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Notes on post-metamorphic colour pattern changes in the Hula painted frog (*Latonia nigriventer*): How realistic is re-identification of juveniles?

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Capture-mark-recapture (CMR) approaches have been extensively used to infer behavioural or movement patterns, population dynamics, or even life cycles of target species since the late 19th century. The prerequisite for a successful application of CMR approaches is the reliable re-identification of individuals (e.g., Lebreton et al., 1992; Silvy et al., 2012). This is often achieved by the application of artificial markings, such as tissue removal (e.g., Brown and Parker, 1976; Twigg, 1978), branding or tattooing (e.g., Lindner and Fuelling, 2002; Van den Hoff et al., 2005), tagging (e.g., Gibbons and Andrews, 2004), dyeing or painting (e.g., De Sousa et al., 2012; Rice et al., 2015) or the use of external equipment (e.g., collars, backpacks, or rings; Spencer, 1978; Bethke et al., 1996; Bennett et al., 2012). Alternatively, the utilisation of natural markings, such as colour patterns (e.g., Kelly, 2001; Arzoumanian et al., 2005; Fritsch and Hobbs, 2007; Rocha et al., 2013), or characteristics of other morphological traits, such as bills, fins, horns, or tusks (e.g., Mukinya, 1976; Scott, 1978; Würsig and Jefferson, 1990; Goswami et al., 2007), can serve to characterise and re-identify individual animals. The applicability of each method depends on the size and lifestyle of the target species and, for natural markings, on the individual uniqueness of these markings or structures.

In many amphibians, distinctive natural markings, such as spots or stripes, are either lacking or too inconspicuous to distinguish between individuals, and CMR studies focusing on amphibian species therefore often apply tissue removal techniques (e.g., toe or tail clipping; Phillott et al., 2007; Grafe et al., 2011; Polich et al., 2013) or tagging techniques (e.g., passive integrated transponder [PIT] tags or implant elastomers; Schulte et al., 2007; Heard et al., 2009; Connette et al., 2011; Sapsford et al., 2015). Invasive marking techniques have the potential to inflict pain and stress on the individuals and may also negatively affect their behaviour or even decrease their chances of survival (e.g., Barron et al., 2010; Walker et al., 2011). Applying a non-invasive individual identification approach is, therefore, preferable and, indeed, there are numerous studies, especially of newt and salamander species, that

Figure 1. Adult female Hula painted frog (*Latonia nigriventer*; SVL 74.6 mm). Photo by Bina Perl.

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Table 1. Size categories used for the correlation analysis.

Category	Size range (mm)	No. of analysed individuals
C1	20–29.9	5
C2	30–39.9	2
C3	40–49.9	2
C4	50–59.9	4
C5	60–69.9	5
C6	70–79.9	5
C7	80–89.9	5
C8	90–99.9	5
C9	100–109.9	5
C10	110–119.9	5
C11	120–129.9	2
Total		**45**

successfully utilise individuals' unique ventral or dorsal patterns for individual recognition (e.g., Bradfield, 2004; Plăiaşu et al., 2005; Mettouris et al., 2016). However, the wider applicability of this approach across amphibian species is hampered by the scarcity of studies demonstrating pattern constancy, even in taxa assumed to have a highly individualised colour pattern.

It should be understood that to be utilised for reliably re-identifying individuals for capture-mark-recapture studies, a colour pattern should be unique and constant through time. In several amphibian species, post-metamorphic changes in colour patterns do occur during ontogenesis (e.g., Lantz, 1953; Fernandez and Collins, 1988; Beukema, 2011; Gollmann and Gollmann, 2011; Vaissi et al., 2017), but there is no consensus how strongly these changes affect re-identification of individuals.

The recently rediscovered Hula painted frog, *Latonia nigriventer* Mendelssohn & Steinitz, 1943, from northern Israel (Fig. 1) is a critically endangered species, and studying populations of this species requires alternatives to invasive marking techniques in view of its alarmingly low population size (Perl et al., 2018) and legislative protection. The species' conspicuous ventral spot pattern has been used for the identification of recaptured individuals, followed by genetic confirmation through DNA sequencing from mostly saliva samples (Perl et al., 2017, 2018). Our observations indicated that post-metamorphic juveniles with a snout–vent length (SVL) of < 35 mm have fewer spots than large adults (SVL > 100 mm), which might have an impact on the use of the spot pattern for re-identification.

