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Embryological development of the high-altitude killifish *Orestias ascotanensis* Parenti 1984 (Teleostei: Cyprinodontidae)

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Abstract *Orestias ascotanensis* Parenti is a critically-endangered fish species endemic to the Ascotán salt pan springs. Given the importance of reproduction and early development in effective conservation of fish populations, this study describes the embryonic development of *O. ascotanensis* under laboratory conditions. Between 2014 and 2015, 50 individuals were captured and maintained under controlled conditions, including temperature, photoperiod and feeding regimen, to induce spawning for artificial fertilization. Females were subjected to hormone therapy with Ovaprim®. Water temperature was maintained at 20 °C (± 1 °C), conductivity at 2800–3600 μS·cm^{-1} and photoperiod at 16:8 (light hours: dark hours). Males showed changes in color and courtship behavior, and females developed a slightly bulging belly. Eggs were 1.55–2.5 mm in diameter, with abundant yolk and dozens of adhesion filaments. Embryonic development lasted 14–18 days; it was divided into five periods, which can be sub-divided into 21 stages, from fertilization to hatching. *O. ascotanensis* showed indications of partial spawning species, including oocytes at different stages of development, relatively large eggs as compared to adult fish length and low batch spawning. Characteristics such as partial spawning, highly-pigmented eggs and larvae and advanced larval development at hatching could be adaptations to the extreme conditions of the salt pan springs, including high levels of ultraviolet radiation and salinity as well as significant daily temperature changes.

Keywords Extremophiles · Altiplano · Salt pan springs · Artificial reproduction

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Introduction

Teleost fish have developed reproductive strategies to maximize the number of offspring produced over the lifecycle, including specific anatomical, physiological and behavioral characteristics. Studies have also shown that teleost reproduction is closely synchronized with environmental characteristics, some of which stimulate hormonal mechanisms associated with the hypothalamus-hypophysis-gonad axis (Murua and Saborido-Rey 2003).

Environmental signals such as photoperiod and daily temperature oscillations may regulate annual reproductive cycles. These variables may also produce an environmental selection pressure on reproductive traits and the timing of spawning (Carrillo and Rodriguez 2001). At least in part as a response to these environmental pressures, fish throughout the world exhibit diverse reproductive styles. Some species reproduce only once and then die, while others experience multiple reproductive periods (Wootton 1999). Moreover, there are two main types of spawning, total and partial, each with a different synchrony of oocyte

maturation (Tyler and Sumpter 1996; Murua and Saborido-Rey 2003). Partial spawners live mainly in middle latitudes, with spawning sometimes extending throughout the year (Comte and Vila 1992). Spawning is external in most fish (Miller and Nummela 2009). Eggs may be demersal or pelagic, although most freshwater fish have demersal eggs, whether free or adherent to vegetation. Cyprinodontiformes or "killifish," which have adherent demersal eggs, show interesting reproductive adaptations, such as parthenogenesis, ovovivipary and slowing of development during dry periods (Leis and Carson-Ewart 2000; Furness 2016; Podrabsky et al. 2017; Arezo et al. 2017; Dominguez-Castanedo et al. 2017). These adaptations may be related to paleoclimatic events in the region (Bao et al. 1999); Parker and Cornfield (1995) have suggested that the ancestors of the Cyprinodontiformes migrated from the Thetis Sea during the time of Gondwana.

The small, high-mountain freshwater cyprinodontid *Orestias ascotanensis* Parenti 1984 tolerates water with high salinity, low oxygen concentration (< 7 $mg{\cdot}L^{-1}$), extreme daily temperature changes (−2 to 25 °C) and ultraviolet radiation above 2000 kWh. The salt pan springs in which this fish is distributed have low depth and high salinity (3000–4000 $\mu S{\cdot}cm^{-1}$) (Keller and Soto 1998). These fish group in small schools around macrophytes. It is the southernmost species of killifish described (Vila et al. 2010). Six other endemic *Orestias* species have also been described in restricted geographic areas; all are classified as in danger of extinction. The small size of these fish populations place them at risk, especially in light of the escalating reduction of their habitats due to a negative hydrological balance and high demand for water in the region (Vila et al. 2007; Morales et al. 2011; Ministerio de Medio Ambiente 2015). Elucidating the reproductive and embryonic adaptations of this species to extreme environmental conditions, with the goal of restoring their populations, would benefit both scientific and conservation-related interests.

