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Fading on the beach: pigmentation plasticity adjusts coloration to substrate type in coastal Western spadefoot toads

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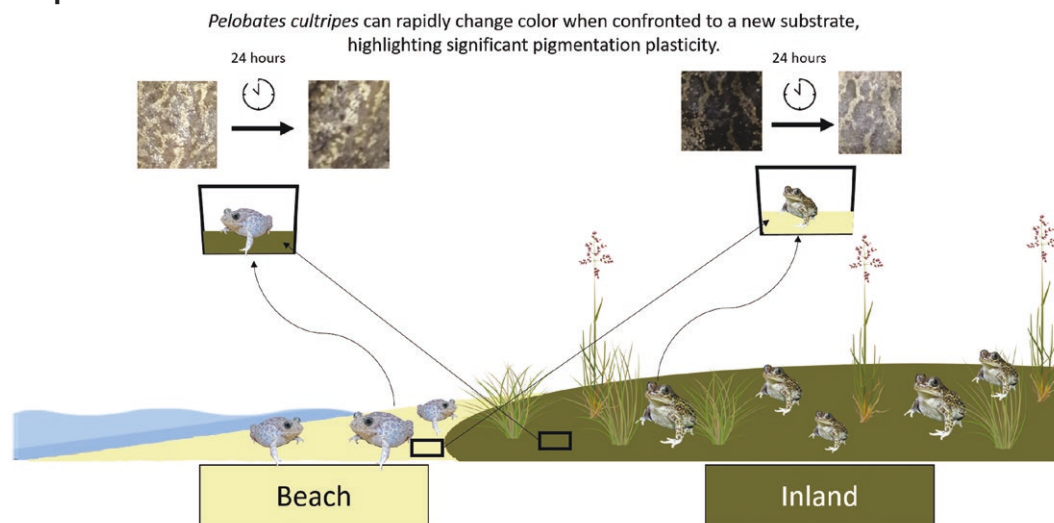
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Abstract

Animal species exhibit a wide range of coloration and patterns, which are under strong selection and often linked to individual quality, influencing mate choice, recognition, signaling, and interspecific interactions such as predator avoidance. In amphibians, coloration can vary based on individual traits as well as environmental conditions, including the coloration of their environment. In this study, we described the dorsal coloration (body coloration reticulated by dark patches) in 676 Western spadefoot toads (*Pelobates cultripes*) from the French Atlantic coast, comparing color variations across different substrates. In addition, we assessed color change in 18 toads transferred between bright and dark substrates. We demonstrated that the dorsal coloration varies based on capture locations (beach vs. inland) and associated substrate types (bright vs. dark), suggesting background color matching. We showed more pronounced effects in females, which might reflect varying predation risks. Experimentally, we showed that individuals can rapidly adjust their coloration to match the substrate within 24 h. Rapid color changes in response to substrate type indicate significant pigmentation plasticity. Bright individuals from sandy substrates showed less dorsal background (body) color change than dark inland individuals, while patch coloration responded differently depending on the substrate of origin. These findings highlight the complex interactions between substrate type, sex, and pigmentation plasticity. These interactions have potential costs and benefits, which might be linked to melanin production, which warrant further investigation.

Key words: amphibians, background color matching, inland, patches, sex-dependent, substrate.

Graphical Abstract



Pelobates cultripes are paler on the beach as compared to inland, matching the color of these substrates.

This ability to adapt to the substrate color depends on distance to the ocean and sex, highlighting different ecological and physiological trade-offs that may impact predator avoidance and signaling.

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Animal species display a large diversity of coloration and patterns (Protas and Patel 2008), and these traits are known to be under strong selection (Svensson and Wong 2011). Coloration has been shown to be linked to individual quality (Bradbury and Vehrencamp 1998; Martín and López 2009; Endler and Mappes 2017; Weaver et al. 2018), and thus is involved in mate choice, recognition, and signaling (Hubbard et al. 2010; Sztatecsny et al. 2010; Secondi, Lepetz, and Théry 2012; Heath et al. 2013). Coloration is also an important feature for interspecific interactions (e.g., aposematic and cryptic coloration used for predator avoidance; Hubbard et al. 2010; Duarte et al. 2017; de Alcántara Viana et al. 2022; Postema et al. 2023). Importantly, although coloration can remain constant throughout the life of an organism, temporal variations of pigmentation occur widely across the animal kingdom (Protas and Patel 2008), and many species have been shown to be able to change colors across variable time scales (Stuart-Fox and Moussalli 2009; Waring 2013; Duarte et al. 2017). For example, coloration can vary according to individual characteristics, such as age, sex, reproductive status, morphology, or body condition (Hamilton and Zuk 1982; Sztatecsny et al. 2010; Martin et al. 2013; Olsson et al. 2013; Tartu et al. 2023), food type (McGraw and Hill 2006; Ruell et al. 2013) but also to thermoregulation (Protas and Patel 2008; Stuart-Fox and Moussalli 2009; Sztatecsny et al. 2010).

