Effects of background color on pigmentation, morphological traits, and behavior in the European tree frog (*Hyla arborea,* Hylidae, Anura) tadpoles

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Abstract

Amphibian tadpoles are capable of avoiding threats (predators, UV radiation, etc.) through changes in coloration, behavior, and shape. In this paper, we tested how quickly European tree frog (*Hyla arborea*) tadpoles can change body pigmentation to achieve crypsis and whether color change is reversible. Additionally, we tested how different environmental background colorations affect the body length, shape, and ontogenetic trajectories of tadpoles. We also analyzed if tadpoles can relate to their coloration and choose the appropriate background to enhance crypsis. For this purpose, we reared tadpoles on white and black backgrounds for 36 days. Halfway through the experiment, half of the tadpoles from each treatment were placed on the alternative background. Our results suggest that *H. arborea* tadpoles are capable of rapidly responding to color changes in their environment, however, color-matching with the white background is poor. These quick color changes are reversible. Rearing in different background

coloration and rapid color changes do not affect tadpoles' length variation but affect tadpoles' shape. Tadpoles introduced to the white background at the start of the experiment developed deeper tail fins and more pronounced snouts. We also found that *H. arborea* tadpoles actively choose an appropriate background to achieve maximum crypsis. This study represents the basis for the future analysis of adaptive coloration in tadpoles as it has a very complex function in anurans.

Introduction

Phenotypic plasticity, especially in traits that enhance the defensive ability of the organisms against predators is widespread in aquatic vertebrates, such as fish and amphibians (e.g., Brönmark & Miner, 1992; McCollum & Leimberger, 1997; Van Buskirk & Schmidt, 2000). Even though plasticity is costly and it is hard quantifying the cost (Van Buskirk & Saxer, 2001), the most observed plastic traits in amphibians are coloration and shape.

Defensive coloration is a widespread phenomenon in animals and can manifest as camouflage, aposematism, and mimicry (Endler, 1978; Stevens & Merilaita, 2009). Animals can camouflage from predators in three main ways: background matching coloration or crypsis (color and pattern matching between animals and a random sample of the background); masquerade (resemblance of some part of the background e.g., leaf or rock); dazzle or motion camouflage (impairing estimates of trajectory and speed of movement by the predator) (Endler, 1978; Stevens & Merilaita, 2009). Crypsis is the most used concealing technique among insects, fish, and amphibians (Sumner, 1940; Brakefield, 1996). For many amphibians, crypsis is the primary line of defense against predators (Wells, 2007). In addition to crypsis, amphibians can alter their color for thermo- and hydro regulation,

and protection against UV radiation (Hoppe, 1979; King et al., 1994; Rodrígez-Rodrígez, et al., 2020). However, it seems that color change is most influenced by crypsis (Kats & Van Dragt, 1986).

Amphibians can change coloration as tadpoles and as adults (Thibaudeau & Altig, 2012; Hoffman & Blouin, 2000) rapidly or slowly. Rapid color changes are achieved through re-arranging pigment particles inside chromatophore cells (Bagnara & Hadley, 1973) – tadpoles get darker by pigment aggregation and lighter by pigment dispersion. This kind of change is reversible and is important for animals living in heterogeneous habitats with temporal and spatial changes in background coloration. This is especially crucial for amphibian tadpoles who are daily migrating from deeper to shallower parts of ponds and back (Beiswenger, 1975) where light conditions change, and surfaces can differ in coloration patterns. On the other hand, slow and permanent changes in coloration occur when environmental stimuli are long-lasting and tadpoles start to change pigment content and the number of chromatophores (Sumner, 1940). Many abiotic factors can affect skin pigmentation and the rate of coloration change but the most influential ones are background coloration, light intensity, and ambient temperature (King et al., 1994; Rodrígez-Rodrígez et al., 2020).

Besides color change, another subset of phenotypic responses to environmental stimuli is related to the phenotypic plasticity of tadpoles' external morphology, especially tail-shape plasticity. Depending on the predator type, tadpoles can develop bigger or smaller bodies and deeper or shallower tail fins. When exposed to small and solitary predators that cannot swallow the whole prey, tadpoles develop smaller bodies and deeper tail fins (McCollum & Leimberger, 1997; Wilson et al., 2005; Benard, 2006; Touchon & Warkentin, 2008). In this way, tadpoles are slower but with larger tails, they can attract predator strikes away from the more vulnerable head-body region and increase survival (Van Buskirk & McCollum, 2000; Van Buskirk et al., 2003, 2004; Wilson et al., 2005). In contrast, if tadpoles are exposed to large predators who can swallow the whole prey and predators who hunt in groups, tadpoles develop shallower tail fins (McCollum & Leimberger, 1997; Wilson et al., 2005; Benard, 2006; Touchon & Warkentin, 2008) which enables them to swim faster and escape when threatened (Van Buskirk & McCollum, 2000; Wilson et al., 2005).

Another important aspect of the response to environmental stimuli is a behavioral defense which can be tightly tied to defensive coloration. Behavioral defense in anuran tadpoles includes spatial avoidance, hiding, and decreasing activity levels (Teplitsky & Laurila, 2007). Some tadpoles, when threatened, decrease their activity (Heyer et al., 1975; Azevedo-Ramos, 1992; Nomura et al., 2003; Van Buskirk & Arioli, 2005), while others do not (Heyer et al., 1975; Brodie & Formanowicz, 1983; Azevedo-Ramos, 1992). Tadpoles that decrease their activity levels are cryptically colored (D'Heursel & Haddad, 1999) and with a lower probability to be detected by visual predators (Morey, 1990). On the other hand, tadpoles that maintain the same levels of activity usually have aposematic coloration (D'Heursel & Haddad, 1999).