Methods

Fifty *O. ascotanensis* individuals 20–60 mm in length were captured between April 2014 and March 2015 from two Ascotán salt pan springs in the high Andean region (576966–7,623,591 UTM MGS 84; elevation 3716 m) (Fig. 1). Fish were obtained using manual nets with the permission of the Subsecretaría de Pesca, Chile, under exempted resolution #1103, passed April 2014.

O. ascotanensis reportedly reproduces over a six-month period, from September through March (austral spring and summer) under natural conditions. Observations of the gonads during this period indicated partial maturation (Fig. 2), during which males acquired a yellow coloration.

Fish were kept in aquaria with controlled temperature at 20 °C (±1 °C), a conductivity of 2800–3600 $\mu S{\cdot}cm^{-1}$ and a 16:8 light:dark photoperiod. Fish were fed twice per day with Sera Vipan, Sera FD Tubifex and Sera mosquito red larvae, supplemented with *Eisenia fetida* Savigny and *Artemia salina L*. To record courtship behavior, individuals were photographed with a digital Nikon COOLPIX AW100 waterproof camera.

After one to three months of acclimation, females with indications of sexual maturity, such as courting behavior or a rounded abdomen, were treated with *Ovaprim*® (SGnRHa + Domperidona) at 1.5 $\mu L gr^{-1}$ body weight via intramuscular injection with a 1-mm tuberculin syringe. Fish were previously anesthetized with *Katmandú*® clove oil at 2.25 $\mu L{\cdot}ml^{-1}$. The dry method was used to obtain gametes by abdominal massage, using 2–3 males per female. Subsequently, oocytes and semen were placed in Petri dishes, and dechlorinated water was added. Fertilized eggs were maintained at 20 °C (±1 °C). Water was changed and oxygenated daily, and dead eggs and embryos were removed. Embryos ($n = 15$–20) were observed and photographed with a Nikon SMZ445 microscope, EZ4HD digital microscope and Leica DM500 microscope coupled with a Leica ICC50HD digital camera.

Results

Courtship After acclimation, males acquired a yellow-orange coloration ventrally. Two to five male fish swam after the females, rubbing the mouth on the ventral side of the female (Fig. 2).

Ovary The pear-shaped single ovary was white and highly pigmented, with oocytes at various sizes and

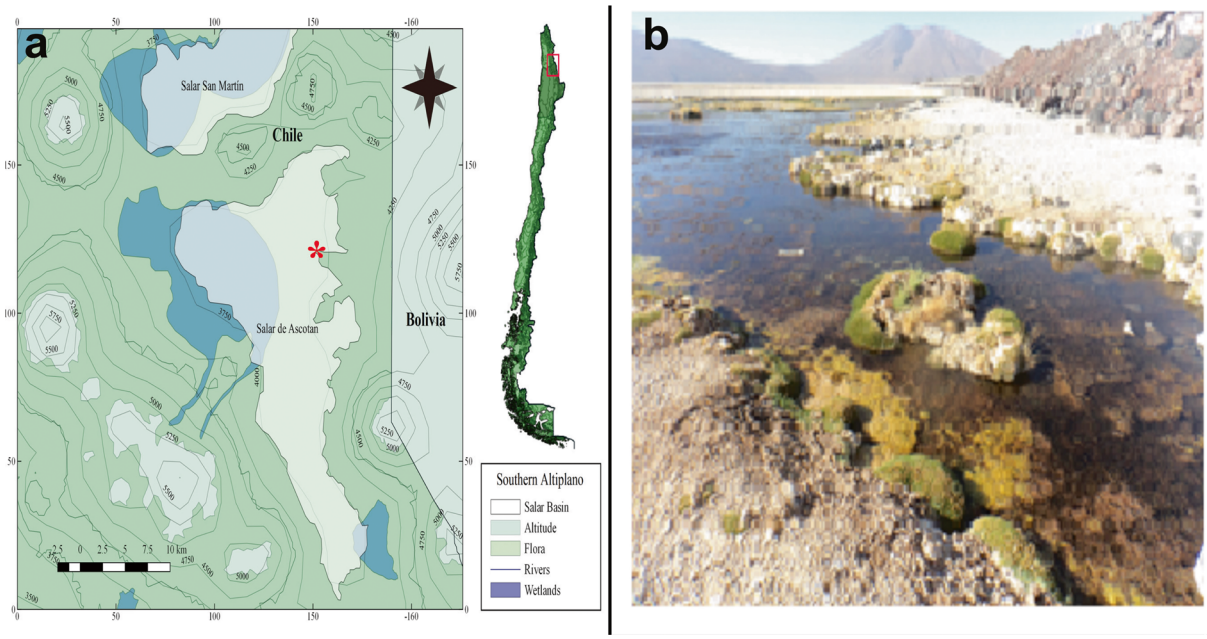


Fig. 1 **a** Map of the Ascotán salt pan (QGIS 3.0.1 software); asterisks (*) indicate the location from which the samples were taken. **b** Detail of the spring (*) containing *O. ascotanensis* populations