To minimize detection by predators that hunt visually, organisms can achieve concealment by displaying preferences for backgrounds that match their own coloration (Endler 1978; Green et al. 2019; Stevens and Ruxton 2019). Yet, because a single pigmentation pattern may not provide crypsis across spatially and temporally variable environments (Magellan and Swartz 2013), organisms can also achieve concealment by adjusting coloration dynamically (Protas and Patel 2008), a process which can occur across different timescales—from rapid changes (as observed in cuttlefish, chameleons, and many fish) to slower modifications, observed in a wide range of animal groups (see Duarte et al. 2017). Potential costs associated to these strategies are very different. Indeed, the former strategy implies that individuals have to find a color-matching environment which should induce strong (micro-) habitat selection and behavioral adjustments (Doligez et al. 2008). The latter allows individuals to move and use diverse habitats but associated dynamic pigmentation adjustments are expected to induce specific costs (Alfakih et al. 2022), notably linked to increase energy expenditure and oxidative stress (Liedtke et al. 2023; Radovanović et al. 2023).

The expression of coloration is particularly important in amphibians (Rojas 2017), which show a strong diversity of colors and contrasting patterns (Rudh and Qvarnström 2013). Furthermore, both coloration (Kraus and Allison 2009; Rudh and Qvarnström 2013) and color vision (Chen et al. 2008) can change across developmental stages, and adults are often shown to express sexual dichromatism (Bell and Zamudio 2012; Lifshitz and St Clair 2016; Ujhegyi and Bókonyi 2020). Due to their lack of protective epidermal structures and their permeable skin (Wells 2007), amphibians are particularly sensitive to changes in their environment. To protect their skin and vital functions, they can adaptively change color in order, for example, to thermoregulate (Nilsson Sköld et al. 2013), this change being largely explained by the high correlation between color (reflectance in the visible spectrum) and near-infrared reflectance (Park et al. 2023). These rapid color changes in some species (Bagnara et al. 1968; Rudh and Qvarnström 2013) might be achieved

by the migration of melanosomes (Nilsson Sköld et al. 2013). For instance, coloration can change in amphibians according to the reflectance of their environment (Polo-Cavia and Gomez-Mestre 2017), and evidence for background matching (i.e., a camouflage tactic for which an organism blends into its environment by resembling the colors and patterns of its surroundings) is found in salamanders and in anurans and is thought to be an adaptation to avoid predation (Kats and van Dragt 1986; Rudh and Qvarnström 2013). In larvae, it has been documented that a clear, high reflecting environment induced depigmentation whereas a dark, low-reflecting environment induced pigmentation over short periods of time (Polo-Cavia and Gomez-Mestre 2017). However, change in coloration might be costly (Polo-Cavia and Gomez-Mestre 2017), inducing increased energy consumption and oxidative stress (Liedtke et al. 2023; Radovanović et al. 2023). In addition, in their natural environment, amphibians can also be exposed to various stressors, such as temperature (Sears and Angilletta 2015), lack of oxygen (Bickler and Buck 2007), and salinity (Hopkins et al. 2016; Lorrain-Soligon et al. 2022), which might as well induce such costs. As a consequence, whether adaptive coloration and dynamic coloration changes, occur in individuals exposed to other stressors, remain to be assessed.

In this study, we investigated variation in dorsal coloration of Western spadefoot toad (*Pelobates cultripes*) individuals of 3 populations from the French Atlantic coast. These coastal habitats provide contrasting substrate types according to the distance to the ocean from light sandy beach to dark soil substrate, thereby allowing to test whether toads' coloration vary according to substrate type, along a gradient of additional stressors, notably linked to the exposure to salinity (Lorrain-Soligon et al. 2022). In addition, to investigate the plasticity of the dorsal coloration of toads, we captured individuals from either bright or dark substrates, and transferred half of each batch to bright or dark substrates for 24 h, to assess whether rapid color change can occur. We hypothesized that (1) individuals in their natural environment will express background matching (Kats and van Dragt 1986; Rudh and Qvarnström 2013; Polo-Cavia et al. 2016). As adaptive coloration has been linked to oxidative damage (Sotoudeh et al. 2019; Chang et al. 2021; Radovanović et al. 2023) and metabolic costs (Polo-Cavia and Gomez-Mestre 2017), it might also be linked to mass, size, body condition, or sex (linked to differing energetic demand of each sex) that are expected to integrate such costs (e.g., Heusner 1982; Markó et al. 2011; Brenes-Soto et al. 2017; Zhu et al. 2023), which also varied according to distance to the ocean due to the increasing cost of salinity closer to the coast (Lorrain-Soligon et al. 2022, 2024). As such (2), we expected that males (for which energetic costs of reproduction are weaker), and larger individuals (or individuals in better condition) should improve background matching coloration, and that this ability should increase along with distance to the ocean (decreasing exposure to salinity). Finally, we predicted (3), that individuals held in captivity should adjust their color pattern to that of the substrate as color can change rapidly in amphibians (Polo-Cavia and Gomez-Mestre 2017).