We here present the results of our investigation to determine if the number and pattern of ventral spots in *L. nigriventer* change during ontogenesis, and whether and how this might impact the applicability of spot pattern comparisons for re-identification of individuals. We first tested if the number of ventral spots is correlated to body size, and we subsequently compared spot counts of juveniles with those of large adults, as well as those of animals of different sex. We further report on one case, in which a small post-metamorphic individual *L. nigriventer* was recaptured and successfully re-identified as a sexually mature male two years later, on the basis of its unique and individually highly characteristic ventral spot pattern.

Materials and Methods

In order to test whether the amount of ventral spots in *L. nigriventer* is correlated to individual body size (SVL), we assigned all captured individuals with SVL > 20 mm to eleven size groups of a 10 mm range (C1–C11; see Table 1 for details). To avoid any bias caused by the low number of individuals captured in some of the size categories, we randomly chose a maximum of five individuals per category using the random number

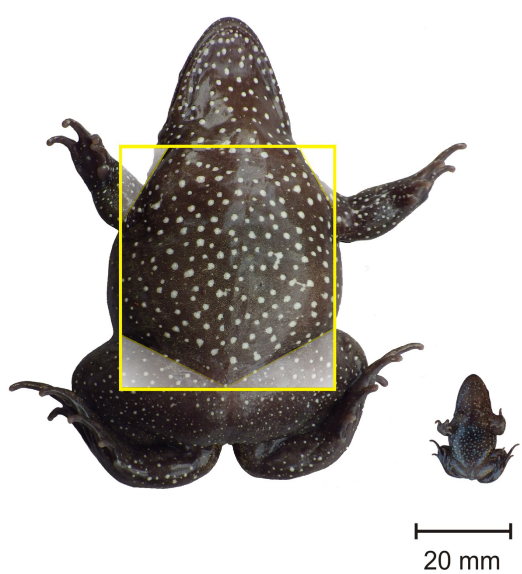

Figure 2. (Left) Adult female (SVL 95.4 mm) and (right) juvenile (SVL 22.5 mm) Hula painted frogs (*Latonia nigriventer*) in ventral view (sized to scale). The ventral area used for spot pattern comparisons is indicated by a yellow frame; shaded areas were excluded. Photos by Bina Perl.

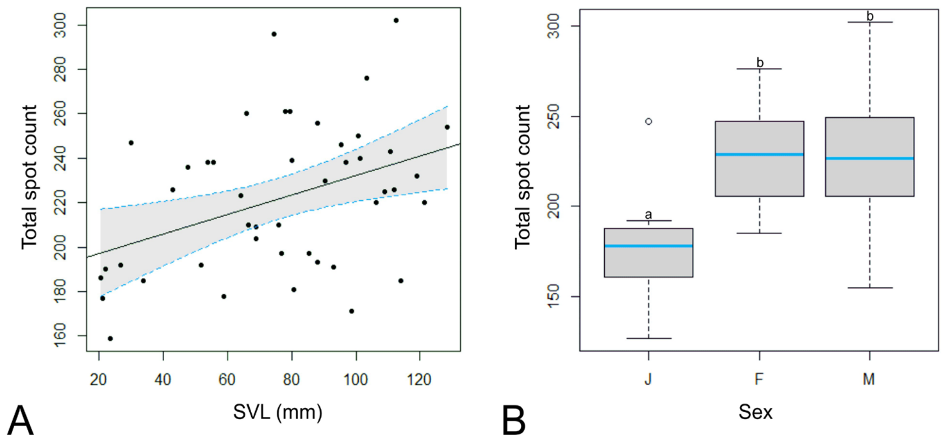

Figure 3. Comparison of the ventral spot pattern displayed by adult and juvenile Hula painted frogs (*Latonia nigriventer*) individuals; (A) Correlation between SVL and number of ventral spots ($n = 45$; $r_s = 0.355$, $P = 0.017$) identified using Spearman's Rank Correlation test. 95% confidence intervals are indicated by the shaded area. (B) Total number of ventral spots in large-sized females, large-sized males and small juveniles. Different lower-case letters indicate significant pairwise differences ($n = 60$; $P < 0.001$, Bonferroni-corrected Mann-Whitney U test). J = Juvenile, F = Female, M = Male.

