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A polymorphism in oocyte pigmentation in natural populations of the glass frog *Espadarana prosoblepon* (Centrolenidae)

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ABSTRACT The adaptive role of amphibian oocyte melanic pigmentation and its molecular control are still elusive. Here we present evidence of a polymorphism in egg pigmentation in the emerald glass frog *Espadarana prosoblepon*. In Ecuadorian natural populations of this species, females can lay dark brown or pale eggs that develop into normal pigmented tadpoles and adults. This trait is a sex-limited phenotype which is inherited like a recessive allele that we called *pale eggs like (pel)*. The *pel* phenotype is exclusive of oocyte cortical melanic pigmentation, which is reduced in comparison to wild type *(wt)* dark pigmented oocytes. Consequently, *pel* early embryos are paler in appearance, with reduced melanic pigmentation distributed to early blastomeres and embryonic ectoderm. However, these embryos form normal melanocyte derived pigmentation. Finally, we discuss the origin of this polymorphism and propose the use of *E. prosoblepon* as a model to study the adaptive role of egg pigmentation.

KEY WORDS: *glass frog, oocyte, melanin, pigment, embryo, Centrolenidae*

Introduction

Amphibian oocytes are the only vertebrate gametes capable of synthesizing the pigment melanin (Wallace and Selman, 1990). However, almost every extant clade of anurans has species that produce whitish, seemingly unpigmented eggs, even in the most basal groups (Desnitskiy, 2012). This apparent dichotomy of the egg pigmentation character has allowed generalizations regarding its adaptive role; yet very little is known regarding the state of this character. Indeed, from studies in a few species, now we know that the pattern of melanic pigmentation in amphibian eggs could vary among species, and the lack of pigmentation could be the result of multiple distinct cellular phenotypes regarding melanin synthesis and pigment localization (see below). Even less is known regarding variability of egg pigmentation within species. Here we report the existence of a polymorphism in oocyte and early embryo melanic pigmentation within natural populations of an amphibian, the emerald glass frog *Espadarana prosoblepon* (Centrolenidae).

In pigmented amphibian eggs, melanin is packed in organelles called melanosomes that are actively localized towards the animal hemisphere, leaving a clear, unpigmented and heavier vegetal pole, rich in yolk, oriented downwards due to gravity, (Elinson, 1980). In *Xenopus laevis*, oocytes accumulate melanosomes across the cortex during vitellogenesis (Dumont, 1972). In this species, the melanogenic pathway is activated independently within the oocytes, through unknown mechanisms. Within the oocyte cytoplasm, melanin is synthesized and packed in melanosomes that are actively transported towards the animal cortex (Kidson and Fabian, 1989). After fertilization, oocyte melanosomes are distributed in every cell that inherit the cytoplasm from the animal pole (mostly ectodermal), to later be found in internalized tissues like the notochord and neural tube after gastrulation (Eppig, 1970; Eppig and Dumont, 1971; Hughes, 1963; Kordylewski, 1983). The oocyte melanosomes can remain in the developing amphibian embryo until later embryo or tadpole stages, when they are actively extruded, as shown in *X.*

Abbreviations used in this paper: PUCE, Pontificia Universidad Católica del Ecuador; *wt*, wild type *E. prosoblepon* ; *pel*: *pale egg like* phenotype.

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laevis (Uehlinger *et al*., 1971).

In nature, the character state "whitish eggs", may be the result of at least four distinct cellular phenotypes within the oocyte: a completely lack of melanosomes (*Gastrotheca riobambae*; del Pino *et al*., 1986), a reduction in pigmentation (*Agalychnis spurrelly;* Schmid *et al*., 2018), a failure in apical melanosome transport (*Engystomops* species; Lee *et al*., 2009) or a combination. The absence or reduction in melanic pigmentation in the oocytes of an anuran doesn't imply albinism since they generate pigmented neural crest-derived melanocytes and normal retina pigmented epithelia (Romero-Carvajal *et al*., 2009; Salazar-Nicholls and del Pino, 2015; Schmid *et al*., 2018). Still, the mechanisms that reduce the amount and localization of melanin in whitish eggs are poorly understood (Lee *et al*., 2009). Also, potential tools to analyze this phenomenon have been understudied, like the *Xenopus* albino embryos, which lose their melanosomes early in development through still unknown mechanisms (Hoperskaya, 1975). The *X. laevis* "*pale eggs*" mutation (*pe*) causes a reduction in oocytes melanic pigmentation in the animal pole; yet, *pe* embryos *d*eveloped normally in spite of its whitish appearance. Older *pe* embryos were indistinguishable from wild types when neural crest-derived melanocytes developed, similar to other species with whitish eggs (Droin and Fischberg, 1984). This mutation was lost without being mapped to a gene. From this limited genetic evidence and a few descriptions of oocyte phenotypes, we could classify whitish eggs in unpigmented eggs, which lack of melanosomes, and pale eggs, with reduced pigmentation. Through this manuscript, we will use the term "whitish" for amphibian eggs with reduced or absent melanic pigmentation on which this phenotype hasn´t been described.

In eggs and early embryos, melanic pigmentation could be playing important adaptive roles as protection against UV irradiation, and as camouflage against predators. Melanin is a known barrier against UV damage, and it could protect eggs and early embryos from irradiation (Blaustein and Belden, 2003; Blaustein *et al*., 2003; Licht and Grant, 1997). Melanin in the animal pole seems to protect sensitive molecules, since amphibian embryos irradiated from the vegetal pole fail to form a neural tube and axial structures (Chung and Malacinski, 1975; Manes and Elinson, 1980). Accordingly, it is common to generalize that amphibians with pigmented eggs lay clutches in exposed areas while amphibians with whitish eggs have evolved adaptations to conceal the eggs and avoid irradiation (Altig and McDiarmid, 2007). *Xenopus laevis, Rana*, *and* others, lay dark pigmented eggs in exposed aquatic clutches, while *Eleutherodactylus* and *Gastrotheca* lay whitish eggs that are concealed in subterranean nests or in a skin pouch, respectively (Elinson and del Pino, 2012). Multiple studies in other species, however, show that tolerance to environmental UV radiation is highly variable, even among species with pigmented eggs (Anzalone *et al*., 1998; Blaustein *et al*., 1994; Palen *et al*., 2005). The existence of other physical, molecular, and behavioral adaptations to avoid UV-B damage, suggest there might be other explanations to the adaptive value of egg pigmentation (Marquis *et al*., 2008; Palen and Schindler, 2010; Palen *et al*., 2005).

Another suggested role for egg pigmentation is to camouflage eggs against predators. Though there is no experimental data regarding the influence of egg pigmentation over egg predation, whitish tadpoles could be more visible to predators, as shown in albino spadefoot tadpoles (Childs, 1953). Indeed, parental care adaptations and enclosed nesting seem to occur more often in some families where whitish eggs occur. Glass frogs (Centrolenidae) are unique in this aspect since most of the species produce whitish eggs; yet, arboreal clutches are the main reproduction mode (Guayasamin *et al*., 2009). It has been suggested that these arboreal eggs are mostly deposited in the underside of leaves to avoid UV radiation; however, in *Hyalinobatrachium fleischmanni* this is an adaptation against predation (Delia *et al*., 2010). Hence other selective pressures, different to UV protection, could be influencing the presence, absence and the amount of egg pigmentation in amphibians.

We describe in the present manuscript a group of polymorphic populations of *E. prosoblepon* that, similar to the *pale eggs* mutation in *X. laevis*, has reduced oocyte and early embryo pigmentation. We were also able to determine the heritability of this trait in a genetic analysis in a laboratory population. Accordingly, we have called this *E. prosoblepon* phenotype *pale eggs like* or *pel,* a genetic trait that appears to be widespread across geographically distant populations of *E. prosoblepon.* A similar polymorphism has been reported once before in *Dendrobates auratus* without further population analyses (Flores *et al*., 2012). Hence, ours constitutes the first report of a widespread polymorphism in egg pigmentation in natural populations of an amphibian.