stages of development (Fig. 3a, b). Oocytes varied from <0.3– mm to 20 mm in diameter.

Eggs Eggs were denser than water, irregularly circular and creamy whitish, measuring 155– to 285 mm ($\bar{X}$ 149 ± 047) with an oil drop 400–430 μm ($\bar{X}$ 41,275 ± 98) in diameter. After fertilization, the eggs sunk and developed dozens of sticky filaments that adhered to the macrophytes. Embryonic development followed the model described by Kimmel Ballard et al. (1995), including five periods cleavage, blastulation, gastrulation, differentiation and morphogenesis subdivided into 21 stages. The timing of each embryonic stage was calculated based on the emergence of the respective diagnostic characteristics (Table 1).

Fig. 2 Courtship; sexual dimorphism (color and size) is apparent

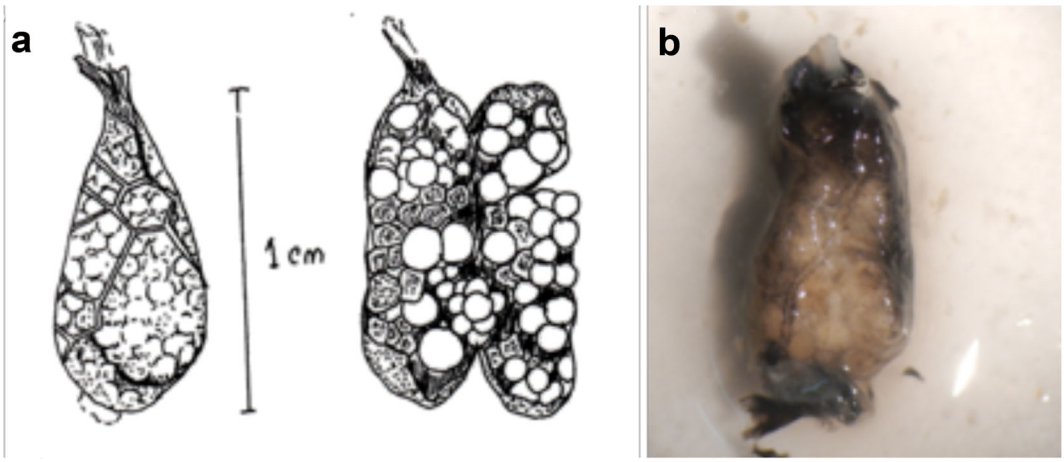


Fig. 3 **a** *O. ascotanensis* ovary and oocytes of varying size. **b** Photograph illustrating the external appearance of the female *O. ascotanensis* gonad

STAGE 1: Undivided egg

The zygote is formed after fertilization, followed by formation of the perivitelline space, 125–180 μm ($\bar{X}$ 148.35 ± 16.18) in length. After 1 h 40 min, the embryonic disc is a large opaque cell, 650 μm in diameter. The oil drop is 415 ± 9.86 μm in diameter. Vegetal and animal poles are established, defining

Table 1 *Orestias ascotanensis*: embryonic development at 20 °C (±1 °C)

Period	Stage	Description	Hours post-fertilization (hpf)
Zygote	1	Undivided egg	1⅔
Cleavage	2	2 cells	2–2½
	3	4 cells	3½–4
	4	8 cells	4½–5½
		16 cells	5½–6
	6	32–64 cells	6½
Blastulation	7	Blastula	7½–8
	8	30% epiboly	20
Gastrulation	9	50% epiboly	25
	10	Embryonic shield	28
	11	70% epiboly	31
Differentiation and morphogenesis	12	Brain vesicles	50
	13	Pigmentation	70
	14	Optic cups, heart beats and first muscular contractions	92
	15	Otic vesicles and vitelline blood vessels and circulation	96
	16	Heart development	140
	17	Pectoral fins	166
	18	Body cavity	190
	19	Crystalline lens and digestive tube	216
	20	Sclera, cornea and liver	288
	21	Gill arches and caudal fin	308
		Hatching	336–432

the anterior and posterior axis of the embryo: the cephalic region at the animal pole (AP) and the caudal region at the vegetal pole (VP) (Fig. 4a).