An interesting, yet rarely explored question of amphibian behavioral threat avoidance is whether tadpoles and adults can recognize and choose a background that enhances their crypsis ability. Empirical evidence of individual preference for the appropriate background is mainly found in insects (Kettlewell, 1955; Boardman et al., 1974; Gillis, 1982; Anhesjö & Forsman, 2006), and for amphibians is usually assumed (Eterovick et al., 2010; Nomura et al., 2013). However, Polo-Cavia and Gomez-Mestre (2017) found partial evidence that newt tadpoles prefer background matching their induced phenotypes thus increasing crypsis abilities.

Frogs of the *Hyla* genus are well known to change colors in response to different factors (e.g., Wente & Phillips, 2003; Stegen et al., 2004; Degani & Biton, 2013; Choi & Jang, 2014; Kang et al., 2016). The European tree frog (*Hyla arborea*) has the potential to be an excellent model species to test hypotheses of the adaptive significance of colors as their adults have a great color-changing ability, and are superior in color-matching compared to closely related species (Nielsen, 1980). However, there are almost no papers dealing with color change in *Hyla* tadpoles (e.g., D'Heursel & Haddad, 1999; Kruger & Morin, 2020). Therefore, our study aims to identify the effects of dark and light background colors on the European tree frog (*Hyla arborea*) tadpoles' body coloration, body length, body and tail shape, and behavior during their early development. We tested (1) whether the background coloration can (and how quickly) induce tadpoles' color change for background matching, (2) if induced tadpole coloration is reversible, (3) if different background colors affect variation in tadpoles' body length, body/tail shape, and ontogenetic trajectories, and (4) if tadpoles exhibit cryptic behavior.

Material and methods

Study animals and experimental design

Hyla arborea eggs were collected from Reva pond, located in the vicinity of Belgrade in April 2021. The eggs were transported and reared at the Department of Evolutionary Biology, Institute for Biological Sciences, University of Belgrade. The experiment started when tadpoles reached Gosner stage 25 (Gosner, 1960). We haphazardly chose 80 specimens for the experiment. All of the remaining tadpoles were returned to Reva pond. Each of the experimental tadpoles was housed alone in plastic containers ($15.5 \times 14.5 \times 6.5$ cm) filled with one liter of dechlorinated tap water and reared at constant room temperature (20ºC) and natural photoperiod (14L/10D h). Containers were placed in the laboratory with windows and air conditioning but were not directly exposed to the sunlight or air conditioning airflow. Containers were arranged in four 4×5 blocks (two blocks of black boxes and two blocks of white boxes) and the blocks and boxes changed place haphazardly every four days. Containers were cleaned and the water was changed every four days, too. Tadpoles were fed *ad libitum* every two days with commercial fish food tablets (Tetra TabiMin®, Tetra GmbH, Melle, Germany). Containers were checked daily for the well-being of the animals.

On the first day of the experiment, all individuals were haphazardly assigned to one of two treatments: D – dark background treatment with 40 containers wrapped in a black paper sleeve placed on a black surface imitating dark conditions and L – light background treatment with 40 containers wrapped in a white paper sleeve placed on a white surface imitating light conditions. After twenty days of the experiment, we inverted the rearing conditions of tadpoles, and half of the sample (20 individuals) from the D treatment was moved from black to white boxes (DL treatment), and half animals (20 individuals) from the L treatment were switched from white to the black boxes (LD treatment). The remaining 20 individuals from the D and L treatments were kept in their original conditions (now DD and LL treatments) (fig. 1).

The collection of *H. arborea* eggs from the natural population was approved by the Ministry of Environmental Protection of the Republic of Serbia (permit no. 353-01-2716/2020-04). The experimental design and procedures were approved by the Ethical Committee of the Institute for Biological Research "Siniša Stanković", University of Belgrade (permit no. 01-739). All experimental procedures complied with the European Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes.

Pigmentation

To establish whether background coloration affects variation in *H. arborea* tadpole skin pigmentation, high-resolution images of the dorsal side of 50 animals in total were taken periodically (table 1). The images were taken using a DSLR camera Nikon D7500 equipped with AF-S DX Micro 40mm f/2.8G NIKKOR lens, mounted perpendicularly to the animals and at a fixed height.

Photographs were processed in Adobe Photoshop CC 2015 (Adobe System Inc., 2015). We defined pigmentation as a percentage of dark pixels on the tadpole's body following the methodological approach described by Mayani-Parás et al. (2015). We detected the available RGB range and used the mean value as a threshold for the given treatment/date group – pixels with lower RGB values were described as light and with higher values as dark. The body part was selected using the magic wand tool from the whole image, and the percentage of dark pixels was read from the histogram.
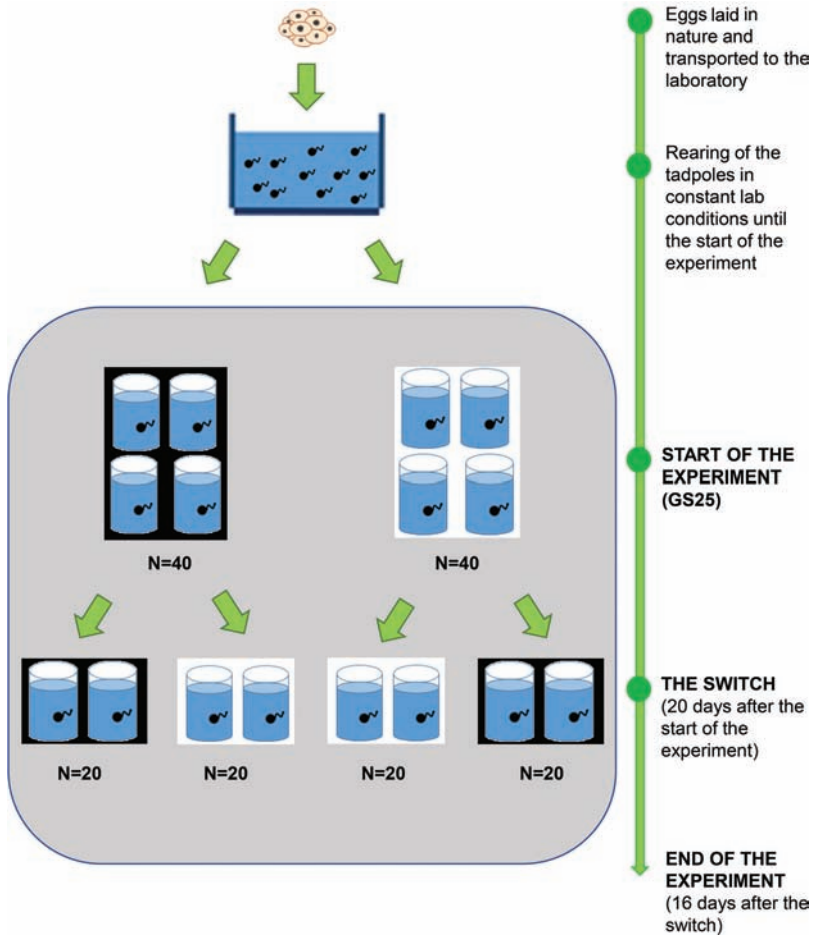
FIGURE 1 Experimental design of the study. N – sample size; GS – developmental stage by Gosner, 1960