Results

In 2014, during a field trip to "Los Cedros Biological Reserve" we collected two amplectant pairs of *E. prosoblepon* that later laid eggs in plastic containers. Both couples were found on vegetation over streams. Embryos in early cleavage stage showed drastic differences in the amount of pigmentation. One of the couples laid dark pigmented eggs (Fig. 1A). Within 24 h, while still being kept in the container, all the embryos reached to tailbud stage maintaining their dark appearance. Hatched tadpoles were transported to the Balsa de los Sapos Laboratory (BDLS) where only few survived and metamorphosed to froglets like the one shown in Fig. 1C. The other couple laid eggs with dark pigmentation only in the most animal region (Fig. 1D). Importantly, after 24 hours this pigment had banished or diluted, and tailbud stage embryos where completely pale (Fig. 1E). The few metamorphosed froglets from this clutch that survived had the normal pattern of pigmentation described for *E. prosoblepon* adults (see discussion). The presence of melanic pigmentation in embryos (Fig. 1D), tadpoles (not shown), and the presence of melanic pigmentation in the skin and eyes of adult frogs discarded the possibility that the pale embryo phenotype had its origin on an albinism allele (Fig. 1C, 1F). This also suggested that the phenotype derived from reduced pigmentation in the oocyte, similar to the *pe* mutation previously recovered in *Xenopus* (Droin and Fischberg, 1984). Because of this, we called this phenotype *pale egg like* (*pel*) and started to consider this a polymorphism of the dark pigmented eggs (*wt*).

The pel *phenotype is a sex-limited genetic trait possibly caused by a recessive allele*

While searching for *pel* individuals in other populations in Ecuador, we were able to perform a pedigree analysis in a family from the Manta Real location (Fig. 1G). A single couple of founders collected in the field laid two egg clutches (C1 and C2) with dark pigmented embryos. These embryos were incubated until hatching and reared under homogeneous laboratory conditions to obtain

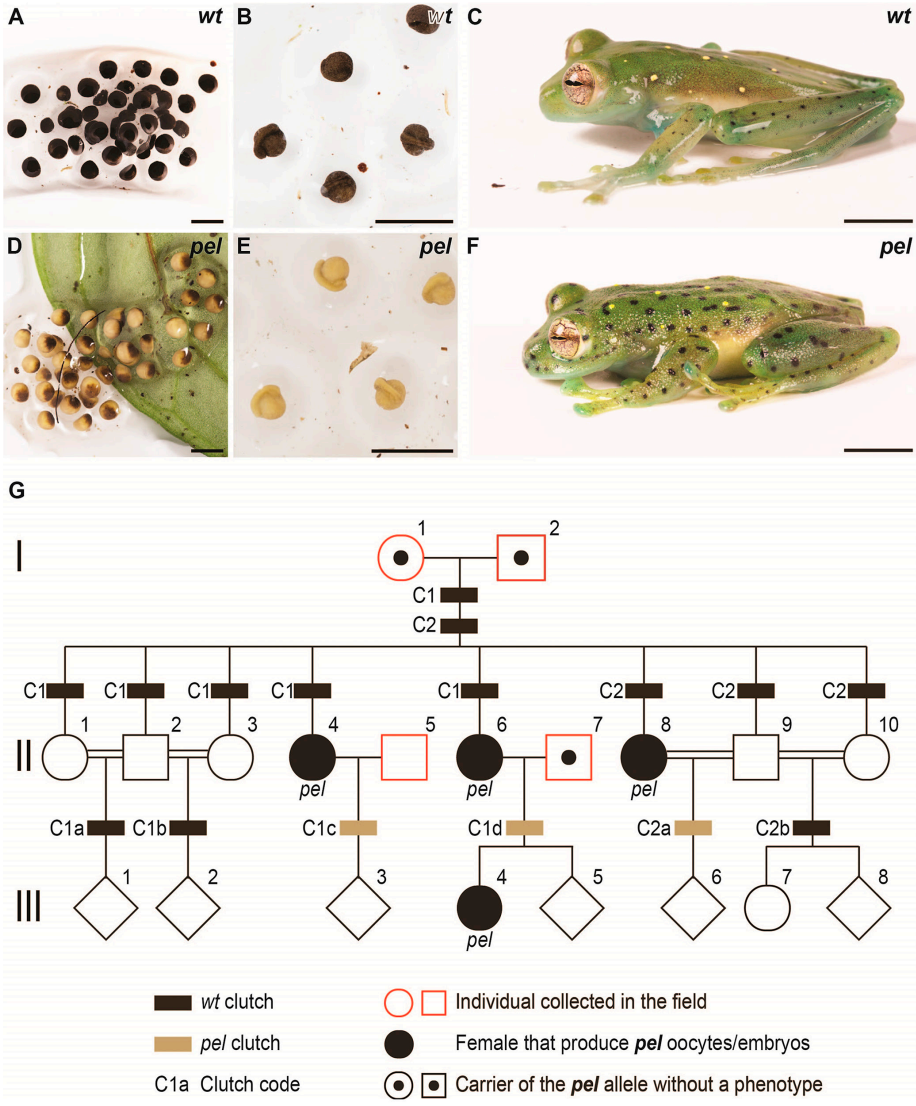


Fig. 1. The *pale eggs like (pel)* phenotype is a genetic trait that produces pale embryos in *Espadarana prosoblepon* from Ecuador. (A) *Dark brown* (wt) *clutch collected at Los Cedros location, on 2014. Embryos within the clutch are in an early blastula stage with approximately 32 cells.* **(B)** *Tailbud stage embryos within the clutch. Image taken 48 hrs after the image in A.* **(C)** *Adult frog developed from the dark brown clutch shown in A. Image taken approximately 2 years after clutch collection.* **(D)** pel *embryo clutch collected, close to the clutch in A, during the same filed trip. Embryos within the clutch are in an early blastula stage with approximately 32 cells.* **(E)** *Tailbud stage embryos from the clutch shown in (D). Image taken 48 hrs after collection.* **(F)** *Adult frog developed from the* pel *clutch shown in (D). Image taken 2 years after clutch collection. These* E. prosoblepon *frogs developed dark colored spots in the skin, similar to the frogs from the clutch in (A-C). Embryos in (A,D) were reared* ex-situ *at the BDLS laboratory in separate containers, with the same food, photoperiod and environmental conditions.* **(G)** *The pedigree analysis shows the recurrence of the* pel *phenotype in a family reared in captivity. The founders (F0) are a couple collected from the field as adults that laid two clutches with dark pigmented eggs (C1 and C2). Three out of six females reared (F1) have the* pel *phenotype (Dark circles) and produced* pel *embryos (C1c, C1d and C2a). The F2 is mostly composed of newly metamorphed froglets without distinguishable sexual features except F2-4 and F2-7, which are females. The color of the oocytes was assessed through the transparent abdominal skin. The time from F0 to the sexual maturity of F1 is 22 months, approximately. The time from F1 to the sexual maturity of F2 is 26 months.* wt, *dark brown oocyte phenotype or "wild type".* pel, Pale eggs like *phenotype. The bars in (A, B, D, E) are 2 mm. The bars in (C, F) are 5 mm.*

F1 metamorphs and adults. Out of approximately 50 eggs laid in total, we were able to rear successfully eight F1 (I) fertile adults, six females and two males. Females carrying *wt* or *pel* oocytes could be easily distinguished due to the characteristic skin transparency of *E. prosoblepon* frogs, which allowed us to see egg color in the abdomen of live females. We identified three *pel* females (II4, II6 and II8), and three *wt* females (II1, II3 and II10). We could not find any clear trait associated to the *pel* phenotype in males; hence, we concluded the *pel* phenotype is a sex-limited genic trait. Family II9-II10 (female with *wt* eggs), produced one female with *wt* eggs. Family II6-II7, a *pel* female and a founder male from the same location, produced one female with *pel* oocytes (III-4), which could confirm the presence of multiple male carriers of the *pel* allele in this population.

The pel *phenotype is widespread in natural populations of* E. prosoblepon

The inheritability of the *pel* phenotype led us to test the commonality of this potential allele. To do this, we evaluated other populations of the species by subsequent field trips and by studying dissected ovaries of adult females preserved at the QCAZ museum. Unexpectedly, we found the same polymorphism in egg clutches collected during field trips to Los Cedros and Junín sites (Fig. 2 A,B). Also, six out of 17 *E. prosoblepon* females preserved at the QCAZ museum, from distinct collection sites in Ecuador, had ovaries with mature *pel oocytes* (oocyte diameters between 1.9 and 2 mm; Fig. 2D). The remaining females had dark pigmented mature oocytes (Fig. 2C).