Cleavage

STAGE 2: Two cells

The first division of the blastodisc occurs 2–2.5 h post fertilization. Cleavage is meroblastic and vertical, generating two symmetrical blastomeres 420 ± 2.48 μm in diameter. The oil drop is partially divided in some embryos (Fig. 4b).

STAGE 3: Four cells

The second cleavage is perpendicular to the first, giving rise to four cells measuring 200 ± 6.56 μm in diameter.

STAGE 4: Eight cells

The third cleavage cycle occurs after 4.5 h, parallel to the first, generating eight cells 100 ± 0.85 μm in diameter in one layer. The oil drop is partially divided.

STAGE 5: 16 cells

The fourth cleavage cycle occurs at 5.5–6 h, generating 16 cells 90 ± 3.47 μm in diameter in one layer. The oil drop is partially divided.

STAGE 6: 32–64 cells

The fifth and six cleavage events give rise to 32 and 64 cells, respectively. These cleavage cycles occur with less synchrony than the previous cycles; therefore, the cells vary in size, gradually shrinking as they divide, with cells 40–70 μm in diameter ($\bar{X}$ 53.7 ± 8.27). The cells are compacted into more than one layer.

Blastulation

STAGE 7: Blastula

Hundreds of compact cells undergo repeated divisions to form the blastula. After 7 h 30 m, cells measure 30 ± 0.85 μm in diameter (Fig. 4c).

STAGE 8: 30% epiboly

After repeated divisions, individual cells can no longer be distinguished in the optical microscope. As epiboly proceeds, the blastula folds down and extend superficially towards the equator of the egg. At this stage, epibolic movement is about one-third complete.

Gastrulation

STAGE 9: 50% epiboly

The epibolic movements displace the cells near the vegetal pole towards the equator, thickening the blastoderm and forming the germinal ring.

STAGE 10: Embryonic shield

Through epiboly, involution and convergence, cells migrate to form the dense triangular embryonic shield (Fig. 4d).

STAGE 11: 70% epiboly

The epibolic movements displace the cells, covering 70% of the embryonic shield, which extends and enlarges through the anterior posterior axis by cell migration. The oil drops coalesce.

Differentiation and morphogenesis

STAGE 12: Brain vesicle development

By 50 h after fertilization, the embryo has expanded, and defined structures have developed, including rudimentary brain vesicles which will form the forebrain, midbrain and telencephalon in the anterior, median and posterior regions, respectively. The primordial optic vesicles develop lateral to the cerebral vesicles as cylindrical structures 215 ± 3.3 μm in length. The embryo surrounds half the yolk (Fig. 4e).

STAGE 13: Pigmentation

The embryo surrounds 3/5 of the yolk and has developed the first somites. Brain and optic vesicles have enlarged, and the median region has an intense yellow color. The first star chromatophores, 30–40 μm in diameter ($\bar{X}$

34.6 ± 2.64), are present. Some are dark gray (melanophores) and some white, turning intense orange during direct exposure to light (iridophores). The chromatophores are concentrated in the brain vesicle, while somites are relatively uniform throughout the yolk and around the cephalic region. Pigmentation is heavy but varies among embryos.

STAGE 14: Optic cup formation, first heart beats and muscular movements

After 90 h, the embryo covers 3/4 of the yolk, and optic cups are established as oval structures 260 ± 6.19 μm in diameter. The heart primordium starts beating. This primordium has a tubular form and is 270 ± 3.8 μm in length; it is transparent with two small white vascular valves moved by heart muscle contractions. The tail begins to move.

STAGE 15: Optic cup and vitelline blood vessel development

The forebrain, midbrain and rhombencephalon are differentiated, and the optic cups enlarge to 320 μm ($\bar{X}$ 299.25 ± 12.66) before becoming pigmented. Embryos acquire a yellow-orange color produced by xanthophores and erythrophores. The otic vesicles are oval, transparent structures, 80 ± 7.35 μm in diameter, with two otoliths each. The vitelline vessels begin transporting blood from the aorta to the heart (Fig. 4f).