TABLE 1 The complete schedule when appropriate data was collected during the experiment.

Day	0	4	12	20	24	28	36
Body pigmentation	✓	✓	✓	✓	✓	✓	✓
Body length	✓	✓	✓	✓	✓	✓	✓
Body shape	×	×	×	✓	×	×	✓
Ontogenetic trajectories	×	×	×	✓	×	×	✓

Body length, shape, and ontogenetic trajectories

To describe if the development in different background coloration conditions has an effect on the variation of tadpole size and shape we used traditional and geometric morphometrics. On several occasions, body length measurements were obtained (table 1) from dorsal side pictures. For the calculation of the body length, we used the tpsDig

program (Rohlf, 2006) and the tmorphgen from the Integrated Morphometrics Program (IMP) package (Sheets, 2000).

For body shape and ontogenetic trajectory analysis specimens were photographed laterally on two occasions – right before "the switch" – when only two treatments existed (D and L) and at the end of the experiment – when four treatments existed (DD, DL, LL, and LD) (table 1). For this purpose, animals were placed in tiny glass tanks with the camera placed in front at a fixed distance. Lateral images were taken only on two occasions to prevent inflicting excessive stress on the animals. For the lateral images, animals were placed in a small glass tank and multiple images of every tadpole were taken before they reached the bottom of the tank so the shape of the tadpoles will not be affected.

The body shape was described by placing 3 landmarks and 17 semi-landmarks on lateral images using the tpsDIG program (Rohlf, 2006) (fig. 2). For positioning the landmarks and semi-landmarks we followed Pujol-Buxó et al. (2020). The position of the semi-landmarks was predetermined in the MakeFan6 program from the IMP package (Sheets, 2000). Centroid size (CS) was calculated in images using the CoordGen from the IMP package (Sheets, 2000). Generalized Procrustes Analysis (GPA), shape analysis, and visualization of the shape variation between groups were performed in the MorphoJ program (Klingenberg, 2011).

To analyze the ontogenetic trajectories of tadpoles from different treatments we used phenotypic trajectory analysis (Adams & Collyer, 2007, 2009; Collyer & Adams 2007, 2013). We analyzed total trajectory distances which represent the amount of shape change exhibited by the tadpoles from different treatments and trajectory correlation (angles) which represent the direction of the exhibited changes.

Background choice behavior

We performed a short-term laboratory experiment to examine if background choice differed between tadpoles from different treatments and if this was affected by previous experience with the background. After 36 days of rearing in different background
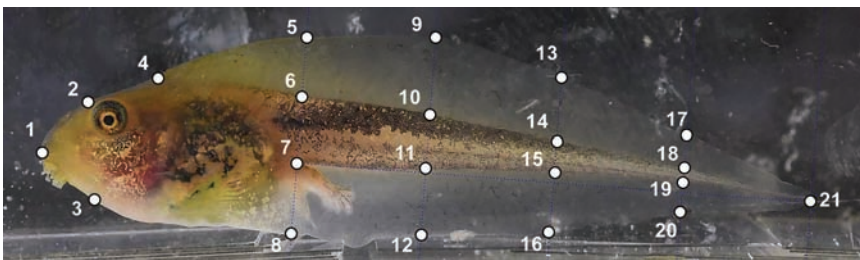

FIGURE 2   Position of landmarks (L) and semi-landmarks (SL): L 1 – the tip of the snout, L 2 & 3 – dorsal and ventral points of anterior eye edge, L 4 – the intersection of head-body and dorsal edge of the tail fin, L 7 – the intersection of head-body and the ventral edge of the tail muscle, SL 5, 6 & 8 – the dorsal side of the tail fin, the dorsal side of the tail muscle, the ventral side of the tail muscle, the ventral side of the tail fin, all in the same vertical line as L 7, SL 9–12 – the dorsal side of the tail fin, the dorsal side of the tail muscle, the ventral side of the tail muscle, the ventral side of the tail fin ¼ the distance between L 7 and L 21, SL 13–16 – the dorsal side of the tail fin, the dorsal side of the tail muscle, the ventral side of the tail muscle, the ventral side of the tail fin ½ the distance between L 7 and L 21, SL 17–20 – the dorsal side of the tail fin, the dorsal side of the tail muscle, the ventral side of the tail muscle, the ventral side of the tail fin ¾ the distance between L 7 and L 21, L 21 – the tip of the tail

coloration conditions, 50 tadpoles, that were photographed for the analysis of the pigmentation were individually introduced in 20l containers ($42 \times 32 \times 21.5$ cm) filled with dechlorinated tap water. One half of every container was painted white, while the other was painted black. After several minutes of tadpole acclimatization to the new environment, we started taking pictures from above the containers at one-minute intervals for 30 minutes. From these pictures, we were able to calculate how many times were tadpoles recorded in each background and how many times they changed the background. After the experiment, tadpoles were brought back to their original containers and fed.