The phylogenetic relationships among the specimens collected and museum samples confirms that every analyzed specimen belongs to the same species, showing low phylogenetic divergence between populations and no relationship between genetic structure and egg pigmentation (Fig. 2E). Our results support the monophyly of *E. prosoblepon* and show that the Ecuadorian populations form a well-supported clade, sister to a clade from Central America. In addition to Junín and Los Cedros, we found polymorphism for egg color in Río Chillayacu, more than 400 km south from Junín. The *pel* phenotype was also found in females from Jama Coaque, Manabí Province, a dry shrub coastal locality, environmentally different to the cloud forest characteristic of the other sites (Fig.

2 E,F). These results suggest *pel* is a widespread polymorphism within in the Ecuadorian populations of *E. prosoblepon*.

The* pel *phenotype in* E. prosoblepon *is the result of reduced melanic pigment in the oocyte cortex

Through a small, non-lethal, surgical procedure, we were able to extract pieces of ovaries containing mature oocytes to characterize the *pel* phenotype in oocytes from *E. prosoblepon* and *Hyalinobatrachium fleischmanni* females. Mature oocytes were analyzed using paraffin sectioning without counterstain to characterize the pigmentation phenotype. The mature, post-vitellogenic oocytes of *E. prosoblepon* have a diameter of approximately 2 mm (Fig. 3 A,D). Wild type (*wt*) mature oocytes have characteristic dark pigmentation easily distinguishable from previtellogenic oocytes, which lack of pigment, and early vitellogenic oocytes that have some pigment but are much smaller (Fig. 3A). In large vitellogenic oocytes, the vegetal pole is undistinguishable from the animal pole due to the amount of pigmentation. Paraffin sections without a counterstain clearly show the restricted accumulation of dark melanic pigment within the cortex and the lack of pigment in the white, yolk rich, cytoplasm (Fig. 3B). The dark pigment in oocytes apparently is the result of both, the presence of melanosomes with darker pigment and the dense accumulation of these organelles in the outermost region of the cortex (Fig. 3C). Importantly, there seems to be less melanosomes in the vegetal cortex of the *wt* oocytes compared to the animal cortex (Fig. 3C). The animal cortex in oocytes was defined as the cortex closer to the germinal vesicle in a large vitellogenic oocyte.

Oocytes with the *pel* phenotype have a brown-yellow appearance, and pigmentation is clearly located towards the animal pole, leaving a less pigmented vegetal pole (Fig. 3 D-F). In paraffin sections of *pel* oocytes, dark pigmentation is still restricted to the cortex, although pigmentation was less noticeable (Fig. 3E). The animal cortex in *pel* oocytes seems to have more pigment, and the brown-yellow appearance seems to be the result of less melanosomes, dispersed within the outer cortex, that carry a clearer melanic pigment (Fig. 3F). To compare, the mature oocytes of the centrolenid *H. fleischmanni* are completely unpigmented and have no visible pigment during any stage of oogenesis (Fig. 3G), within the oocyte (Fig, 3H), or in the animal cortex (Fig. 3I).

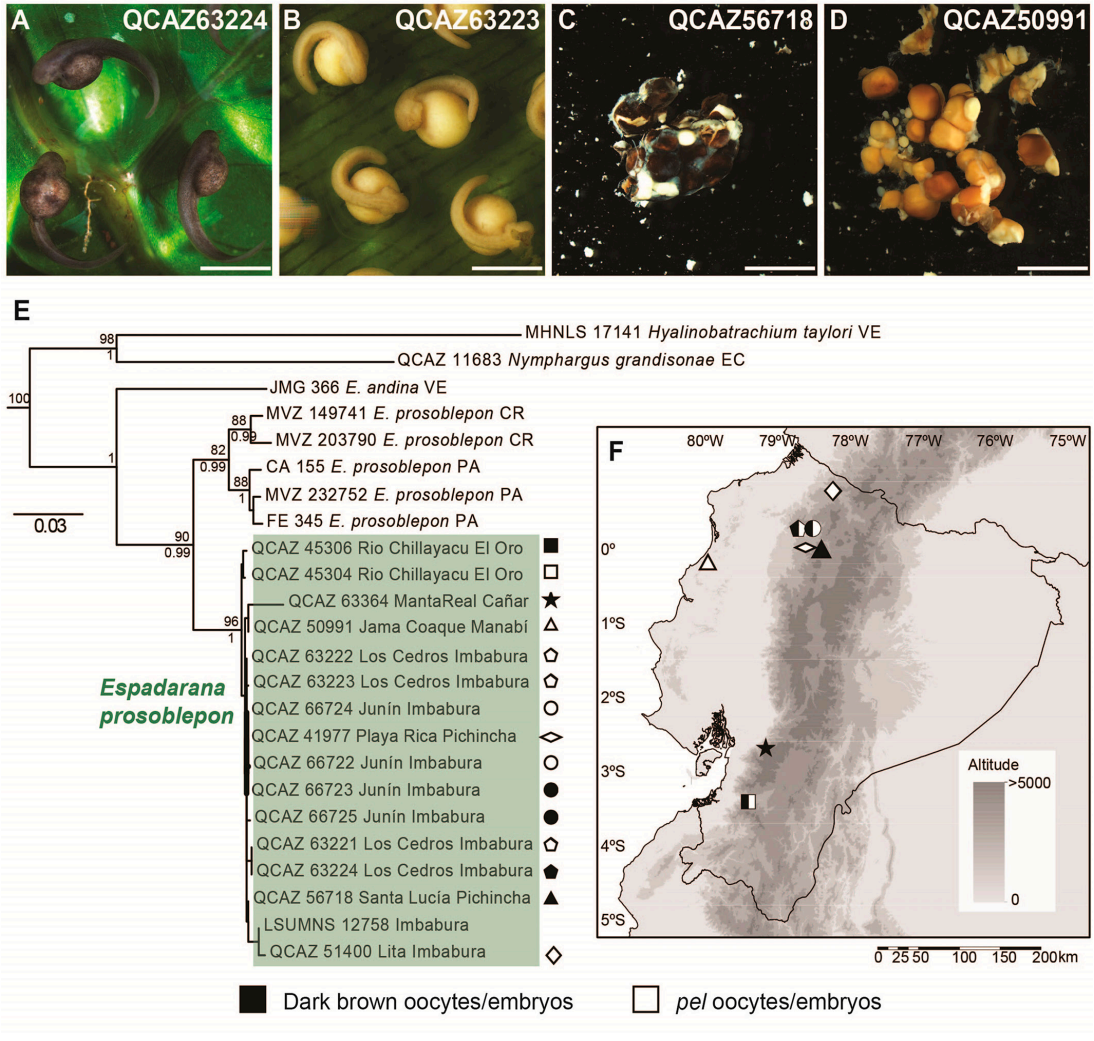


Fig. 2. The *pel* phenotype is common in Ecuadorian populations of *Espadarana prosoblepon*. **(A)** *Dark brown embryos collected at stage 23 (maximum gill extension) from Los Cedros in 2016.* **(B)** pel *embryos collected at stage 19 (heart beat) from Los Cedros on 2016.* **(C)** *Dark brown ovary dissected form an* E. prosoblepon *museum preserved female.* **(D)** pel *ovary dissected form an* E. prosoblepon *museum preserved female. The bars in (A-D) are 4 mm.* **(E)** *Best stepwise Maximum Likelihood tree for the mitochondrial gene NADH dehydrogenase 1 (*ND1*) nucleotide sequences from* E. prosoblepon *preserved tissue in the QCAZ museum (log likelihood = -4115.1963). Collection sites are enlisted in table S1. Sequences used for the analysis are enlisted in table S2. The tree shows a distinctive Ecuadorian population of* E. prosoblepon, *which differentiates from the Panama (PA) and Costa Rica (CR) populations, which conform the well-supported sister clade. The shapes to the right of the code are used to show the collection site on the map in B. If the shape is dark filled it represents a dark brown ovary obtained from a dissection of a preserved specimen or a dark brown embryo preserved for DNA analysis. White filled shapes represent a* pel *ovary dissected or a* pel *embryo preserved for DNA analysis. Images for some of the samples are in Figure 1.* **(F)** *Collection sites within the Ecuadorian territory. In the map, half-dark filled shapes represent locations on which both phenotypes were recorded.*

pel *embryos have reduced egg pigmentation, but produce normal melanocyte derived pigmentation*

Espadarana prosoblepon embryos from Los Cedros and Junín localities, and embryos later obtained from *ex situ* breeding at BDLS were observed and photographed from early stages of cleavage to the hatching of tadpoles to describe the *pel* phenotype. Embryos from *wt* and *pel* clutches develop normally and are able to hatch as tadpoles approximately 4 days after oviposition. In *wt* embryos, dark oocyte pigmentation is retained throughout embryo development (Fig. 4 A-F). Different to mature oocytes, the melanic is concentrated in the animal pole (Fig. 4 A-B). Animal pole derived blastomeres from these embryos will retain this dark pigmentation, which results in dark heterogeneously pigmented embryonic epidermis in further stages (Fig. 4 C-E). In later embryos, neural crest derived melanocytes and eye pigmentation are not distinguishable from the epidermal dark pigmentation (oocyte-derived), and newly hatched tadpoles maintain a dark appearance (Fig. 4 D-F).

