Rudh and Qvarnstrom 2013). During breeding, females hide at the bottom of vernal pools while males chorus en masse at the surface (Banta 1914). Females make short, infrequent trips to the surface and one or more males typically attempt to amplex the female quickly (Berven 1981). This behavior likely leaves little time for either sex to use color as a visual signal of condition. Dichromatism in Wood Frogs may therefore be useful for discriminating between the sexes quickly but may not necessarily be a signal of condition.

While sexual dichromatism is often attributed to sexual selection, it also potentially represents combined pressures from both natural selection and sexual selection (Bell and Zamudio 2012). For instance, coloration can be used as a form of crypsis to avoid predation or as a cue for sexual signaling (e.g., Robertson and Rosenblum 2009). We can identify two possible, non-mutually exclusive hypotheses for the color differences in Wood Frogs.

First, sexual dichromatism might represent sex-specific selection to avoid predation. Prior work found that adult female Wood Frog coloration matches terrestrial forest leaf litter better than adult male coloration (King and King 1991). This likely helps females avoid depredation during terrestrial movements. Males, on the other hand, may be under selection to be darker. Wood Frogs are explosive breeders and during breeding events in ponds, males call at the water's surface while females remain at the bottom of the pond (Banta 1914). In aggregate, male vocalizations, which only occur during breeding events in ponds, may increase male conspicuousness and therefore the risk of predation. Prior work has noted that males are dark and hard to see at the surface of ponds when they are calling but small disturbances adjacent to breeding ponds quickly result in silence (Banta 1914). While vocalizing may increase the probability of being predated upon, the camouflage provided by darker yellow or tan coloration may offset that cost. Selective pressures against predation may therefore be higher for adult females in the terrestrial environment but higher for males in the aquatic environment while breeding.

Second, sexual dichromatism may be a form of sexual signaling that facilitates the ability of males to discriminate other males from possible mates. Under severe time constraints during explosive breeding events, males must discriminate other males from females and prevent themselves from being amplexed by other males. While female Wood Frogs typically do not reach maturity until their second year post-metamorphosis, most males reach maturity as early as the spring after their first winter (Berven 1982). Prior observations and experimental work has shown that male Wood Frog size, but not age, limits reproductive success such that 1-y old males are equally as likely to successfully mate as a similarly-sized older male (Berven 1981). Males may be under strong selective pressure within their first year to become visually distinct from females to maximize their chances of successfully breeding. Doing so may readily allow males to not only distinguish females from other males but also avoid being amplexed by other males, which regularly occurs during the breeding season (Banta 1914; Berven 1981). This is similarly thought to be the ultimate mechanism driving sexual dichromatism in the Moor Frog (*Rana arvalis*), another explosive-breeding ranid frog (Ries et al. 2008; Sztatecsny et al. 2012).

Because sexual dichromatism emerges not long after metamorphosis, reproductively important

physiological pathways are likely beginning to differentiate between the sexes early in development, long before the first reproductive event. Hormonal regulation is a likely proximate mechanism for this color change. For example, in male Stony Creek Frogs (*Litoria wilcoxii*), dichromatism is induced by epinephrine, a neurohormone, but not the reproductive hormone testosterone (Kindermann et al. 2014). However, this color change is rapid, occurring within minutes, as compared to our findings in Wood Frogs where dichromatism develops over weeks or months. Reproductive hormones are perhaps more likely mechanisms and seem to play roles in sexually dimorphic coloration across different amphibian taxa (Sever and Staub 2011; Tang et al. 2014). The best example of hormonal control of dichromatism is in Reed Frogs (*Hyperolius argus*) where both sexes metamorphose at the same color, but females develop different coloration within several months after metamorphosis. Experimental work on this species indicates that estrogenic hormones play a key role in the development of sexual dichromatism (Hayes and Menendez 1999). Because estrogenic contaminants are known to impact gonadal differentiation and sex-specific development in Wood Frogs (Tompsett et al. 2013; Lambert 2015), such endocrine disrupting chemicals may also impact the development and degree of sexual dichromatism. Given amphibians are frequently exposed to estrogenic contaminants in human-altered landscapes (Lambert and Skelly 2016), future work should evaluate whether these contaminants impact sexual dichromatism and, by extension, mate recognition and reproductive success.