The next day, after the tadpoles rested, we repeated the experiment. This time, in each 20 l container we added 0.5 l water from the tank where adult *Triturus macedonicus* newts are fostered. The water containing *Triturus* chemical cues was taken from the 250 l tank (radius: 600 mm, height: 900 mm) where 15 newts were housed. Newts are known predators of frog eggs and tadpoles (Griffiths & Mylotte, 1987; Henrikson, 1990). In this way, we wanted to see how the presence of predator chemical cues will affect the cryptic behavior of *H. arborea* tadpoles.

Statistical analysis

We used repeated measurements ANOVA with the date as the dependent variable and treatment as a factor to compare body pigmentation among groups. Firstly we compared D and L treatments before the switch and secondly, we compared four treatments DL, DD, LD, and LL after the switch. Furthermore, the differences between the four treatments were analyzed using the Tukey post hoc test. According to the Kolmogorov-Smirnov test, our data had a normal distribution of residuals, but Mauchly's test of sphericity showed that our data had unequal variances, so we used the Huynh-Feldt estimate of epsilon for

the correction of ANOVA's overall significance value (Shin, 2009). All statistical tests for analysis of pigmentation were done in SPSS Statistics 26 (IBM Corp., 2019) software.

Body length data were collected on six occasions, so we used repeated measurements ANOVA with the date as the dependent variable and treatment as a factor. Again, firstly we compared the D and L treatments before the switch, and secondly, we compared four treatments after the switch. Data had a normal distribution but unequal variances, so the Greenhouse-Geisser estimate of epsilon was used for the correction of ANOVA's P-value (Shin, 2009). All analyses were done in SPSS Statistics 26 (IBM Corp., 2019).

For analyzing variation in body shape between treatments in the two time points, firstly we performed GPA followed by Procrustes ANOVA. To account for any allometric effect we performed regression with log CS. To determine between-group differences and visualize shape variation Canonical Variate Analysis (CVA) on regression residuals was conducted. All of the specified analyses were done in MorphoJ (Klingenberg, 2011).

To quantify the shape change patterns between treatments in the two time points we use phenotypic trajectory analysis with different attributes of ontogenetic trajectories (distance and correlation) calculated using *trajectory.analysis* function from R package RRPP (Collyer & Adams, 2018, 2019) in RStudio 2021.09.2 (RStudio, PBC, 2009–2022).

To account for any differences in behavior among treatments, as a result of developing in different background coloration conditions, data collected during the behavioral experiment were analyzed using the G test (Woolf, 1957). We compared how many times on average tadpoles from each group were detected in each background. Additionally, we compared how many times on average tadpoles from each group changed the background.

Results

Pigmentation

Tadpoles placed in the L treatment developed lighter pigmentation, while tadpoles from the D treatment become much darker. The maximum dark pigmentation in the D treatment was reached on day 20 with a 24% pigmentation increase from the beginning of the experiment (pigmentation on day 0 of the experiment was 69% of dark pixels). On the other hand, the minimum pigmentation of tadpoles from the L treatment was registered already on day 4, with a 19% pigmentation decrease. Repeated measurements ANOVAs for body pigmentation revealed that groups had significantly different skin pigmentation only four days after the start of the experiment (F = 43.308; P < 0.001), and the coloration of the tadpoles continued to change between groups and dates (F = 1.4; P < 0.001). Interestingly, after day 4, animals from the L treatment grew somewhat darker, but animals from the D treatment were significantly darker than those in the L treatment on every occasion (P < 0.001 for all three comparisons) (figs. 3 and 4).

After the switch to the alternative background, there were no statistically significant differences in pigmentation of DD treatment between the dates until the end of the experiment (F = 0.148; P = 0.862). Specimens from LL treatment continued to become insignificantly darker as they grew bigger but were still much lighter than specimens from DD treatment (P < 0.001 for all three comparisons). Alternatively, animals from DL and LD treatments started changing pigmentation levels immediately after they were introduced to alternative treatments. After only four days (day 24 of the experiment) specimens from DL and LD treatments already achieved pigmentation levels of their counterparts from LL and DD treatments (DL/LL, P = 0.595;
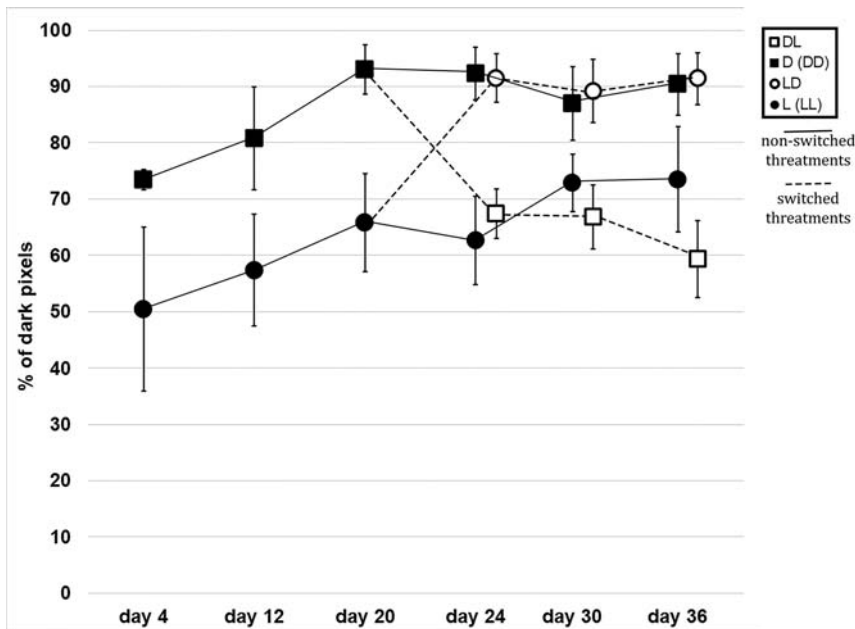