Materials and Methods

Study species

The Western spadefoot toad, *Pelobates cultripes*, is a nocturnal amphibian species, distributed in the region of the Iberian Peninsula and in some localities on the Mediterranean and

Atlantic coast of France (Lizana et al. 1994; Leclair et al. 2005; Thirion 2014; Dufresnes et al. 2019). Adults of this species spend the majority of their time on land where they shelter buried in loose soil (Speybroeck et al. 2018). They hide during the day and are active at night to forage. The species differs from the other Eurasian spadefoots by metatarsal spades being entirely black and a flat skull (Dufresnes et al. 2019). The dorsal background coloration of toads can be yellow, gray, or brown, reticulated by dark patches (Dufresnes et al. 2019). Males and females do not present sexual dichromatism (Dufresnes et al. 2019).

Study sites

Three sites were surveyed between 07/09/2022 and 23/11/2022, on the western coast of France, in areas previously identified as being favorable to the presence of the species: the “Réserve Naturelle Nationale de Moëze-Oléron” (45°53'33.36" N, 1°04'59.16" W, hereafter MO), the “Réserve Naturelle Nationale du marais d'Yves” (46°2'40.735" N, 1°3'16.906" W, hereafter MY), and the “Réserve Naturelle Nationale de la casse de la Belle-Henriette” (46°20'37.32" N, 1°21'59.76" W, hereafter BH, see Lorrain-Soligon et al. 2024). The 3 coastal habitats investigated span a gradient of approximately 1,000 meters landward. This gradient encompasses a diverse range of substrates and ecological environments, from the light-colored sands of the beach to the darker, organic-rich soils found further from the sea. On the beach, the habitat is dominated by a fine-grained, pale sandy substrate, which is light beige in color, with no vegetation. This habitat is intertidal, more salty, and imposes specific costs due to exposure to salt. Indeed, *P. cultripes* found closer to the seashore expressed an increase plasmatic osmolality (Lorrain-Soligon et al. 2022). In the upland habitat (inland), the substrate shifts dramatically in both color and composition. The soil is much darker, ranging from brown to black, reflecting high organic content, with stable and dense vegetation cover (shrub, grasses, as well as trees). Unlike the beach, the upland habitat is sheltered from direct marine influence. Importantly, there is no transition between the beach and inland habitats, as the habitat abruptly changes at their boundary.

Field surveys

Field surveys were performed during the non-reproductive period (fall), a period during which terrestrial activity is dedicated to foraging (Duguet and Melki 2003). Field surveys were carried out at night, between 9 PM and 4 AM when environmental conditions (temperature and humidity) favored their activity (Priol 2015). Surveys occurred on 3 separate occasions, spaced 1–2 weeks apart, with teams consisting of 5 to 10 persons.

We captured a total of 676 adult individuals (85 in BH [27 males and 58 females], 174 in MO [73 males and 101 females] and 417 in MY [176 males and 241 females]). Sex was assessed based on the presence of secondary sexual characters (Speybroeck et al. 2018). Each individual was measured (snout-to-vent length [SVL], using a ruler [± 0.5 mm]) and weighted (± 1 g using a Pesola scale).

To assess coloration, we used the color gradient function of W3 schools (https://www.w3schools.com/colors/colors_gradient.asp; see Supplementary Appendix A). The dorsal area of Western spadefoot toads is composed of a background

coloration reticulated by darker patches (see Dufresnes et al. 2019 and Supplementary Appendix A for pictures). Consequently, we produced 2 independent color gradients, one for the background (hereafter “body” for simplicity) and one for the darker patches (hereafter ‘patch’ for simplicity; see Supplementary Appendix A for pictures), and each color scale was graduated from 1 to 10 (Supplementary Appendix A). To determine the starting and final colors of the gradient, we selected the darkest and brightest individuals based on photographs taken from the field. These extreme values were chosen to capture the full range of color variation for both body and patch coloration. To minimize bias introduced by environmental background colors, all toads were consistently placed on a white background (white plastic bin) during classification, and the color gradient was positioned on the side of the toad. These measures were taken to ensure reliable and standardized color classification across all individuals in the study. The matching color score for both the body and the patches was recorded (ranging from 1 to 10). Although different evaluators classified the color of individuals, all evaluators underwent a rigorous calibration process prior to classification. This process reduced subjectivity and improved consistency in color assessments. Precise locations of capture were recorded with a GPS (Garmin GLO) in order to assess distance to the ocean at high tide, using the NNJoin extension on Qgis 3.10. We also noted if individuals were captured on the beach (bright sandy substrate) or inland (darker soil substrate).