STAGE 16: Heart development

The embryo surrounds 5/6 of the yolk sphere, and the end of the tail moves right and left. The mouth fissure has developed, and the optic cups measure 380 ± 4.9 μm in diameter. The retina is pigmented, and the posterior eye structures develop. The optic cups have an iridescent cover after 166 h. The heart has chambers, a venous hole, the atrium and ventricular and arterial bulbs. Frontally from the head, the atrium and ventricle are 110 and 180 μm ($\bar{X}$ 144.0 ± 23.75) in length, respectively. Erythrocyte circulation begins 123 h post fertilization, and the heart takes on a red coloration due to blood flow.

Fig. 4 **a** STAGE 1: Zygote with blastodisc (30x): (c) chorion, (ed) embryonic disc, (pvs) perivitelline space, (od) oil drop, (AP) animal pole, (VP) vegetal pole, (y) yolk. **b** STAGE 2: Two blastomeres (30x): (b1) and (b2) blastomeres, (c) chorion, (pvs) perivitelline space, (od) oil drop, (AP) animal pole, (y) yolk. **c** STAGE 6: 32–64 cells (30x): (bl) blastomeres, (c) chorion, (pvs) perivitelline space, (od) oil drop, (AP) animal pole, (VP) vegetal pole (y) yolk. **d** STAGE 10: Embryonic shield (30x): (c) chorion, dorsal (D) and ventral (V) axis, (e) embryonic shield, (pvs) perivitelline space, (od) oil drop, (AP) animal pole and (VP) vegetal pole. **e** STAGE 12: Dorsal view of brain vesicles (30x): (ov) optic vesicles and (bv) brain vesicles. **f** STAGE 16: First chromatophores. (30x): (c) chorion, (i) iridophores (m) melanophores, (sm) somites, (fb) forebrain, (mb) midbrain, (rc) rhombencephalon, (oc) optic cup. **g** STAGE 17: Dorsoposterior view (35x): (oc) optic cup, (sv) sinus venosus, (at) atrium, (vt) ventricle. **h** STAGE 18: Frontal view (35x): (oc) optic cups, (ov) otic vesicle, (cc) corporal cavity, (dt) digestive tube. **i** STAGE 19: Lateral view of *O. ascotanensis* (35x): (cc) corporal cavity, (fb) forebrain, (mb) midbrain, (rc) rhombencephalon, (l) lens, (ov) otic vesicle. **j** STAGE 20: *O. ascotanensis* embryo, lateral view (35x): (cr) cornea, (l) lens, (sc) sclera, (m) mouth, (yv) yolk vessels, (ys) yolk sac. **k** STAGE 21: *O. ascotanensis* embryo, frontal view (35x): (ba) branchial arcs, (h) heart, (im) inferior maxilla, (sm) superior maxilla, (p) pigmentation. **l** Hatched *O. ascotanensis* embryo (35x): (ys) yolk sac, (c) chorion, (e) eye, (t) tail