FIGURE 3   Variation in body pigmentation between different background coloration treatments during ethe xperimental time in *H. arborea* tadpoles. DL – dark-light treatment; D – dark treatment; DD – dark-dark treatment; LD – light-dark treatment; L – light treatment; LL – light-light treatment
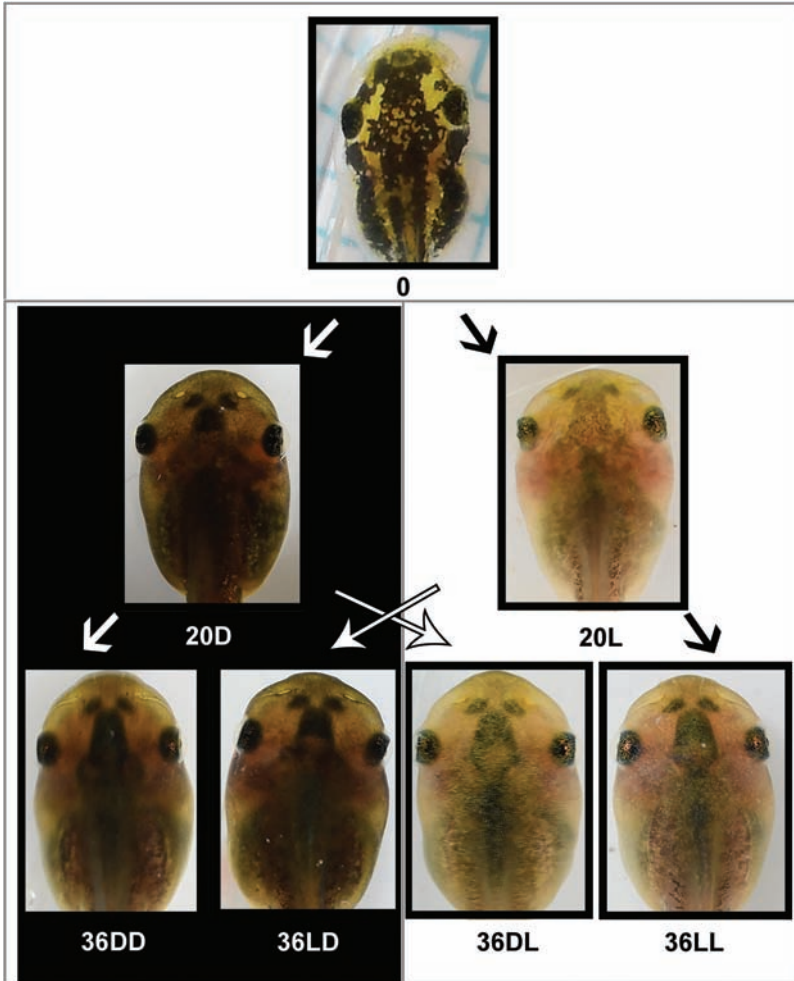
FIGURE 4   The tadpole body coloration by treatment: day 0 – the start of the experiment, average pigmentation 69% of dark pixels, no treatment groups; day 20 of the experiment (day 20) – two treatment groups, Dark and Light, average pigmentation D – 93% and L – 62% of dark pixels; day 36 – the end of the experiment (day 36) – four treatments, DD – dark-dark treatment, LD – light-dark treatment, DL – dark-light treatment, LL – light-light treatment, average pigmentation DD – 90%, DL – 60%, LD – 91%, LL – 70% of dark pixels.

LD/DD, P = 0.485) which also resulted in a significant difference between them (P < 0.05). Tadpoles from LD treatment increased pigmentation level by 25% and until the end of the experiment did not change pigmentation, hence no difference between LD and DD groups was observed. Tadpoles from DL treatment initially decreased pigmentation level by 26% and slowly continued to get lighter. On day 28 of the experiment, there was no difference in pigmentation levels between DL and LL groups (P = 0.052), but by day 36 the DL group was significantly lighter than the LL group (P < 0.001) (figs. 3 and 4).

Body length, shape, and ontogenetic trajectories

Repeated measurements ANOVAs showed that treatment did not affect body length variation (F = 1.087; P = 0.369). The only difference was
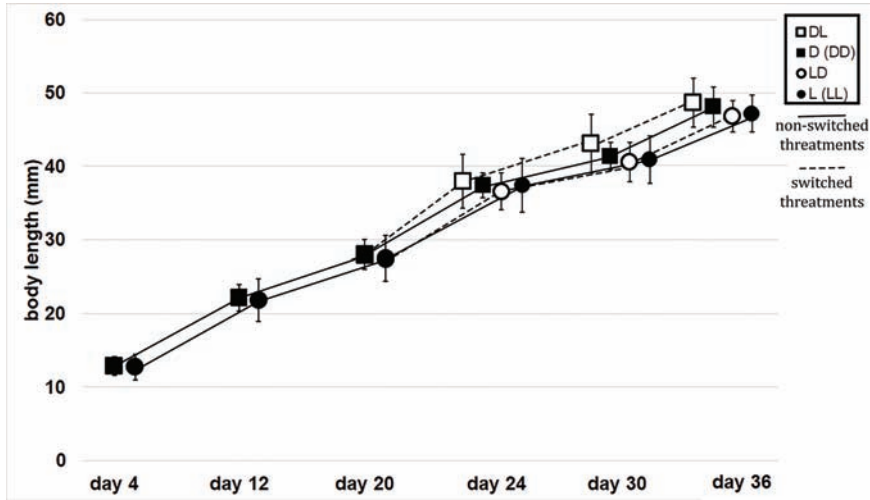
FIGURE 5   Body length variation between different background coloration treatments during experimental time
in *H. arborea* tadpoles. DL – dark-light treatment; D – dark treatment; DD – dark-dark treatment; LD –
light-dark treatment; L – light treatment; LL – light-light treatment.

observed between the days of the experiment
(F = 924.854; P < 0.001) (fig. 5).