In *pel* embryos, dark oocyte pigmentation is only retained in some animal pole blastomeres, leaving an unpigmented vegetal pole (Fig. 4 G-I). This pigment is apparently diluted during subsequent stages, which results in a pale embryonic epidermis (Fig. 4J). During the heart beat stage, a few differentiating melanocytes can be distinguished in the dorsal trunk region, posterior to the head (Fig. 4K). Following the normal progression of melanocyte devel-

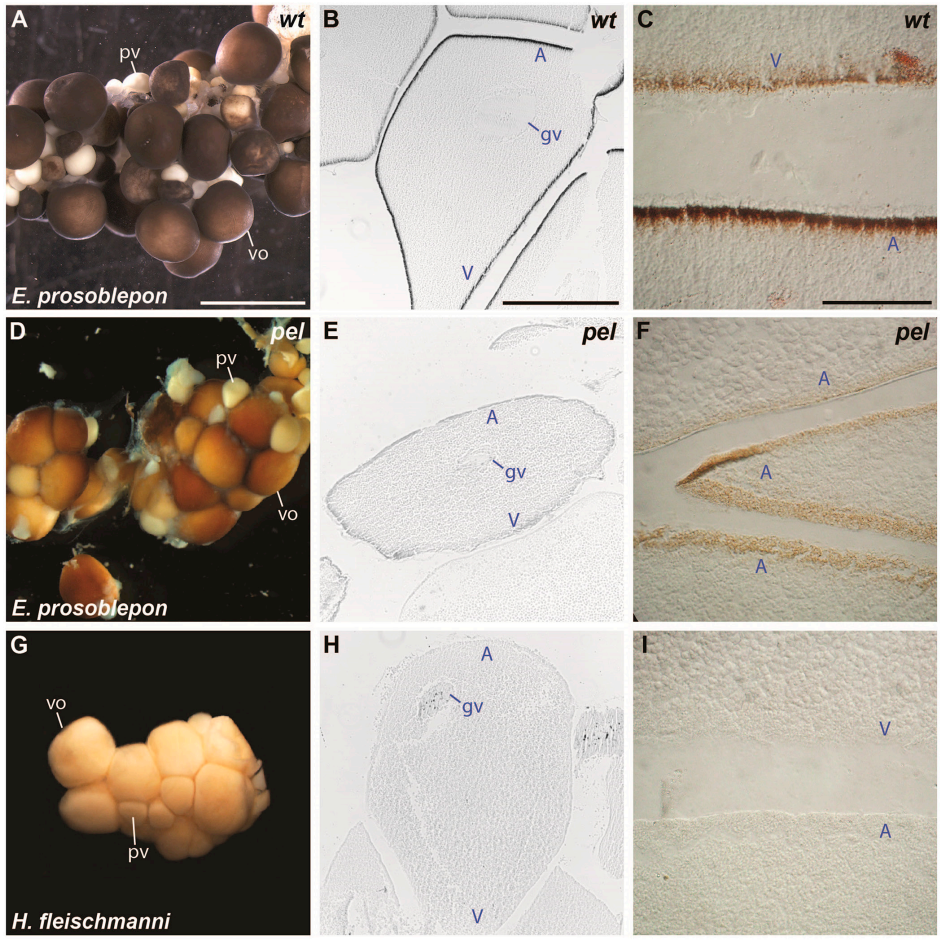


Fig. 3. The *pel* phenotype in ovaries and oocytes from *E. prosoblepon* females is distinct to the unpigmented egg phenotype of *Hyalinobatrachium fleischmanni* (Centrolenidae). (A) *Ovary with the dark brown phenotype from an* E. prosoblepon *female of the "Manta Real" population (Southern Ecuador). Vitellogenic oocytes are dark brown while previtellogenic eggs are smaller and unpigmented.* **(B)** *Dark pigment is located through the cortex of a vitellogenic oocyte.* **(C)** *In the animal pole, melanosomes are tightly packed within the cortex producing dark brown pigmentation. The vegetal pole of an adjacent oocyte also shows pigmentation with less amount of melanosomes.* **(D)** *Ovary with the* pel *phenotype from an* E. prosoblepon *female from the same population of A.* **(E)** *Representative vitellogenic oocyte from the ovary in D. Dark pigment is also located through the cortex of the oocyte, yet the cortex appears to be less pigmented and wider.* **(F)** *Three variations of animal cortex from* pel *oocytes. The animal cortex in* pel *still has dark pigmentation with a light brown-yellow appearance.* **(G)** *Ovary with white eggs from a* Hyalinobatrachium fleischmanni *female (Centrolenidae).* **(H)** *Pigment is not present in any portion of the vitellogenic oocyte.* **(I)** *The cortex of* H. fleischmanni *vitellogenic oocytes lack of melanic pigmentation. (A,D,G) Images are portions of ovaries surgically extracted from females collected in the field and maintained at BDLS (imaged at the same magnification). (B,E,H,C,F,I) Microscopy images of paraffin sections acquired using Nomarski's DIC microscopy. (B,E,H) Acquired under the same illumination setup using a 5X objective and a high sensitivity monochrome microscopy camera. (C,F, I) Acquired under the same illumination setup using a 40X objective and a color microscopy camera. Abbreviations: A, animal pole; gv, germinal vesicle; pv, previtellogenic oocyte; V, vegetal pole; vo, vitellogenic oocyte. Bars: (A) 4 mm; (B) 1 mm; (C) 200 μm.*

opment in amphibians, next stages in *pel* embryos show progressive increase of melanocytes, with a higher density in the most dorsal anterior region. Eye retinal pigmentation is also distinguishable in *pel* embryos, compared to *wt* embryos (Fig. 4 M,N). Newly hatched tadpoles have a dark appearance due to melanocyte derived pigmentation but overall paler when compared to *wt* tadpoles (Fig. 4L).

Altogether, our results provide strong evidence of the existence of a common allele in Ecuadorian populations of *E. prosoblepon* that affects oocyte pigmentation but not neural-crest derived melanogenesis.

Discussion

This manuscript presents the first evidence of polymorphisms in egg pigmentation in an amphibian. *Espadarana prosoblepon* has previously been described as a species that produces dark eggs (Savage, 2002). From museum specimens and field collected clutches, we could conclude that egg pigmentation in *E. prosoblepon* is polymorphic, and that most females produce dark-brown eggs. However, we don´t have enough field data to quantify the commonality of the *pel* phenotype in comparison to *wt* eggs. The same occurs with most amphibian species on which egg pigmentation is generally thought to be a monomorphic and stable character. Flores *et al.,* (2012) reported a similar variation in *Dendrobates auratus* (Dendrobatidae). These embryos from a single couple developed normally, the hatched tadpoles were similar in size to other dark-pigmented tadpoles of the same species, and the froglets were normal-colored (Flores *et al*., 2012). While this population might have a similar polymorphism, it has not been reported yet. Variations in egg pigmentation were also reported in clutches of *Notophthalmus viridescens* without further analyses (Bishop, 1941).