Individuals held in captivity

On 09/06/2023 and 12/06/2023 in MO, 18 individuals were captured (10 from bright substrates [light individuals, 2 males and 8 females], and 8 from dark substrate [dark individuals, 1 male and 7 females]). As above, their color was assessed upon capture using the color scales. Precise locations of capture were recorded with a GPS (Garmin GLO). They were placed in a transport box (14 × 16 × 9 cm) and brought back to the reserve premises. They were then individually transferred to a box (24 × 34 × 13 cm) containing either sand (washed sand for masonry, bright substrate, 5 light individuals originating from bright substrate, and 4 dark individuals originating from dark substrate) or black soil (Solabiol organic potting soil, dark substrate, 5 light individuals originating from bright substrate, and 4 dark individuals originating from dark substrate). Individuals were kept in captivity for 24 h, and their final coloration was assessed using the color scales (as described above). They were then released at their exact location of capture.

Statistical analyses

All statistical analyses were performed using R 3.6.3 (R Core Team 2020) and Rstudio v1.1.419. We computed Linear Models (LMMs) and Gamma Generalized Linear Models (GLMMs) using the *lme4* package (Bates et al. 2015). For all test computed, models accuracy was tested using the `check_model` function from the performance package (Lüdtke et al. 2020). The normal distribution of residuals from linear models were checked using a Shapiro test (`shapiro.test` function, implemented in the R environment, R Core Team 2020). For all tests, significance level was set at 0.05, and best variables were retained using a top-down selection based on *P*-value (`drop1` function from the R environment). The data obtained are presented as mean \pm standard error (SE, Figures 1 and 2).

Field surveys

First, to understand if the difference in coloration between patch and body was different between coastal and inland individuals, we used a Gamma GLMM with coloration score as a dependent variable, and body part (patch or body), location (beach or inland) and their interaction as explanatory variables, and site (BH, MO, and MY) as a random effect.

We investigated separately the variations in patch and body coloration according to distance to the ocean (as a continuous variable), to their location (on the beach or inland), to their sex (males or females), to the interaction between location and sex, as well as to mass, SVL and body condition (as the residuals of the regression between $\log_{10}[\text{body mass}]$ and $\log_{10}[\text{SVL}]$), using Gamma GLMMs with a log link, and site (BH, MO and MY) as a random effect.

Individuals held in captivity

We investigated the change in patch and body coloration (patch or body coloration from day 2 – patch or body coloration from day 1), according to the substrate coloration (bright or dark) of the experimental container. For bright and dark individuals (individuals' initial coloration), we fitted separate LMs (linear models), with change in coloration as a dependent variable, and substrate coloration as an explanatory variable. Separate models were fitted due to low number of individuals.

Results

Field surveys

Individual patch coloration was darker than body coloration, this contrast being higher in beach population (body coloration: 4.50 ± 0.10 SE; patch coloration: 8.45 ± 0.11 SE) as compared to inland ones (body coloration: 6.43 ± 0.07 SE; patch coloration: 9.45 ± 0.03 SE; [Table 1](#), [Figure 1](#)).

Body coloration score and patch coloration score varied according to location (being darker for inland individuals), and the interaction between location and sex ([Table 1](#), [Figure 1](#)). On the beach, body coloration and patch coloration were darker in males as compared to females while coloration scores were similar between males and females from inland locations ([Supplementary Appendix B](#), [Figure 1](#)). Overall, coloration of males and females was similar. Patch coloration score also increased with distance to the ocean ([Table 1](#)). Neither distance to the ocean, body mass, SVL nor body condition influenced body coloration score, and neither body mass, SVL nor body condition influenced patch coloration score.

Individuals held in captivity

In bright individuals (captured on sandy substrate), body coloration did not change according to substrate type ([Figure 2A](#), [Table 1](#)), but patch coloration became darker in individuals placed on dark substrate ([Figure 2C](#), [Table 1](#)).

In dark individuals (captured inland), body coloration became lighter on bright substrate as compared to dark substrate ([Table 1](#), [Figure 2B](#)), but patch coloration did not change according to substrate coloration ([Figure 2D](#), [Table 1](#)). An

example of a change in coloration of a dark individual placed on a bright substrate is given in [Figure 2E](#).