Procrustes ANOVA showed a significant dif-
ference in body shape between treatments at
two time points (F = 2.84; P < 0.001). A closer
investigation with CVA showed how speci-
mens from each group are distributed in a
morphospace bounded by CV1 and CV2 axes
(fig. 6). The CV1 axis explains 36.40% and the
CV2 axis explains 22.90% of overall shape var-
iation. Generally, specimens who started the
experiment in dark treatment (groups 20 D,
36 DD, and 36 DL) were separated along the
CV1 axis with more rounded snouts and shal-
lower fin tails from those who started in light
treatment (20 L, 36 L, and 36 LD). CVA also
revealed individual differences between treat-
ments at different time points: after twenty
days of the experiment, D and L had signifi-
cantly different shapes (P < 0.05), and at the
end of the experiment the only statistically
significant difference in shape was registered
between DL and LL treatment (P < 0.05).

Ontogenetic trajectories are shown in fig.
7. Phenotypic trajectory analysis revealed

that the longest trajectory distance (the larg-
est amount of shape change) between day
20 and day 36 was exhibited by DL treatment
(trajectory distance 1574.0960) followed by LD
treatment (trajectory distance 1171.6769), DD
treatment (trajectory distance 970.4235), and
LL treatment (trajectory distance 712.8309).
A comparison of the absolute differences
in trajectory distances between treatments
revealed a significant statistical difference in
trajectory distances only between LL and DL
treatments (P = 0.015). Comparison of corre-
lation (angles) between trajectories revealed
that the direction of the shape changes only
marginally differed between DD and DL
treatment (P = 0.05).

Background choice behavior
The last background coloration in which the
tadpoles were reared had a significant effect
on background choice behavior. DD treat-
ment spent the most time in black conditions
(90% of the time), followed by LD treatment
(63% of the time), LL treatment (40% of the
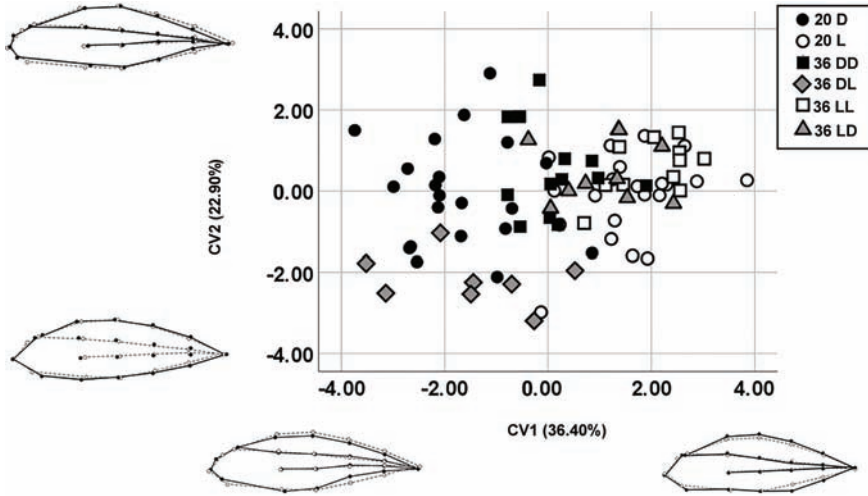time), and the least amount of time in black

FIGURE 6   Mean body shape of each treatment in two time points (after 20 days of the experiment – two treatments, and after 36 days/at the end of the experiment – four treatments) visualized in the canonical variate space (CV1 vs. CV2). 20 D – dark treatment after 20 days; 20 L – light treatment after 20 days; 36 DD – dark-dark treatment after 36 days; 36 DL – dark-light treatment after 36 days; 36 LL – light-light treatment after 36 days; 36 LD – light-dark treatment after 36 days.
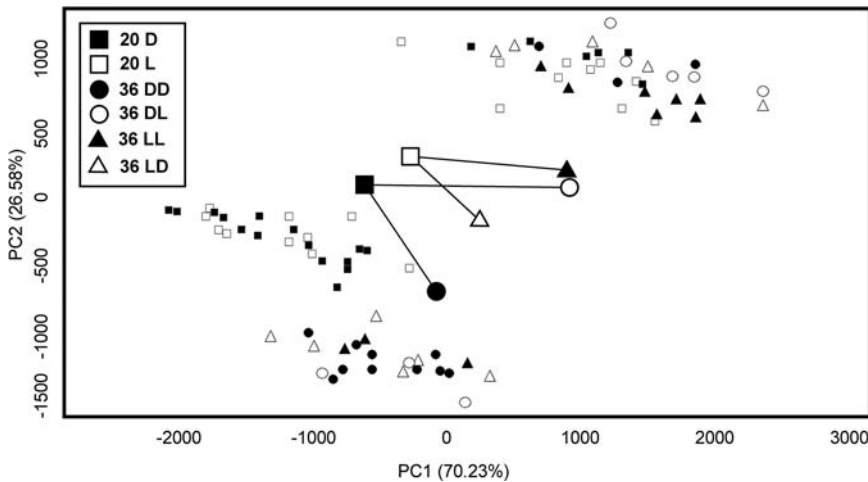