The evolution of egg pigmentation in Centrolenidae and other anurans

There is a possibility that the dark pigmented character might not be fixed in the *Espadarana* group, and the existence of polymorphisms like *pel* might be an evidence of a “soft reversal” to a plesiomorphic state (Kornet and Turner, 1999). To

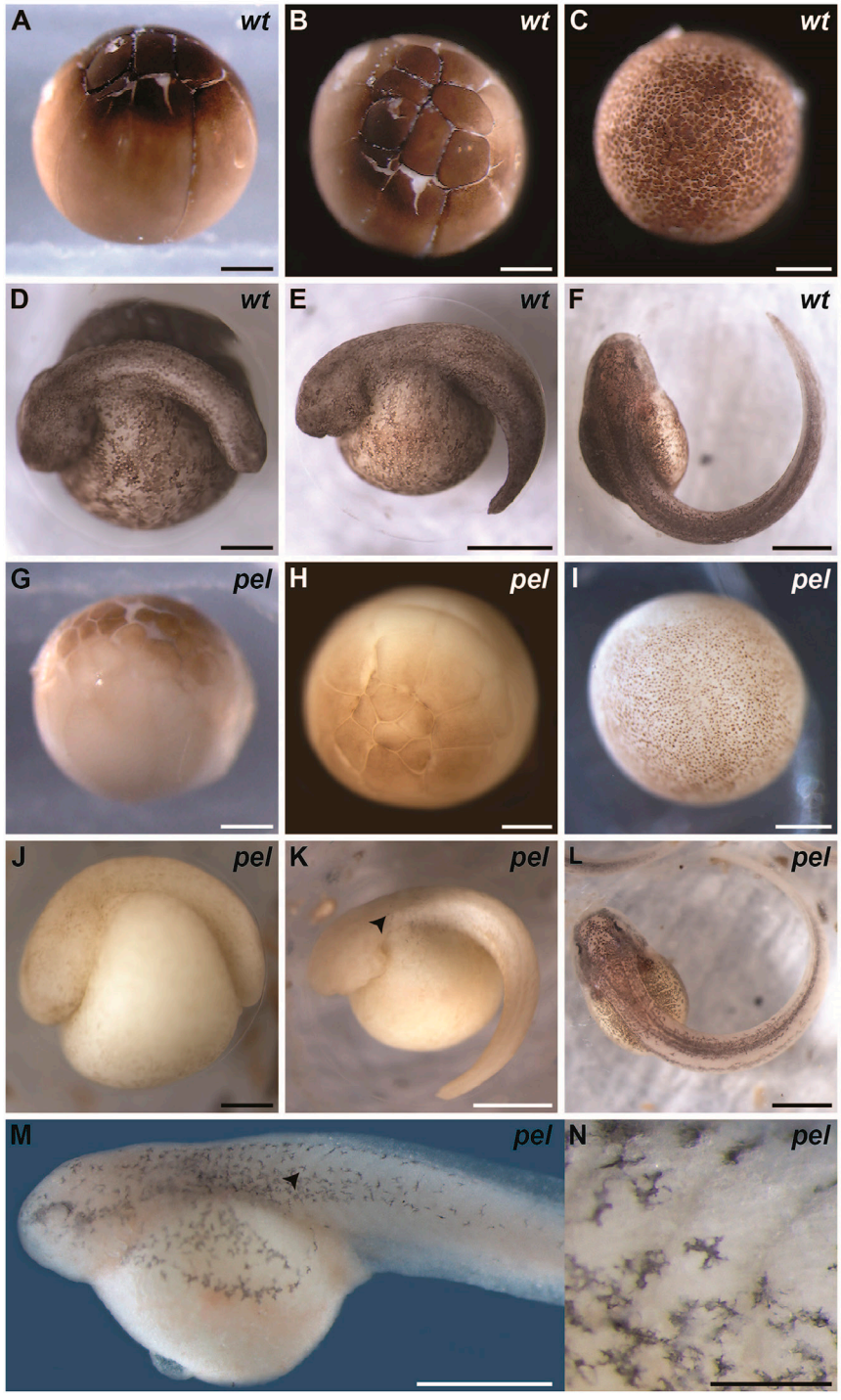


Fig. 4. Development of *E. prosoblepon* embryos with dark brown (*wt*) (A-F) and *pel* (G-N) phenotypes. *Embryos in (A-C) and (G-I) were fixed on the field and imaged later on.* **(A)** *Lateral view of a 16-cell embryo. The dark brown cytoplasmic pigment is more concentrated in the animal pole and decreases toward the vegetal hemisphere.* **(B)** *Animal view of the embryo in A.* **(C)** *Animal view of a small cell blastula (late-blastula). Dark brown pigment is retained on every animal cell.* **(D)** *Dark brown embryo at tail bud stage (St. 18).* **(E)** *Lateral view of an embryo in heart beat stage.* **(F)** *Dorsal view of a tadpole at hatching.* **(G)** *Lateral view of a 32-cell embryo with the* pel *phenotype. There is less dark pigment in the animal cells and no pigment is visible towards the vegetal hemisphere.* **(H)** *Animal view of the embryo in (G).* **(I)** *Animal view of a small cell blastula (late-blastula)* pel *embryo. Dark brown pigment is retained on every animal cell.* **(J)** *Lateral view of a* pel *embryo at tailbud stage (st. 17). No remaining oocyte pigment is visible at this stage* **(K)** pel *embryo at heart beat stage (St. 19). The arrow shows a differentiating melanocyte originating from the dorsal anterior region.* **(L)** *Dorsal view of a tadpole at hatching. Dark pigment derived from the neural crest covers the dorsal part of the tadpole.* **(M)** *Lateral view of a* pel *embryo at gill circulation stage (St. 20). The arrow indicates a melanocyte amplified in N.* **(N)** *Neural crest derived pigmented cells originated from the dorsal part of the embryo present the characteristic shape of a melanocyte. Bars: (A-D, G-J, N) 500 μm; (E,F, K-M) 2 mm.*

understand the state of egg pigmentation in glass frogs (Centrolenidae) we performed a literature review regarding reported egg color and site of oviposition in 56 species (Fig. 5A). The character reconstruction analyses for egg color pigmentation in Centrolenid frogs show that whitish eggs are the ancestral character, since it is present across glass frogs lineages, including the most recent common ancestor of the Centrolenids (proportional likelihoods = 0.71 for "whitish"). Every member of the Hyalinobatrachinae clade has whitish eggs, possibly unpigmented like in *H. fleischmanni* (Fig. 3G-3I). Dark colored oocytes appear 10 times throughout this glass frog phylogeny, independently, in the Centrolenini and Cochranellini clades. Among the Centrolenini clade, only two species have dark pigmented eggs: *Centrolene geckoideum* and *C. buckleyi* (Fig. 5A). The majority of species with dark pigmented eggs have been reported in Cochranellini, where seven out of 19 assessed species have dark pigmented eggs, including several species in the genus *Espadarana*, *Cochranella*, *Sachatamia*, and *Rulyrana* (Fig. 5B). Within *Espadarana* there is generic variability in egg color pigmentation. For example, whitish eggs are recorded in *E. andina* (Rojas-Runjaic *et al*., 2012), and dark pigmented eggs in *E. callistomma* (Salazar-Nicholls and del Pino, 2015) and some populations of *E. prosoblepon* (this study). The presence of a polymorphism in the *Espadarana* clade and the relatively recent divergence between the *E. prosoblepon – E. callistomma* supports the hypothesis that egg pigmentation is an unfixed evolutionary character on which "reversals" to the ancestral state are possible (Kornet and Turner, 1999). These analyses also suggest that the *pel* polymorphism might be common in other *Espadarana* species. Yet, there is no data regarding the amount and distribution of oocyte melanin in other *Espadarana* species. Importantly, these results should be interpreted with caution, since most reports come from observations rather than from statistically supported surveys, and the analysis only covers 56 of the 161 currently known species. To compare, in Bufonidae and Leptodactylidae, dark pigmentation is a plesiomorphic character: most species have dark pigmented eggs, and whitish eggs have appeared independently, at least three times, in these families (Grosso *et al*., 2019, 2017; Vera Candioti *et al*., 2016).

The genetic and molecular basis of a polymorphism in egg pigmentation

Polymorphic characters are the result of variations in the population's allelic frequencies, variation in environmental selective pressures, or both (Leimar, 2009). Here we have shown that pale eggs in *E. prosoblepon* are the result of a genetic trait, *pel,* which causes a sex-limited phenotype, since males don't produce oocytes. The fact that 50% of the females carried the *pel* phenotype leads us to conclude that the founders are possible carriers of a recessive allele, assuming *pel* is caused by a single allele inherited through mendelian mechanisms. This also allowed us to discard, preliminarily, the possibility of a maternal effect gene or an unknown environmental factor, since the *pel* phenotype appeared in 50% of an F1 progeny reared under the same controlled laboratory conditions.