generator function in MS Excel. Subsequently, the number of spots on the ventral side of each individual (excluding limb patterns distal to the limb insertion and anterior to the scapula; Fig. 2) were counted by the same person. Spearman's rank correlation test was employed to examine the relation between SVL and number of ventral spots. In a second analysis, based on the data of the 20 largest captured females and the 20 largest captured males (SVL 100–129.9 mm), as well as the 20 smallest captured juveniles (SVL 16.2–33.8 mm), we tested for general differences in the ventral spot counts of (1) large-sized females (mean SVL 107.7 mm), (2) large-sized males (mean SVL 108.6 mm), and (3) small juveniles (mean SVL 23.4 mm). Statistical analysis was performed by pairwise Mann-Whitney-U-test using Bonferroni correction. Furthermore, we compared the ventral spot pattern on images taken from a post-metamorphic individual of *L. nigriventer* that was initially captured with a SVL of 21.2 mm and recaptured as a sexually mature male with a SVL of 80.8 mm after two years.

In all cases, we assessed the number of ventral spots using photographs of the ventral patterns of each individual (Fig. 2). For taking the photographs, live individuals were positioned on their dorsal side, after which they tended to remain motionless for up to about 30 s. All photographs were taken against the same neutral grey background from a 90° angle. In order to avoid unwanted light reflections on the frogs' skin, individuals were shielded from direct sunlight. We quantified the spots by counting and marking them manually utilising ImageJ software (Abràmoff et al., 2004; Schneider et al., 2012). All statistical analyses were performed using R 3.1.1 (R Core Team, 2013) including the R package 'stats'. Plots were generated with the R package 'graphics'.

Results

Our results revealed a relatively weak but significantly positive correlation between SVL and number of ventral spots ($r_s = 0.355$, $P = 0.017$, Spearman's Rank Correlation Test; Fig. 3A). The comparison between sexes (large adult females vs. large adult males) revealed no significant differences in the number of ventral spots ($P = 1.0$; Bonferroni-corrected Mann-Whitney-U-test; Fig. 3B). However, the differences between females and juveniles, as well as males and juveniles, each were strongly significant ($P < 0.001$ in both comparisons; Bonferroni-corrected Mann-Whitney-U-test; Fig. 3B).

We found that the configuration of the ventral pattern of the individual captured shortly after metamorphosis and recaptured as a sexually mature male two years later, did not substantially change during these two years (Fig. 4), even though the blurriness of the pattern decreased markedly over the years. Overall, the spot configuration remained the same and the individual was unequivocally re-identified. The fewest changes