FIGURE 7   Ontogenetic trajectories of each treatment in two time points (after 20 days of the experiment – two treatments, and after 36 days/at the end of the experiment – four treatments) visualized in the space of principal components (PC1 vs. PC2). 20 D – dark treatment after 20 days; 20 L – light treatment after 20 days; 36 DD – dark-dark treatment after 36 days; 36 DL – dark-light treatment after 36 days; 36 LL – light-light treatment after 36 days; 36 LD – light-dark treatment after 36 days.

conditions spent DL treatment (36% of the time) (fig. 8). Pairwise comparisons revealed that DD specimens spent significantly more time in the dark background than specimens from the LL treatment ($P < 0.05$). Also, DD specimens spent significantly more time in the dark background from both switched treatments (DD/LD $P < 0.05$; DD/LL $P < 0.05$).
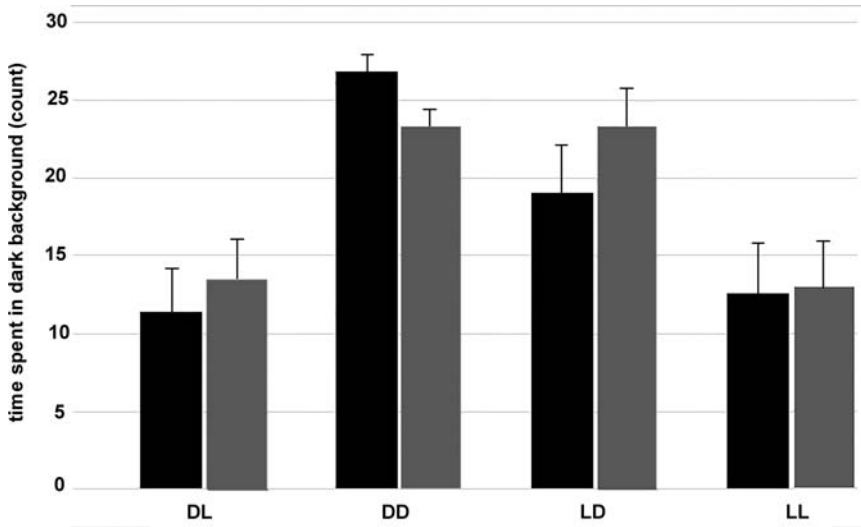
FIGURE 8   How many times on average (with standard error) *H. arborea* tadpoles from different treatments were
            detected in the dark background: without predator chemical cues (black bars), with predator chemical
            cues (grey bars). DL – dark-light treatment; DD – dark-dark treatment; LD – light-dark treatment;
            LL – light-light treatment.

Even though specimens from the LD treatment showed a preference for the dark background, they spent significantly more time in the dark part of the container only from the DL treatment (P < 0.05), while there was no statistically significant difference from the LL treatment (P = 0.09). Also, there was no significant difference in time spent in black conditions between LL and DL treatments (P = 0.76). There was no statistically significant difference between treatments in the number of movements between different backgrounds (P = 0.55).

After the introduction of the predator chemical cues, specimens from different treatments did not spend significantly different amounts of time in black conditions than in the previous experiment (P > 0.55 for all comparisons). However, the LD group spent more time in black and pairwise comparisons revealed that tadpoles from DD and LD treatments spent statistically more time in black than tadpoles from LL and DL (P < 0.55 for all comparisons), while there was no difference between DD and LD and between LL and DL treatments (P > 0.55 for both comparisons) (fig. 8).

Discussion

Our study showed that *Hyla arborea* tadpoles can rapidly change body skin pigmentation when exposed to different backgrounds. The capability of amphibians to change skin pigmentation in different environments is well documented (e.g., Altig & Channing, 1993; Hoffman & Blouin, 2000; Thibaudeau & Altig, 2012; Nilsson Sköld et al., 2013) but rarely is tested how quickly this change occurs. According to our results, only four days after being exposed to the white and black backgrounds, tadpoles exhibited an appropriate color change. Tadpoles from the dark treatment reached the maximum amount of body pigmentation after 20 days, while tadpoles from the light treatment reached minimum body pigmentation already after four

days. The percentages of body pigmentation increase in the dark treatment and decrease in the light treatment were fairly similar. Our finding suggests that changes in skin pigmentation are rapidly occurring and are in concordance with the findings of Polo-Cavia and Gomez-Mestre (2017) and Rodrígez-Rodríguez et al. (2020) who also described rapid changes in skin pigmentation in newt tadpoles *Lissotriton boscai* and frog tadpoles *Alytes dickhilleni* respectively. Rapid skin color changes are also documented in adult frog *Alytes obstetricans* (Polo-Cavia et al., 2016).

In addition, we tested if these color changes are reversible by placing half of the tadpoles on the alternative background. Again, only four days after the switch to the alternative background, tadpoles from DL and LD treatments matched the color of their treatment counterparts (LL and DD respectively), even though the amount of change was considerably larger than after the first four days of the experiment. During this time tadpoles from the two switch treatments reached their maximum and minimum body skin pigmentation. Again, percentages of pigmentation increase and decrease were similar. Quick reversibility of skin coloration was also documented in *Lissotriton boscai* (Polo-Cavia & Gomez-Mestre, 2017). At the end of the experiment tadpoles from the DD and LD treatments had the same level of pigmentation, while tadpoles from DL were lighter than those from the LL treatment. Considering this and taking into account that the minimum pigmentation of LL treatment was reached after four days of the experiment we can conclude that the light background provokes a more extreme reaction. This may be because lightening comes at a lower physiological cost than darkening. Polo-Cavia and Gomez-Mestre (2017) found that the standard metabolic rate of heavily pigmented tadpoles is much higher than that of unpigmented tadpoles. However, we must point out that, even though the *Hyla arborea* tadpoles have the great ability to rapidly respond to background color change, their ability to match white backgrounds is poor. This may be because a completely white background is ecologically irrelevant and cannot be naturally encountered.