A similar allele was recovered once before, in a genetic analysis of mutations arisen from nuclear transplantation in *X. laevis.* Droin and Fischberg (1984) called this mutation "*pale eggs"* and determined that this mutation was caused by a single recessive autosomal allele. We can only hypothesize that the *pel* phenotype is caused by a single allele that follow a mendelian pattern of inheritance since we don't have enough crosses to identify homozygotes or genotype the founders. This is a complicated task, given the long time required to obtain mature adults and the poor success during tadpole rearing. We are currently working on genomic approaches to genotype these individuals and address potential genes and pathways involved in this phenotype.

The potential genes and molecules related to the *pel* phenotype could be part of pathways involved in melanogenesis, in melanosome biogenesis and transport, or in melanosome stability. In amphibian oocytes, the melanogenic pathway is activated independently from other pigmented tissues through mechanisms that are yet to be discovered (Kidson and Fabian, 1989). Accordingly, mutations in genes of the melanogenic pathway that cause organismal albinism also cause unpigmented oocytes, as shown in *Rana pipiens*, and *Ambystoma mexicanum* (Bluemink and Hoperskaya, 1975; Browder, 1972; Eppig and Dumont, 1974; Smith-Gill *et al*., 1972). However, amphibian whitish eggs might not only be the result of a deleterious effect over the melanogenic pathway, but also due to a failure in the stability or transport of melanosomes. In the commonly used *Xenopus* periodic albino mutant early melanocytes and retinal-pigmented cells form melanosomes that are later lost in adults, and the oocytes fail to form mature melanosomes (Hoperskaya, 1975; Seldenrijk *et al*., 1982). Importantly, in this mutant, the enzyme tyrosinase is functional and the mutated gene that causes the phenotype is still unknown (Wyllie and De Robertis, 1976). Another example of a melanosome stability phenotype are the *X. laevis* "*rusty"* mutants, where defects in the process of melanosome extrusion produced highly pigmented embryos and larvae, with no other apparent defect. Oocyte melanosomes in their *wt* siblings were normally extruded from the skin and tissues in later tadpole stages (Uehlinger *et al*., 1971). To date, the *rusty* and the *pale eggs* mutations haven't been mapped to the *X. laevis* genome (Droin, 1992; Rungger, 2002). Less known pathways that control chromatophore cell differentiation may also be involved since *Rana japonica* mutants with reduced chromatophores produce a phenotype similar to *pale eggs* (Sumida *et al*., 2016).

Melanosome transport also affects egg pigmentation. Túngara frogs (*Engystomops*, Leptodactylidae) early embryos are visibly whitish. However, oocyte melanic pigment seems to be retained around the nuclei of early blastomeres, and melanosomes are not located in the cellular cortex (Romero-Carvajal *et al*., 2009). Lee *et al.,* (2009) showed that Shroom2 and Spectrin, proteins that interact with the cytoskeletal melanosome transport system in melanocytes, show colocalization to the perinuclear region in *Engystomops pustulosus.* This suggests that melanosome localization in *Engystomops* eggs might be the result of a differential interaction between the cytoskeleton and the melanosome transport system (Lee *et al*., 2009). In *Engystomops*, however, perinuclear pigment is not dark or abundant, suggesting that these whitish eggs might be the result, not only of a failure in cortical melanosomes transport but also of reduced melanin synthesis. In *Agalychnis spurrelli* there is a reduced amount of melanin correctly localized towards the apical pole and perinuclear pigmentation (Schmid *et al*., 2018). Our results show that, in *pel* oocytes and early embryos less melanosomes are located in the cortical region, with no evidence of perinuclear pigmentation. Further analyses are still needed to understand the ultrastructure and composition of melanosomes in *pel* oocytes in *E. prosoblepon*.

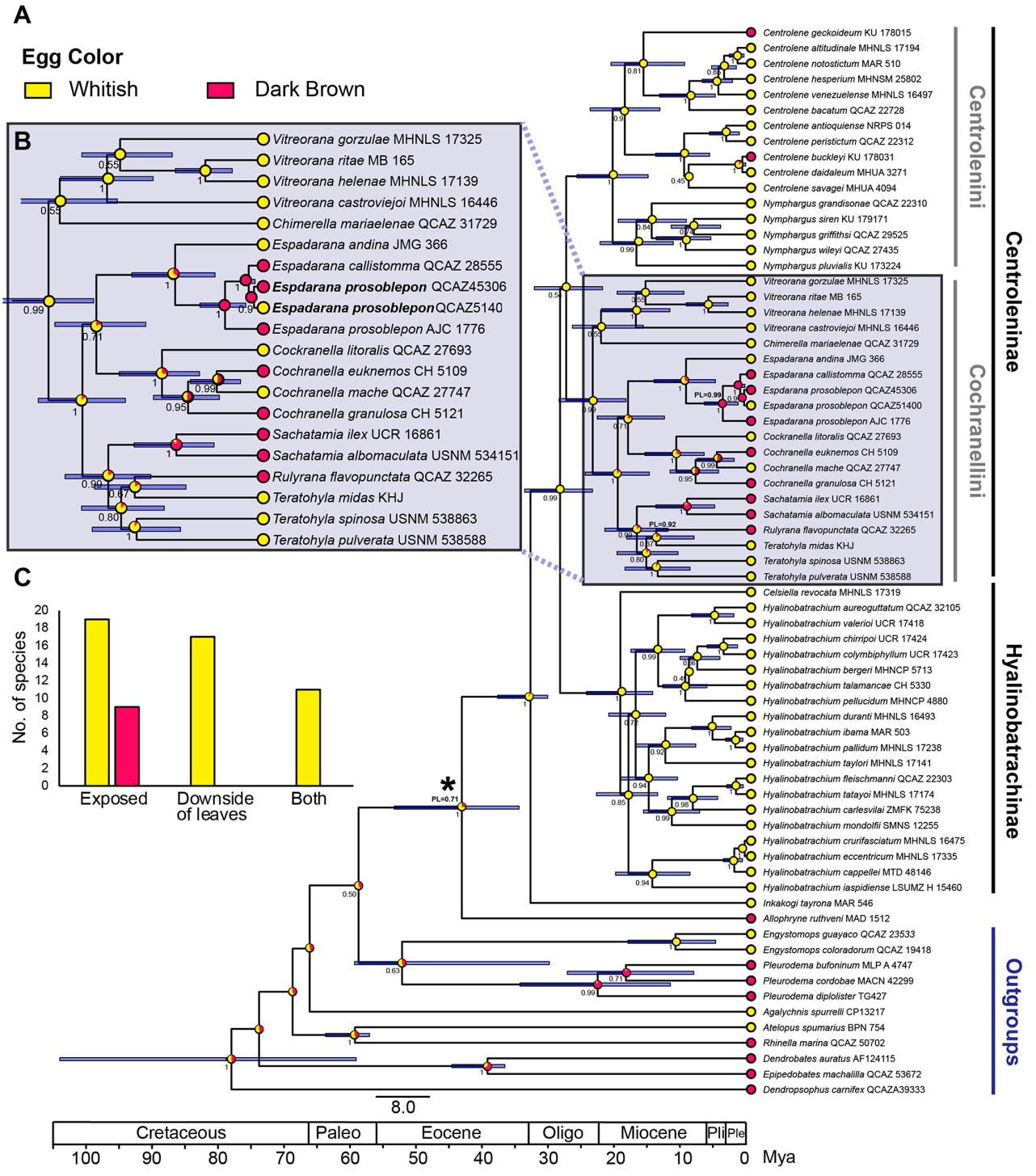


Fig. 5. Whitish eggs are ancestral in Centrolenidae. (A) *Maximum likelihood ancestral character reconstruction of egg pigment coloration for 58 glass frog species, based on relaxed uncorrelated molecular clock using 16S sequences (Table S2). Circle and bar color indicate egg pigmentation, yellow for whitish eggs, magenta for dark brown eggs. Pie charts at the nodes indicate the probabilities of each state based on maximum likelihood estimates. An asterisk denotes the most recent common ancestor of the family. Branch support posterior probabilities are below the branches, and proportional likelihoods (PL) for the dark-brown state for selected nodes are above branches.* **(B)** *Ancestral character reconstruction for the tribe Cochranellini, including several species in the genus* Espadarana. *In this group, the dark brow egg character appeared at least six times during evolution.* **(C)** *Number of centrolenid species for which oviposition site and egg color is known (Table S3). An exposed oviposition site implies mostly the upper side of leaves; however, for a few species there were other exposed sites reported, such as branches, rocks and moss surfaces. The only other reported oviposition site was the underside of leaves. For some species, both sites were reported, either in the same or in distinct publications.*

The adaptive role of egg pigmentation polymorphisms

Melanin based pigmentation represents a tractable character to study evolution and adaptation because its synthesis is controlled by a well-described genetic program (Dubey and Roulin, 2014). In addition, polymorphisms in melanic patterns have been linked to changes in selective pressures and changes in a population's genetic composition, in multiple vertebrate species (Dubey and Roulin, 2014; McKinnon and Pierotti, 2010).