STAGE 17: Pectoral fin development

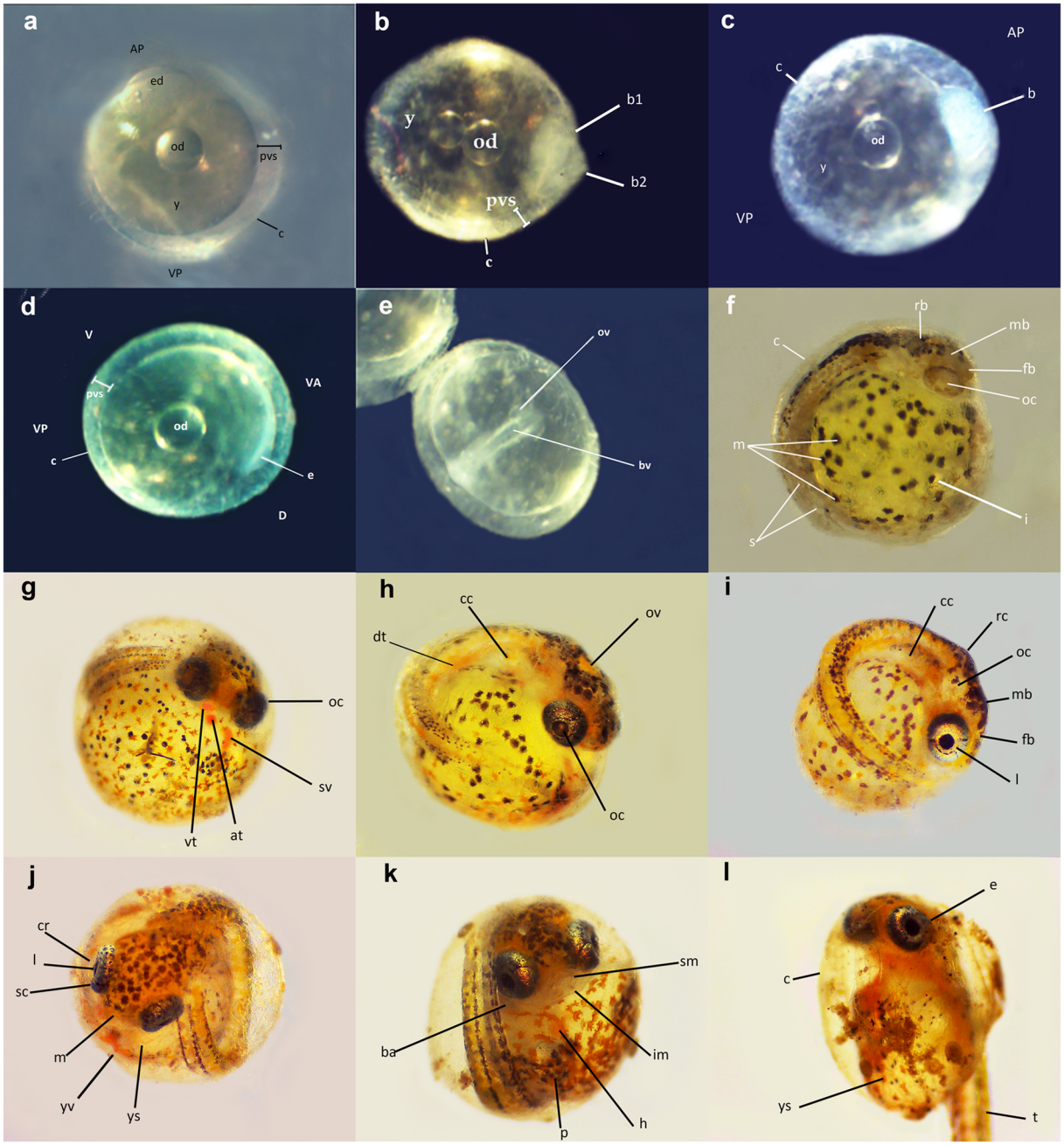
The tail nearly reaches the head, and small, transparent pectoral fins with a rounded border are observed, reaching 0.33–0.38 mm ($\bar{X}$ 0.36 ± 0.01) in length (Fig. 4g).

STAGE 18: Corporal cavity delimitation and caudal fin formation

The embryonic head and tail touch at 192 h, and the caudal fin begins to form. The retina acquires pigmentation and has an iridescent color. The lateral head zone broadens, and the body cavity reaches 8–10% of the total egg area (Fig. 4h).

STAGE 19: Digestive tube and crystalline lens formation

The caudal fin has 3–4 rays 216 h after fertilization. The head, tail and body cavity are growing, covering the last 15% of the yolk sac circumference. This area is gradually becoming pigmented,



acquiring a silver color. The digestive tube is completely developed as a tubular orange structure from the head to the genital pore. The transparent crystalline is 120 ± 13.74 μm in diameter. The circular otic vesicles reach 165 ± 15.92 μm in diameter (Fig. 4i).

STAGE 20: Sclera, cornea and liver

The eyes of the embryo develop the sclera, which is transparent and gelatinous and surrounds the posterior part of the eyes; anterior to the crystalline, the cornea is dome-shaped. At the anterior part of the head, superior

and inferior maxillae are formed, allowing the embryo to open and close the mouth. The liver, an oval structure 175 ± 7.06 μm in length, is observed on the left side of the body. Yolk sac blood vessels branch off. Body pigmentation and muscle size are increasing.

STAGE 21: Branchial arc formation

The embryo moves, rotating 360°. Branchial arches are formed ventral to the otic vesicles, and the operculum moves slightly. Muscles have developed at the base of the eyes, which can rotate 15°. The caudal fin has 7–8 rays. The embryo reaches 725 ± 1.07 μm in length. Pigments migrate from the vitelline vessels to the heart zone (Fig. 4j).