Discussion

In this study, we showed that the dorsal coloration in *Pelobates cultripes* varies with locations of capture (on the beach or inland) which are characterized by contrasting substrate types (bright *versus* dark), this pattern being more marked in females. Experimentally, we showed that individuals can rapidly (within 24 h) adjust their coloration to match the substrate type, yet with body and patches coloration responding differently according to the substrate from which the individuals originated.

Individuals' coloration on different substrates

Individuals captured on the beach were brighter than individuals collected inland. This pattern suggests background color matching which is common in amphibians ([Norris and Lowe 1964](#); [Kats and van Dragt 1986](#); [Polo-Cavia and Gomez-Mestre 2017](#)) and is mainly thought to be an adaptation to avoid predation ([Kats and van Dragt 1986](#); [Rudh and Qvarnström 2013](#)). Indeed, Western spadefoot toads can be preyed upon by a variety of predators (e.g., birds, mammals, and reptiles; [Marangoni and Tejado 2007](#); [Recuero and Salvador Milla 2014](#)), some of which can strongly rely on visual cues to detect their prey (i.e., birds as compared to mammals and snakes). Interestingly, *P. cultripes* bones have been found in owl pellets in our study area (V. Lelong pers. obs.). Importantly, some predators (including owls, [Höglund et al. 2019](#)) can rely on UV signals to detect their prey ([Bennett and Cuthill 1994](#)), and future studies are required to assess whether toads can reflect in the UV spectrum. Darker coloration in inland individuals might help them blend into their specific environments, such as dense vegetation growing over a dark soil, while the same process is achieved with lighter coloration in beach-dwelling individuals occurring over bare sandy substrates. Accordingly, the sex-specific differences in coloration we found on the beach might be a result of different predation risks or reproductive strategies. Males and females might use different microhabitats or have different behaviors that expose them to varying levels of predation. Importantly, this location-specific sexual dimorphism in coloration contrasts with the previously thought absence of sexual dichromatism in this species ([Dufresnes et al. 2019](#)).

Different coloration might also induce specific costs. Indeed, adaptive matching to background color (and particularly to darker background) have been shown to induce oxidative damage ([Sotoudeh et al. 2019](#); [Chang et al. 2021](#); [Radovanović et al. 2023](#)) and metabolic costs ([Polo-Cavia and Gomez-Mestre 2017](#)). Although we did not investigate such markers in the current study, we failed to detect any relationship between color patterns and individual characteristics that are expected to integrate such costs (e.g., mass, size, or body condition, [Heusner 1982](#); [Markó et al. 2011](#); [Brenes-Soto et al. 2017](#); [Zhu et al. 2023](#)). This suggests that the costs associated with coloration might be more nuanced or context-dependent, and additionally, other environmental parameters might be more prominent in inducing these costs (for example salinity, see [Lorrain-Soligon et al. 2022](#)). Interestingly, in our context, lighter background-matching coloration, which is expected to decrease the costs associated