The main selective pressures over egg survival are predation, parasitism, and hazardous abiotic factors like UV radiation. Melanin might be playing multiple adaptive roles guided to the survival of early embryos, mainly by camouflaging embryos from predators and by protecting them against ultraviolet radiation (UV-A and UV-B) (Altig and McDiarmid, 2007; Duellman and Trueb, 1994). UV-B radiation seems to be the most toxic, not only to DNA but also to mRNA and cytoskeletal elements important for embryo development: Axolotl, *Rana,* and *Xenopus* eggs irradiated from the vegetal pole fail to form a neural tube and axial structures (Chung and Malacinski, 1975; Manes and Elinson, 1980). Only one study has shown increased sensitivity of Axolotl albino eggs to UV radiation (Malacinski *et al.*, 1975), and no studies on the effect of UV-B irradiation have been conducted in naturally occurring whitish eggs. Experimental irradiation in pigmented eggs of amphibians has shown variability on the deleterious effects of UV (Anzalone *et al.*, 1998; Licht, 2003; Palen *et al.*, 2005). This variability could be explained by marked interspecific differences in the activity of photolyases, which repair UV-induced DNA damage, and the protectant role of the egg jelly (Blaustein and Belden, 2003; Blaustein *et al.*, 1994; Hays *et al.*, 1996). Although still incomplete, evidence suggests UV protection might not be the main role of oocyte derived melanin. Certainly, controlled experiments of UV irradiation in albino eggs or in polymorphic pale eggs, might help to pinpoint the actual contribution of melanic pigmentation to UV-protection in amphibian oocytes and early embryos.

Much less evidence is available regarding egg pigmentation and the protection against predators. It has been hypothesized that dark pigment would act as camouflage, since predators would not be able to distinguish the eggs from a dark background (Savage, 2002). This hypothesis could help explain the ubiquitous tendency in amphibian eggs to accumulate melanic pigment in the animal pole. It would also explain variability in the amount of oocyte derived melanic pigment among species of Bufonidae and Leptodactylidae (Grosso *et al.*, 2019, 2017; Vera Candioti *et al.*, 2016). Still, there is no experimental evidence on the role of egg pigmentation as camouflage against predators.

The influence of egg pigmentation in the oviposition site

The site of oviposition might influence the survival of the progeny. In amphibians, oviposition adaptations range from aquatic nesting to egg brooding and viviparity (Haddad, Célio; Prado, 2005). Oviposition sites might also contribute to protect eggs against UV radiation and predation. Since egg pigmentation appears to be determined genetically, it is possible that egg pigmentation influences the parental behavior of choosing an oviposition site. This has not been analyzed in amphibians. Our analysis on reported data shows that egg pigmentation is not guiding oviposition behavior, since there are reports of species with whitish eggs depositing eggs in exposed areas, mainly the upper side of leaves (Fig. 5A; Table S3). A similar phenomenon has been reported in Bufonidae, Leptodatylidae and Hylinae (Arboranae), where pale eggs have appeared independently, with no apparent relation to oviposition behavior (Grandison, 1978; Grosso *et al.*, 2019; Heyer, 1969; Nali *et al.*, 2014).

In Centrolenidae, parental guarding of egg clutches could compensate the lack of pigmentation. By reducing selective pressures, parental care could allow for the appearance of potentially deleterious phenotypes like *pel* (McKinnon and Pierotti, 2010). Parental guarding is widespread across Centrolenidae, and some species with dark pigmented eggs like *E. prosoblepon* have reduced parental care, compared to species with whitish eggs (Delia *et al.*, 2017; Jacobson, 1985). In locations where *H. fleischmanni* overlaps with a batrachophagous bat (*Trachops cirrhosus*), nesting behavior and oviposition is enclosed, in the downside of leaves, and in locations outside the distribution of the bat, oviposition occurs in exposed locations (Delia *et al.*, 2010). The study of nesting behavior in the polymorphic populations of *E. prosoblepon* could provide evidence to support this hypothesis. Interestingly, the dendrobatids *Ranitomeya vanzolinii* and *R. imitator* species show biparental extended care of enclosed eggs that are whitish ("cream") in appearance (Brown *et al.*, 2011). This is rare in Dendrobatidae, where most species lay dark pigmented embryos and exhibit parental care (Grant *et al.*, 2006).

To conclude, the adaptive role of egg pigmentation in amphibians remains elusive due to incomplete experimental evidence and the complex interactions between phenotype and behavior that have evolved to ensure the survival of early embryos. Research on predation, survival and behavior related to the *pel* phenotype in *E. prosoblepon* could help us understand the true adaptive role of egg pigmentation.

Materials and Methods

Taxon sampling, collection sites and* ex situ *maintenance

During three expeditions starting in 2014, two sites in the province of Imbabura in the Ecuadorian lowlands were visited for the collection of *E. prosoblepon*. Sampling in Los Cedros Biological Reserve (0,3088 N; 78,77927 W, 1399 m above sea level) resulted in the collecting of two pairs of *E. prosoblepon* in amplexus that deposited dark brown and *pel* egg clutches in captivity. In a second visit to the same site in 2016, we collected two *pel* clutches (field series SC 54161, 16 eggs; SC 54165, 35 eggs) and two wild type, dark pigmented (*wt*) clutches (SC 54236, 24 eggs; SC 54237, 20 eggs). Field series numbers (SC) provide unique identifiers for individuals or clutches collected in the field. A second locality, Junín (0.282939 N, 78.667738 W, 1350 m above sea level), was surveyed in 2017, from which we collected two *pel* clutches (23 and 15 late embryos) and two *wt* clutches (37 early embryos and 35 advanced embryos). Collected adult specimens and eggs were kept alive at "Balsa de los Sapos" (BDLS), an *ex situ* facility at Pontificia Universidad Católica del Ecuador for the study and conservation of Ecuadorian amphibians. "Balsa de los Sapos" also maintains a colony of *E. prosoblepon* individuals from Manta Real (western Andean slope in Cañar province, Ecuador 2.5404 S, 79.3570 W, 330 m above sea level) and *Hyalinobatrachium fleischmanni* (Chocó Forest, Esmeraldas Province, Ecuador, 78.62405, 1.04186). Romero-Carvajal *et al.,* (2009) described in detail conditions for ex situ maintenance at BDLS. Embryos, tadpoles and adults of *E. prosoblepon* of both phenotypes were reared under the same light, food and temperature conditions. Additional specimens of *E. prosoblepon* for the analyses of ovaries and for DNA extraction were obtained from museum herpetological collections deposited at the Museo de Zoología of the Pontificia Universidad Católica del Ecuador (QCAZ; Table S1). The following collecting permits from the Ecuadorian Ministry of Environment

allowed this study: 005-14 IC-FAU-DNB/MA, 002-16 IC-FAU-DNB/MA, 003-17 IC-FAU-DNB/MA

Oocyte collection, tissue fixation and histology

Surgical procedures on living frogs were performed following international IACUC guidelines for *Xenopus laevis* oocyte collection (NIH, 2019). Females were placed in autoclaved large glass petri dishes with enough *s*terile 1X Steinberg's solution to cover the abdomen without submerging the nostrils. Drops of 0.5% Tricaine methanesulfonate (MS-222) were slowly added to the solution until a surgical level of anesthesia was confirmed (app. 6 drops within an hour). To extract pieces of ovaries, we performed a 5 mm incision in a ventro-lateral location of the abdomen, near one of the ovaries. To suture, we used absorbable violet braided (Vicryl 6-0). After the surgery, frogs were rinsed in sterile 1X Steinberg's and transferred to a larger recovery container with enough ventilation and humidity. Anesthesia recovery, survival and feeding were recorded for every intervened individual after a week.