Hatching

Hatching takes place at 14–18 days. The embryo rotates energetically and releases from the chorion (Fig. 4k, l).

Discussion

High Andean "killifish" Cyprinodontiforms live under extreme climatic and aquatic conditions and are cited as evolving since the Mesozoic era in the Tethys Sea (Parker and Cornfield 1995; Keller and Soto 1998). Extreme environmental characteristics and the seismic history of the region have repeatedly fragmented the freshwater systems, favoring great ecological differentiation among fish fauna (Morales et al. 2011; Vila et al. 2013).

The genus *Orestias* is an ancient fish with characteristics that reflect its harsh habitat. Altiplano systems offer extreme climatic conditions, including ultraviolet light, severe daily temperature fluctuations and high salinity. For example, *Orestias ascotanensis* live in the ponds formed by the springs that feed the Ascotán salt pans, where salinity can reach 3600–4000 $\mu S \cdot cm^{-1}$, and water temperatures have been reported to oscillate from 2 °C at night to 25 °C at midday (Keller and Soto 1998). *Orestias* reproduce from September through March, when water temperatures reach 18–22 °C and the light period is longer and more intense. These fish group around macrophyte mats, where they feed and reproduce, with eggs adhering to the plants.

As a primitive fish, *O. ascotanensis* develops a stock of oocytes 0.3–2.5 mm in diameter. The mature eggs are proportionally large (1.55–2.5 mm) in relation to the size of the adult fish, which reach a maximum length of 90 mm (Vila et al. 2010). Asynchronous development has been reported as a survival mechanism in fish (Comte and Vila 1992). This is probably due to the environmental characteristics of their natural habitat, which produces signals–especially light and temperature— that regulate the reproductive cycle and timing of oocyte maturation and/or spawning (Arenzon et al. 2002).

Other *Orestias* species from Lake Titicaca, such as *O. luteus* Valenciennes and *O. olivaceus* Garman, have been reported to have fractional spawning of 50–400 eggs, 1.3–2.3 mm in diameter. *O. agassi* Valenciennes, from Bolivia, also produces small broods, spawning in basins during the rainy summer months. Similar to *O. ascotanensis*, the eggs are heavier than water, adhesive, transparent and pale yellow. These fish spawned at night among the coastal macrophytes (Dejoux and Iltis 1991).

As previously noted, partial spawning is a mechanism that may increase the total number of oocytes produced over a spawning period, especially among small fish (Vazzoler 1996). The rather large size of the *Orestias* oocytes likely enhances fish survival (Wootton 1999). The timing of embryonic development in *O. ascotanensis* is similar to species such as *O. agassi, O. luteus* and *O. ispi* (Lauzanne 1982) lasting at 16–28 days, under temperatures of 13–22 °C. The extreme environmental conditions in the Altiplano freshwater systems —such as severe cold, intense rains during the "Altiplano winter" and significant temperature changes between midday and midnight— likely influence the timing of fish development, larval yolk sac size, total larval length and survival time (Pepin 1991; Arenzon et al. 2002; Kupren et al. 2011). The embryonic development of *O. ascotanensis* is distinguished from other fish by the early formation of pigment cells on about the second day of development, as well as notable pigmentation during subsequent stages. The presence of early melanophores in zebrafish (*Danio rerio* Hamilton) has been described as an adaptive response against damage produced by ultraviolet radiation (Mueller and Neuhauss 2014). Similarly, early pigmentation in the *O. ascotanensis* embryo may confer protection against the high levels of ultraviolet radiation in the Altiplano. Additionally, the marked development of the circulatory system, with a significantly elevated number of red blood cells, is likely a response to the low oxygen pressure (Arany et al. 1996; Wu 2002).

The larvae are totally developed and ready to feed at hatching. Larval survival directly affects adult recruitment and maintenance of small fish populations (Saborido-Rey 2008). The timing and sites of reproduction seem to be related to the availability of macrophytes that offer refuge and food for the fish (Vila et al. 2007).

At present, southern Altiplano fresh water systems are diminishing significantly, due to high demand for water and climate change. Advancing techniques related to artificial reproduction, including studies on larval development and gamete cryopreservation, would contribute to the genetic diversity and conservation of critically-endangered endemic fish (Mijkherjee et al. 2002).

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Compliance with ethical standards

Ethical approval All procedures involving animals were performed in accordance with the standards of the Universidad de Chile Bioethics Committee.

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