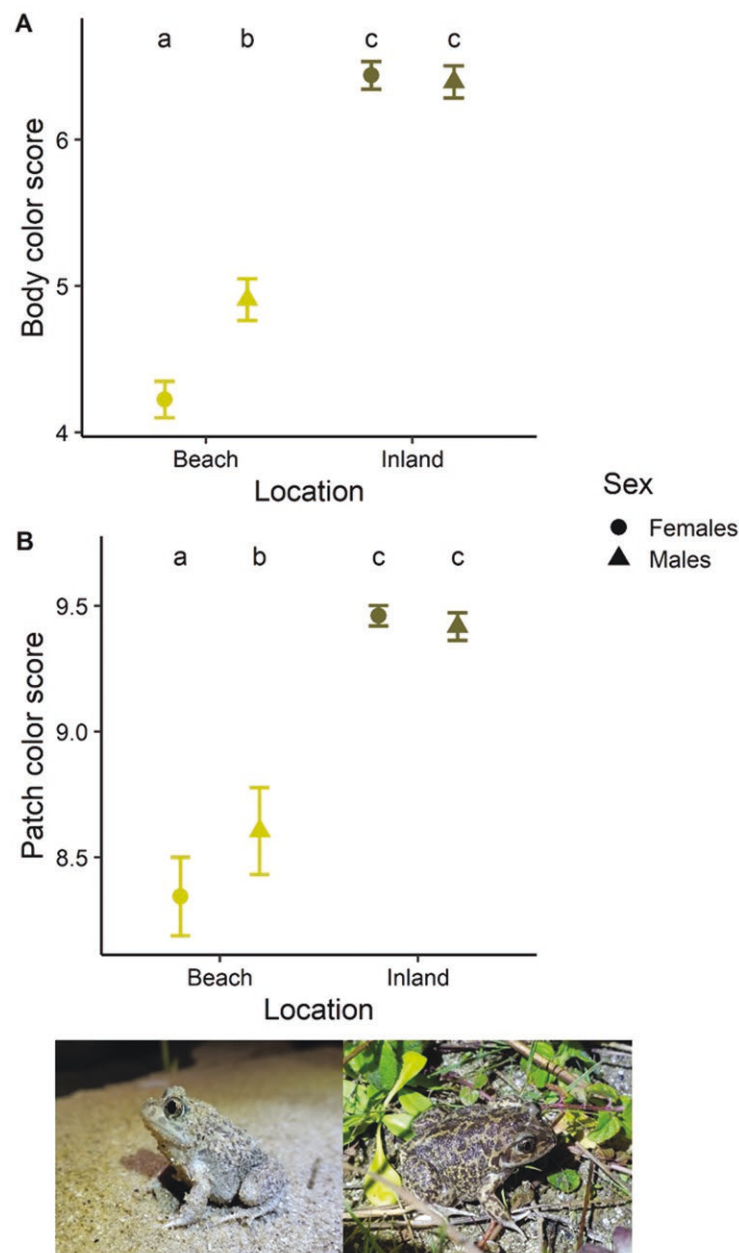


Figure 1. Body (A) and patch (B) color score in relation to capture location (on the beach or inland) and sex in *Pelobates cultripipes* found in the Atlantic coast of France. Mean \pm SE. Different letters represent a significant difference at $\alpha = 0.05$. Photo credit: Théo Gicquel de Menou

with darker coloration (Polo-Cavia and Gomez-Mestre 2017), may be advantageous in beach dwelling Western spadefoot toads for another, unrelated reason. Indeed, in this species, individuals located on the beach are strongly exposed to higher salinity which induces specific costs (higher plasma osmolality, Lorrain-Soligon et al. 2022) affecting their growth rate, body size, and condition (Lorrain-Soligon et al. 2022, 2024). This dovetails relatively well with the fact that beach dwelling females were paler than males, insofar as females invest a larger amount of energy in reproduction as compared to males (Wells 2007). Expected increased salinization in coastal environments may increase exposure to salinity in inland individuals thereby inducing potentially additive or synergetic costs with those induced by darker coloration. Future studies should usefully test for the effects of these combined environmental stressors.

Change in coloration

Our experimental test on individuals placed on different substrate types highlighted rapid changes in body and patch coloration. This indicates that pigmentation plasticity allows individuals to rapidly adjust their coloration to that of their environmental background (Kats and van Dragt 1986; Polo-Cavia et al. 2016; Polo-Cavia and Gomez-Mestre 2017). Importantly, we found different changes in body and patch coloration depending on the habitat of origin. Indeed, bright individuals from sandy substrates did not exhibit significant changes in body coloration score based on substrate type, whereas dark inland individuals showed a marked decrease in body coloration score when placed on bright substrates. Conversely, patch coloration scores increased for bright individuals on dark substrates but did not significantly vary for dark individuals on different substrates. The differential