Museum specimens of adult females were also evaluated for the description of egg phenotypes. The QCAZ museum collection contains 191 specimens of *Espadarana prosoblepon*, including females and oocytes. Thirty-five females were dissected, and the ovaries with oocytes were extracted and preserved in 90% Ethanol. Each set of oocytes from females was measured to establish maturation stage (oocyte diameter around 2 mm) and photographed. Egg pigmentation phenotype was established using only mature oocytes.

Embryos staging was done according to Salazar-Nicholls and del Pino (2015). Before fixation, egg jelly was manually removed from embryos using fine tweezers. Embryos and surgically collected oocytes were fixed in 4% Formaldehyde in PBS for imaging and histological analyses. Paraffin embedding and sectioning were done following standard procedures (Kelly *et al*., 1991). No counterstain was used to avoid interference in the pigmentation assessment.

Live embryos or fixed tissue were imaged using a DP73 color camera attached to a CKX41 Stereomicroscope using the Software *cellSens* (Olympus). Paraffin sections were imaged with Nomarski's DIC illumination using either a Nikon DS-Qi2 monochrome camera or an AxioCam MRC5 color camera attached to a Zeiss Z1 Axio-Observer microscope.

Phylogenetic analyses and ancestral character reconstruction

We carried out two phylogenetic analyses. The first one was done for samples of *Espadarana prosoblepon* and closely related species, to test the hypothesis that the *pel* phenotype might belong to a cryptic or species living in sympatry. The second analysis was done at the family level to trace the evolution of egg pigmentation.

DNA extractions, sequencing and alignment

Total genomic DNA was extracted from 15 specimens of *E. prosoblepon* (Table S2), using a modified guanidinium isothiocyanate protocol (Esselstyn *et al*., 2008). Two mitochondrial DNA regions were amplified: NADH dehydrogenase subunit 1 (*ND1*), and 16S of rRNA (16S). The mitochondrial gene *ND1* was sequenced in two fragments using the primers 16S-frog (5′–TTACCCTRGGGATAACAGCGCAA–3;′ Wiens *et al*., 2005), WL384 (5′–GAGATWGTTTGWGCAACTGCTCG–3′; Moen and Wiens, 2009), tMet-frog (5′–TTGGGGTATGGGCCCAAAAGCT–3′;Wiens *et al*., 2005) and WL379 (5′– GCAATAATYATYTGAACMCC–3′; Moen and Wiens, 2009), following standard amplification protocols (Zhang *et al.,* 2013). The 16S of rRNA (16S) sequences were amplified in two fragments using primers 12Sc(L) (5′–GTRGGCCTAAAAGCAGCCAC–3′), 16Sa (5′–ATGTTTTTGGTAAACAGGCG–3′; Goebel *et al*., 1999), 16SC-16L (5′–GTRGGCCTAAAAGCAGCCAC–3′; Pauly *et al*., 2004), and 16Sbr-H (5′–CCGGTCTGAACTCAGATCACG T –3′; Palumbi *et al*., 1991). For the 16SC-16Sbr combination, we used the following PCR parameters: 94°C (2 minutes), 50°C (30 seconds), 72°C (1 minute) followed by 10 cycles of 94°C (30 seconds), 50°C (30 seconds), 72°C (1 minute) and 22 cycles of 94°C (30 seconds), 55°C (30 seconds), 72°C (1 minute), and a final temperature of 72°C (5 minutes). PCRs were performed in 25 μl volume: 1×PCR Buffer (–Mg), 3 mM MgCl2, 0.2 mM dNTP mix, 0.2 μM of each primer, 0.1 U/μl of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and 1 μl of DNA. PCR products were purified with ExoSAP-IT (Affymetrix, Cleveland, OH). Sequencing was done commercially by Macrogen Korea Inc. (Seul, Korea). Additional sequences of *E. prosoblepon* and outgroup taxa were retrieved from GenBank for phylogenetic analyses (Table S2). Outgroup sampling comprised of 68 taxa including other species of *Espadarana,* species within the Centrolenidae family, and other anuran lineages (Table S2).

DNA sequences were assembled and aligned in Geneious v7.1.7 (Drummond *et al*., 2010) using the MAFFT plugin (Katoh and Standley, 2013), under default settings. Alignment errors were manually adjusted in Mesquite v3.51 (Maddison and Maddison, 2018). PartitionFinder (Lanfear *et al*., 2012) was used to obtain a partition scheme and best nucleotide substitution models for the phylogenetic search using the Bayesian Information Criterion (BIC) as optimality criterion.

Espadarana prosoblepon phylogenetic analysis

Analyses were carried out in Garli v2.0 (Zwickl, 2006) and MrBayes v3.2.2 (Ronquist *et al*., 2012). For the analyses performed in Garli we used default settings, including 10 independent searches starting from random trees, 10 searches starting from stepwise trees, and nodal support was estimated with 200 pseudoreplicates of non-parametric bootstrapping. The 50% majority rule consensus tree was obtained with Mesquite v3.51 (Maddison and Maddison, 2018). For MrBayes we conducted four independent runs, each with five MCMC chains that ran for 1 x104 generations, sampling every 1000 generations. The resulting log files were analyzed in Tracer v1.7. (Rambaut *et al*., 2018) to evaluate convergence and effective sample sizes (ESS). Lastly, 10% burning was applied to the output trees, and the 50% majority rule consensus tree was obtained using Mesquite v3.51 (Maddison and Maddison, 2018).

Ancestral character reconstruction

The selection of taxa for ancestral character reconstruction and phylogenetic analyses was based on availability of sequences in GenBank, phylogenetic relations, and existing information about egg coloration and ovipositional data (58 species and 11 outgroups, Table S2). First, we generated a phylogeny using 16S sequences, a relaxed uncorrelated clock and the Yule speciation prior in BEAST v.1.7.0. (Drummond *et al*., 2012; Table S3). Five points of calibration were included: the split between *Teratohyla* species 2.7 Ma (Castroviejo-Fisher *et al*., 2014), split between *Celsiella* species 5.0 Ma and the *c*rown Centrolenidae 15.0-30.0 Ma (Roelants *et al*., 2007), for the glass frog lineages. For outgroups, we used the age calculated in Guillory *et al*.(2019), 38.5 Ma for Dendrobatidae, and 57.0 Ma for Bufonidae (Baéz and Gasparini, 1979). Beast log files were examined in Tracer v1.7. (Rambaut *et al*., 2018) to ensure effective samples sizes. Output trees were analyzed in TreeAnnotator 2.0.02 (http://beast.bio.ed.ac.uk), using maximum clade credibility after a 10% burn–in.

For the ancestral character reconstruction we performed analyses in Mesquite v.3.51 (Maddison and Maddison, 2018). Egg color was coded as a binary variable: (0) pale and (1) dark. The reconstruction was made using Maximum Likelihood with the Markov k-state single-parameter (mk1) model. Transition between character states across centrolenid lineages were traced in the ultrametric tree previously generated in TreeAnnotator.

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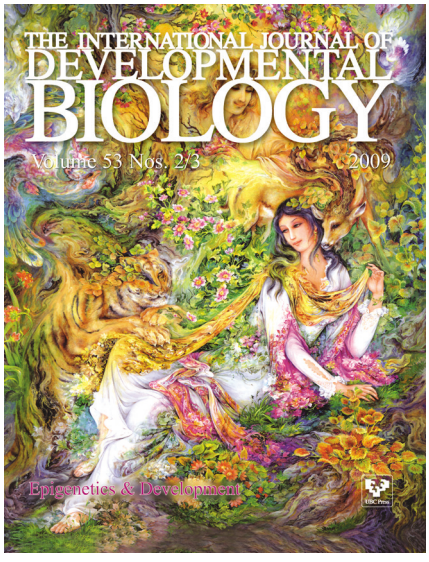
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