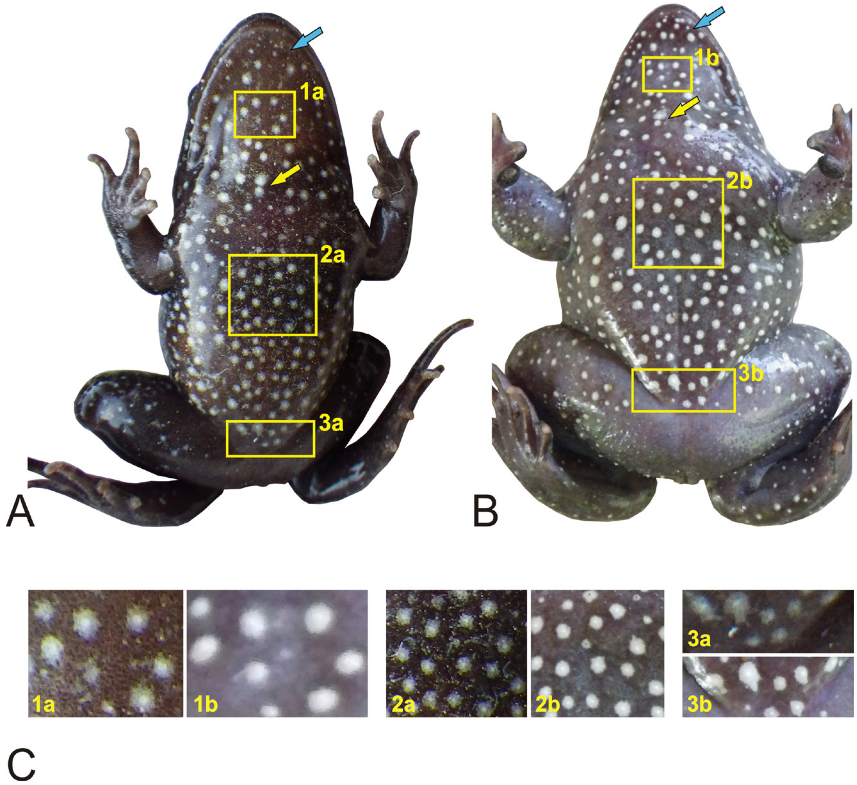

Figure 4. Comparison of the ventral spot pattern displayed by the same Hula painted frog (*Latonia nigriventer*) individual (A) as a juvenile (SVL = 21.2 mm, captured on 5 Feb 2014) and (B) as a sexually mature adult male (SVL = 80.8 mm, captured on 16 May 2016). Yellow frames (1–3) and yellow arrow highlight the same and almost unchanged skin areas in (A) and (B), the blue arrow indicates a skin area where the configuration of the spot pattern slightly changed within the two years between the two photographs. In (C) the framed areas are enlarged and arranged side by side to allow a better comparison. Photos by Bina Perl.

were observed in the pelvic region (see 3a and 3b in Fig. 4), while some differences in the expression of the spot pattern existed in the chin region (blue arrows in Fig. 4). Despite these little differences, individual spot elements could still be traced. Nevertheless, all in all, only 75% of the spots that were discernible in the adult individual were also discernible in the post-metamorph (post-metamorph = 164 spots, adult = 218 spots).

Discussion

The reliable identification of individuals is essential for many population-based studies, and recaptures after long time spans are particularly valuable to assessing growth and survival rates. Natural markings, if present and unique for each individual of the focal species, offer obvious advantages over artificial markings, which are either non-permanent and thus cannot be used for identifying single individuals throughout their lifetime, or are invasive and may adversely affect an individual's health and behaviour (e.g. Walker et al., 2011).

In our study of *L. nigriventer*, we found that the number of ventral spots was correlated to body size, even though the correlation was rather weak. In direct comparison, large females and males had a significantly greater number of spots than small juveniles, while no differences were detected between sexes. However, even though these results suggest a substantial ontogenetic change unlinked to sex, the comparison of the spot pattern exhibited by a single individual that was captured as post-metamorph and recaptured as sexually mature adult showed that the configuration of the spots did not change substantially. Even the shape and the proportions of the spots remained largely the same. However, 25% fewer spots were discernible in the post-metamorph. Taking into consideration that the individual's size increased four-fold in the two years between the two captures, and that the overall spot configuration and proportions did not change markedly, it may well be that the spots were already present in the post-metamorph, yet only noticeable as blurriness.

We acknowledge that the best means to investigate this topic would have been to compare the spot pattern of the same individuals over several years, and with larger sample sizes. However, as a critically endangered species under strong legislative protection, husbandry of *L. nigriventer* is currently not permitted. Although a sample size of one is not enough to draw broad conclusions, our current results suggest that the ventral pattern of Hula painted frogs may not dramatically change during ontogenesis despite an overall increase in visible spots. The general configuration and proportion of spots remain largely the same, and the individual could easily be re-identified, even though some of the smaller spots were not yet fully discernible in the small post-metamorph. Based on our results, and considering that a small juvenile with a SVL of 21.2 mm could be successfully re-identified, we conservatively suggest that the ventral spot pattern in *L. nigriventer* is unique enough to unambiguously identify adult individuals, and probably also individuals with a SVL > 25 mm. However, more data will be required to assess the suitability of the ventral pattern for identifying smaller juveniles with a SVL < 25 mm, as it is unlikely that the spot pattern would already be sufficiently expressed directly after metamorphosis which occurs at 6–9 mm SVL (Perl et al., 2017).

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