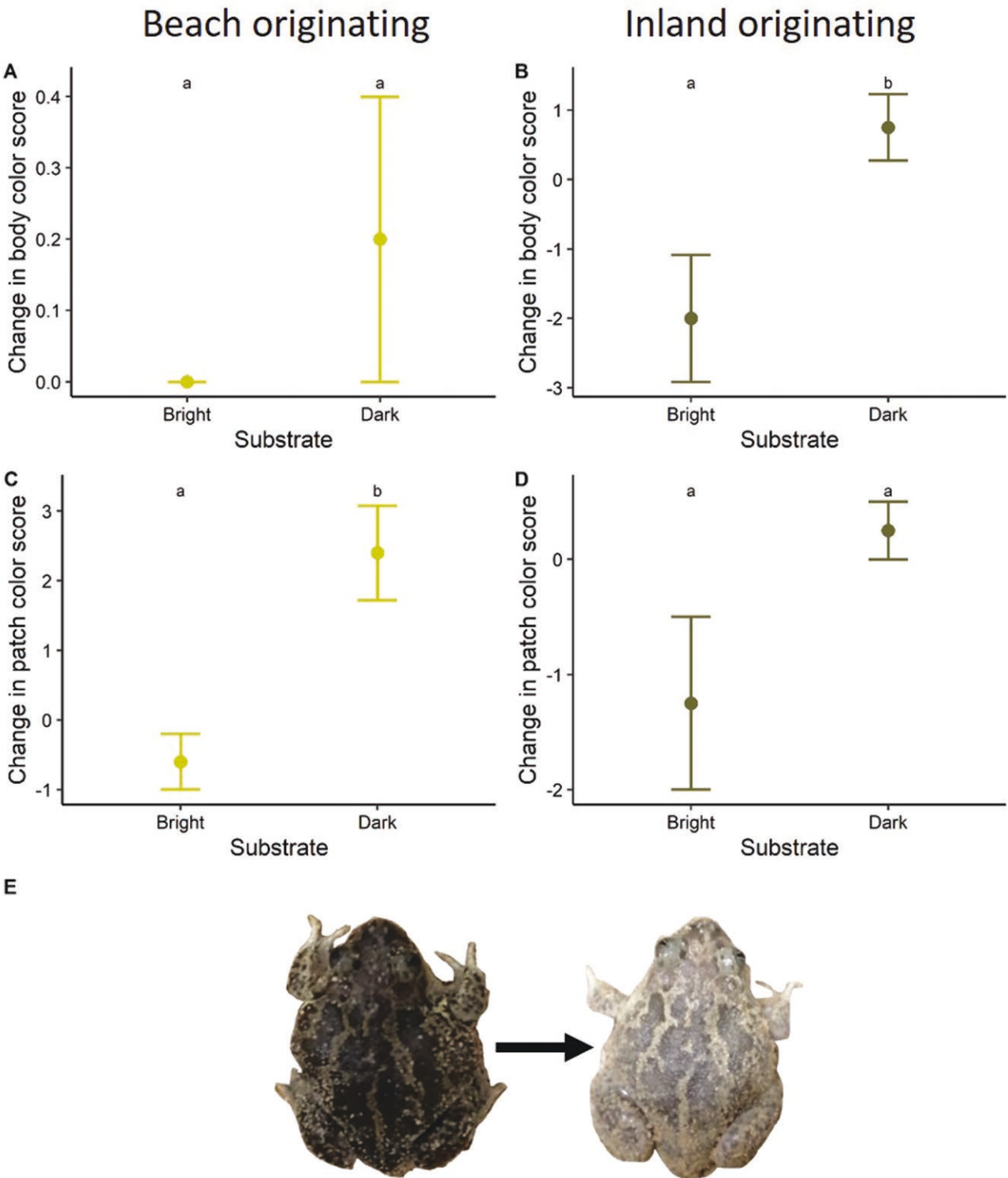


Figure 2. Changes in (AB) body and (CD) patch color score, for beach originating (AC) or inland originating (BD) *Pelobates cultripes* found in the Atlantic coast of France, after 24 h on a bright or dark substrate (mean \pm SE). Different letters represent a significant difference at $\alpha = 0.05$. (E) The picture illustrates an example of the actual change in coloration of a dark individual placed for 24 hours on a bright substrate.

response of body and patch to substrate color highlights a dynamic aspect of toad coloration that could influence crypsis. As the contrast between the patches and body background changes with substrate type, this variation may affect the toad camouflage. Indeed, by darkening patch coloration in dark substrates, paler toads increase the contrast between the patches and the body, which can enhance edge disruption

and facilitate crypsis by disruptive coloration (Endler 2006; Stevens and Cuthill 2006). Changes in contrast could also either enhance or diminish detectability depending on the specific background and the predator's visual system. The substrates used in our experiments were uniform, thereby limiting our ability to explore how "patchiness" respond to more complex, heterogeneous substrates. Future studies

Table 1. Results of the statistical analyses on the different variables studied for *Pelobates cultripes* found on the field or held in captivity. For field individuals, we evaluated the difference in coloration between patch and body, according to the zone prospected (beach or inland), but also the influence of sex, zone and their interaction on coloration of either the patch or the body. For individuals held in captivity, we evaluated the influence of the coloration of the substrate, on the change in their body or patch coloration, separately for beach originating or inland originating individuals. We computed Linear Models (LMMs) and Gamma Generalized Linear Models (GLMMs)