Gonadal differentiation occurs prior to metamorphosis in Wood Frogs (Witschi 1929; Lambert 2015; Warne and Crespi 2015). Males can begin breeding after their first year, but females typically postpone breeding until their second year (Berven 1982). Our work here, in conjunction with the hormonal experiments on Reed Frogs (Hayes and Menendez 1999), suggests that reproductive physiology in amphibians is substantially different between the sexes prior to sexual maturity. While this might not be surprising, sex-specific differences in development or ecology are seldom explored in amphibian studies. Sexual dichromatism at these early life stages might indicate that other traits, like behavior or physiology, may also be sexually dimorphic early in development. In our data, the largest color differences occurred in the second summer, months after the breeding season. However, little ecological data exists for Wood Frogs during this stage of development to suggest why the sexes are so visually distinct at this point.

Regardless of the ultimate mechanism driving sexual dichromatism in Wood Frogs, our data indicate that color patterns diverge between males and females earlier in ontogeny than may have previously been appreciated. Sex hormones, like estradiol or testosterone, are likely responsible for coloration differences corresponding to sexual maturation. As such, sexual dichromatism likely indicates that other biological systems (e.g., physiology or behavior) might diverge between males and females not long after metamorphosis but substantially before the first breeding event. The ecological and evolutionary ramifications of sex-specific phenotypes early in development are rarely considered in most taxa but likely are worthy of further research.

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Literature Cited

Banta, A.M. 1914. Sex recognition and the mating behavior of the Wood Frog, *Rana sylvatica*. Biological Bulletin 26:171–183.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1–48.

Bell, R.C., and K.R. Zamudio. 2012. Sexual dichromatism in frogs: natural selection, sexual selection and unexpected diversity. Proceedings of the Royal Society B 279:4687–4693.

Berven, K.A. 1981. Mate choice in the Wood Frog, *Rana sylvatica*. Evolution 35:707–722.

Berven, K.A. 1982. The genetic basis of altitudinal variation in the Wood Frog *Rana sylvatica*. I. An experimental analysis of life history traits. Evolution 36:962–983.

Boyer, J.F.F., and L. Swierk. 2017. Rapid body color brightening is associated with exposure to a stressor

in an *Anolis* lizard. Canadian Journal of Zoology 219:213–219.

Calisi, R.M., and D.K. Hews. 2007. Steroid correlates of multiple color traits in the spiny lizard, *Sceloporus pyrocephalus*. Journal of Comparative Physiology B 177:641–654.

Costanzo, J.P, R.E. Lee, and M.F. Wright. 1991. Effect of cooling rate on the survival of frozen Wood Frogs, *Rana sylvatica*. Journal of Comparative Physiology B 161:225–229.

Doucet, S.M., and D.J. Mennill. 2009. Dynamic sexual dichromatism in an explosively breeding neotropical toad. Biology Letters 6:63–66.

Gosner, K.L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Hailman, J.P., and R.G. Jaeger. 1974. Phototactic responses to spectrally dominant stimuli and use of colour vision by adult anuran amphibians: a comparative survey. Animal Behaviour 22:757–795.

Hayes, T.B., and K.P. Menendez. 1999. The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic Reedfrog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. General and Comparative Endocrinology 115:188–189.

Howard, R.D. 1980. Mating behavior and mating success in Wood Frogs, *Rana sylvatica*. Animal Behaviour 28:705–716.

Kindermann, C., E.J. Narayan, and J-M. Hero. 2014. The neuro-hormonal control of rapid dynamic skin colour change in an amphibian during amplexus. PLoS ONE, 9, 1–11. https://doi.org/10.1371/journal.pone.0114120.

King, R.B., and B. King. 1991. Sexual differences in color and color change in Wood Frogs. Canadian Journal of Zoology 69:1963–1968.

Lambert, M.R. 2015. Clover root exudate produces male-biased sex ratios and accelerates male metamorphic timing in Wood Frogs. Royal Society Open Science 2:150433.

Lambert, M.R., and D.K. Skelly. 2016. Diverse sources for endocrine disruption in the wild. Endocrine Disruptors, 4, 1–5. http://dx.doi.org/10.1080/23273747.2016.1148803.

Lambert, M.R., A.B. Stoler, M.S. Smylie, R.A. Relyea, and D.K. Skelly. 2016. Interactive effect of road salt and leaf litter on Wood Frog sex ratios and metamorphic sexual size dimorphism. Canadian Journal of Fisheries and Aquatic Sciences 74:141–146.

Langkilde, T., and K.E. Boronow. 2012. Hot boys are blue: temperature-dependent color change in male Eastern Fence Lizards. Journal of Herpetology 46:461–465.