Our results are showing the immense capability of *H. arborea* tadpoles to quickly adapt to different environmental backgrounds, which is vital for organisms living in a heterogeneous environment. Rapid color adaptations are important for amphibians in numerous aspects including enhancing the chances of avoiding threats through crypsis (Heinen, 1993; Morey, 1990; Rodrígez-Rodríguez et al., 2020), protection against UV radiation (Garcia et al., 2004), thermoregulation (King et al., 1994; Rodrígez-Rodríguez et al., 2020), and water-retaining capacity (King et al., 1994). Newly metamorphosized *Bufo americanus* (Heinen, 1993) and adult *Pseudacris regilla* (Morey, 1990) frogs are less captured by garter snakes when found on the matched background. Rodrígez-Rodríguez et al. (2020) described that *Alytes dickhilleni* tadpoles are darker when exposed to shorter photoperiod. They also found that dark *A. dickhilleni* tadpoles have higher body temperatures than pale ones which could lead to higher growth and developmental rate (Sanuy et al., 2008; Castano et al., 2010). In situations when the environmental temperature is very high, King et al. (1994) discovered that *Hyla cinerea* frogs become paler, and in that way absorb less heat. They also theorized that by changing pigmentation levels amphibians can regulate body hydration. When temperatures and the risk of dehydration are high, individuals get depigmented resulting in an increased albedo effect and better chances of water retention (Tracy, 1976). In their work, Garcia et al. (2004) showed that *Ambystoma barbouri* and

Ambystoma texanum tadpoles both get darker when exposed to UV light which enhances their protection against radiation.

Different background coloration treatments did not affect *H. arborea* tadpoles' body length. Bearing in mind that some physiological costs of rapid color change probably exist, according to our results this stress is not great enough to be significant for tadpoles' size variation. However, Holmes et al. (2016) found evidence that white background can be more stressful for amphibians. Adult *Xenopus laevis* female frogs had significantly higher concentrations of corticosterone when reared in the white background than in the black. Also, both males and females *X. laevis* lost more weight in white than in the black background. Furthermore, changes in size were observed in experiments where changes in background coloration were coupled with predator presence (McCollum & Van Buskirk, 1996; Touchon & Warkentin, 2008) and these differences in size were predominantly caused by the presence of the predator. On the other hand, we observed slight tadpole shape changes between treatments. After twenty days, tadpoles from L treatment had slightly but significantly deeper tail fins and a more pronounced snout. At the end of the experiment, sixteen days after the switch we observed that the changing of the background did not affect the tadpoles' body shape. Tadpoles who started the experiment in the white boxes (treatments LL and LD) still had deeper tail fins and somewhat more pronounced snouts than those tadpoles who started in black boxes (treatments DD and DL), remarking that statistical significance was detected only between treatments LL and DL. This difference between LL and DL treatment was also confirmed with ontogenetic trajectory distance data. The development of a deeper tail fin is the usual tadpole response to threat, especially the presence

of a predator (predator-induced shape) (Van Buskirk & Schmidt, 2000, Touchon & Warkentin, 2008). Background coloration can reduce the perceived risk of predation in amphibians (Garcia & Sih, 2003). As we showed *H. arborea* tadpoles are not that good at matching white backgrounds, tadpoles here may have presumed greater predation risk and developed predator-induced tail fin shape. A deeper tail fin reduces overall swimming speed but increases maneuverability and can be used as a lure for predators, deflecting them from the head and body thus increasing chances of survival when attacked (Hoff & Wasserung, 2000; Van Buskirk et al., 2003, 2004). However, even though tadpoles with predator-induced phenotype survive better when exposed to predators, they have a higher mortality rate in environments without real predation risk (McCollum & Van Buskirk, 1996), so there is a big tradeoff between survivorship and rightly perceiving predation risk. In addition, being reared on a completely white background (white is biologically irrelevant and non-existing color) is stressful for the tadpoles (Holmes et al., 2016) and this also might trigger the development of an anti-predator tail shape.

The background choice behavior experiment revealed that *H. arborea* tadpoles reared in constant dark or light conditions actively choose the matching background to enhance crypsis. Tadpoles from the DD treatment spent significantly more time in the black part of the boxes, while the opposite was true for tadpoles from the LL treatment. Tadpoles from the switched treatments spent more time in the last background coloration they were reared in. However, tadpoles from the LD treatment did not show a clear preference for the dark background. When predator cues were introduced in the experiment, all treatment groups showed a significant preference for the last background they were reared in. Our result

suggests that *H. arborea* tadpoles can relate to and choose appropriate surroundings to blend in, even without external pressure from predators and when the predator cues are introduced, this phenomenon is even more pronounced. Partially similar results were found for *Lissotriton boscai* larvae, who without predator cues showed a preference for the background they were last reared, but in the presence of predator cues both unpigmented and heavily pigmented larvae showed a clear preference for the light background (Polo-Cavia & Gomez-Mestre, 2017). Eterovick et al. (2010) in a laboratory experiment found that *Bockermannohyla alvarengai* tadpoles escape to the matching background when attacked. *B. alvarengai* tadpoles also choose a background in a non-random way in natural habitats (Eterovick & Barros, 2003). Another way to achieve crypsis is to decrease movements (Nomura et al., 2013) but we found no difference in movements between treatments in both experiments.

Our experimental findings show that (1) *Hyla arborea* tadpoles can rapidly change body skin pigmentation when exposed to different backgrounds, (2) color changes are reversible, (3) background color matching has no effect on the variation in body length, but it does affect the shape of the body resembling predator-induced one, and (4) the last background coloration in which the tadpoles were reared had a significant effect on background choice behavior.

This study provides a solid basis for future research on the color pattern adaptive function in the European tree frog in the laboratory environment but also in natural ponds. It would be interesting and very beneficial to focus future research on possible trade-offs between different needs regarding color variation as it plays a role not just in predator avoidance, but in UV protection, and thermoregulation.

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