Experiment	Population	Dependant variable	Predictor	Estimate	SE	t-value	P	
Field coloration	All	Coloration	ZoneInland	−0.066	0.004	−18.000	<0.001	
			Body Area Patch	−0.104	0.004	−27.680	<0.001	
			ZoneInland: BodyAreaPatch	0.054	0.004	12.890	<0.001	
		Body coloration	ZoneInland	0.415	0.030	13.991	<0.001	
			SexMale	0.159	0.038	4.173	<0.001	
			ZoneInland: SexMale	−0.165	0.046	−3.573	<0.001	
		Patch coloration	ZoneInland	0.173	0.016	10.600	<0.001	
			SexMale	0.049	0.018	2.695	0.007	
			distance	<0.001	<0.001	−3.935	<0.001	
			ZoneInland: SexMale	−0.065	0.022	−2.961	0.003	
		Laboratory change in coloration	Beach originating	Change in body coloration	Dark substrate	0.200	0.200	1.000
Beach originating	Change in patch coloration		Dark substrate	3.000	0.787	3.810	0.005	
Inland originating	Change in body coloration		Dark substrate	2.750	1.031	2.668	0.037	
Inland originating	Change in patch coloration		Dark substrate	1,500	0.791	1.897	0.107	

should usefully investigate adjustments not only of their overall color but also the pattern or patchiness of their appearance in response to complex—more realistic—substrates.

These results also lead to several hypotheses regarding the mechanisms behind the pigmentation plasticity linked to the habitat of origin. First, the more rapid paling of dark-bodied individuals when placed on bright substrates could be attributed to the lower metabolic cost of lightening compared to darkening (Polo-Cavia and Gomez-Mestre 2017). This process might involve the breakdown or dispersion of existing melanin, while synthesis of melanin required to darken the whole body is more costly and resource-consuming (Talloe et al. 2004; Aspengren et al. 2006; Aspengren et al. 2008; Kindermann and Hero 2016). The physiological processes involved, such as the contraction and relaxation of chromatophores, could be more efficient when reducing, rather than increasing pigmentation (Bagnara et al. 1968; Alfakih et al. 2022). This might also suggest that long term exposure to conditions that do not require high melanin presence tunes down its production. Accordingly, the darkening of the patches (but not body color) in bright-bodied individuals on dark substrates suggests a related, yet opposite mechanism. We showed that patches are darker than the rest of the body, which was already known for the species (Dufresnes et al. 2019). These patches might thus already contain higher levels of melanin (Grether et al. 2004; Kindermann and Hero 2016). The increase in patch coloration might be due to the redistribution or concentration of existing melanin within these structures (Kindermann and Hero 2016), which could be more rapid and less costly than de novo melanin synthesis, required to darken the paler body coloration (Talloe et al. 2004; Aspengren et al. 2006, 2008; Kindermann and Hero 2016). These processes highlight the potential role of initial coloration (and initial pigment presence

and concentration) in determining the magnitude and temporal variation of pigmentation plasticity. To further understand pigmentation dynamics, it would be beneficial to maintain individuals on experimental substrates for extended time periods. This would help determine if the rapid changes we described are maintained over time, or if longer durations can lead to other adjustments (e.g., patchiness), and thus to assess the long-term ability of individuals to adapt to changing environments, according to their initial colorations.

There are some caveats to our investigations notably linked to the methodology we used to quantify coloration. Indeed, up-to-date methodologies include the use of a spectrometer to precisely quantify coloration (reflectance) along a wide spectrum (including UV, see for example Badiane et al. 2022; Tartu et al. 2023). These approaches allow to compare reflectance profile with similar profile obtained from the substrates and to further test detectability by various predators (Stevens et al. 2017; Green et al. 2019; Rodríguez-Gironés and Maldonado 2020). In this respect, we acknowledge that our methodology is too limited to allow such investigations and further studies are required to test whether coloration spectrum (collected through spectrometry) of toads match those of various substrates and decrease actual detectability by predators. We highlight that the methodology we adopted was linked to prior field observations from which we observed apparently 2 different morphs of *P. cultripes* (clear vs. dark) which seemed spatially segregated (sandy beach versus darker inland substrates). Importantly, the level of variation we currently describe (e.g., the continuum of coloration rather than discrete morphs, sex differences and variations between patch and body color) was unexpected. Nonetheless, we emphasize that, although perfectible, the methodology we used should

not generate spurious patterns given the contrasts we found between clear individuals captured on sandy beach and dark individuals captured on darker inland substrates.

Our study thus reveals complex interactions between location, sex, and substrate type in determining body and patch coloration in *Pelobates cultripipes*. These variations likely represent adaptive responses to predation and environmental substrate, with potential costs and benefits that are yet to be fully understood. The sex-specific differences and habitat-dependent changes in coloration might be due to a combination of habitat choice and color change properties, the basis of which (genetic, hormonal, energetic, or a combination thereof) need to be further investigated.

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Authors' Contributions

LLS, FR, and FB conceptualized the study. All authors participated in data collection. LLS and FB analyzed the data and wrote the initial draft. All authors have reviewed and edited the manuscript and approved the final version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Statement

This work was approved by the French authorities under permits R-45GRETA-F1-10 and 135-2020 DBEC.

Data Availability

Data will be made available by the corresponding author upon request.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

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