Layne, J.R., and M.C. First. 1991. Resumption of physiological functions in the Wood Frog (*Rana sylvatica*) after freezing. American Journal of Physiology 261:R134–R137.

Ng, J., A.L. Kelly, D.J. MacGuigan, and R.E. Glor. 2013. The role of heritable and dietary factors in the sexual signal of a Hispaniolan *Anolis* lizard, *Anolis distichus*. Journal of Heredity 104:862–873.

Ries, C., J. Spaethe, M. Sztatecsy, C. Strondl, and W. Hodl. 2008. Turning blue and ultraviolet: sex-specific colour change during the mating season in the Balkan Moor Frog. Journal of Zoology 276:229–236.

Robertson, J.M., and E.B. Rosenblum. 2009. Rapid divergence of social signal coloration across the White Sands ecotone for three lizard species under strong natural selection. Biological Journal of the Linnaean Society 98:243–255.

Rudh, A., and A. Qvarnstrom. 2013. Adaptive colouration in amphibians. Seminars in Cell & Developmental Biology 24:553–561.

Rudgh, A., B. Rogell, and J. Hoglund. 2007. Non-gradual variation in colour morphs of the Strawberry Poison Frog *Dendrobates pumilio*: genetic and geographical isolation suggest a role for selection in maintaining polymorphism. Molecular Ecology 16:4284–4294.

Sever, D.M., and N.L. Staub. 2011. Hormones, sex accessory structures, and secondary sexual characteristics in amphibians. Pp. 83–98 *In* Hormones and Reproduction of Vertebrates, Volume 2. Amphibians. Norris, D.O., and K.H. Lopez (Eds.). Elsevier, New York, New York, USA.

Stevens, M., C.A. Párraga, I.C. Cuthill, J.C. Partridge, and T.S. Troscianko. 2007. Using digital photography to study animal coloration. Biological Journal of the Linnean Society 90:211–237.

Swierk L., S.P. Graham, and T. Langkilde. 2014. The stress of scramble: sex differences in behavior and physiological stress response in a time-constrained mating system. Behavioral Ecology and Sociobiology 68:1761–1768.

Sztatecsny, M., D. Preininger, A. Freudmann, M.C. Loretto, F. Maier, and W. Hodl. 2012. Don't get the blues: conspicuous nuptial colouration of male Moor Frogs (*Rana arvalis*) supports visual mate recognition during scramble competition in large breeding aggregations. Behavioural Ecology and Sociobiology 66:1587–1593.

Tang, Z-J., S-I. Lue, M-J. Tsai, T-L. Yu, V. Thiyagarajan, C-H. Lee, W-T. Huang, and C-F. Weng. 2014. The hormonal regulation of color changes in the sexually dichromatic frog *Buergeria robusta*. Physiological and Biochemical Zoology 87:397–410.

Tompsett, A.R., S. Wiseman, E. Higley, J.P. Giesy, and M. Hecker. 2013. Effects of exposure to 17α-ethynylestradiol during larval development on

growth, sexual differentiation, and abundances of transcripts in the liver of the Wood Frog (*Lithobates sylvaticus*). Aquatic Toxicology 126:42–51.

Troscianko, J., and M. Stevens. 2015. Image calibration and analysis toolbox - a free software suite for objectively measuring reflectance, colour and pattern. Methods in Ecology and Evolution 6:1320–1331.

Warne, R.W., and E.J. Crespi. 2015. Larval growth rate and sex determine resource allocation and stress responsiveness across life stages in juvenile frogs. Journal of Experimental Zoology 323A:179–201.

White, T.E., R.L. Dalrymple, D.W.A. Noble, J.C. O'Hanlon, D.B. Zurek, and K.D.L. Umbers. 2015. Reproducible research in the study of biological coloration. Animal Behaviour 106:51–57.

Witschi, E. 1929. Studies on sex differentiation and sex determination in amphibians II. Sex reversal in female tadpoles of *Rana sylvatica* following the application of high temperatures. Journal of Experimental Zoology 52:267–291.

Yovanovich, C.A.M., S.M. Koskela, N. Nevala, S.L. Kondrashev, A. Kelmer, and K. Donner. 2017. The dual rod system of amphibians supports colour discrimination at the absolute visual threshold. Philosophical Transactions of the Royal Society B 372:1–10.

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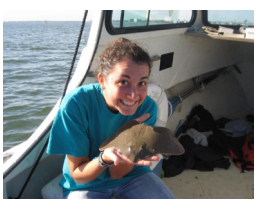

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