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Development of convergent adaptations reveal highly conserved early ontogenetic skull shape in fishes with amphibious vision

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The development of skeletal elements in fish is strongly influenced by the functional demands and environmental constraints they face during different life stages but mostly occurs during their larval development. One example of late modifications within the skeletal system is the adaptation of the skull and eye morphology that allows for amphibious vision in the four-eyed fishes *Anableps* spp. Another species that is equally capable of simultaneous aquatic and aerial vision, *Rhinomugil corsula*, has been widely neglected in this field of research, although it presents great opportunities for comparative analyses on the evolution of this ability. We studied the development of the skull and eyes of *Rhinomugil* based on morphological, morphometric, and histological data. While cross sections reveal that the eyes develop required morphological adaptations for simultaneous amphibious vision in larval life stages, the restructuring of the neurocranium which causes the dorsolateral relocation of the skull and eye occurs only during late juvenile development. In *Rhinomugil* and *Anableps*, restructuring of the skull and eye occurs during similar developmental phases suggesting that the development of the skull shape is widely conserved and cannot easily be changed during larval development.

Keywords Rhinomugil corsula, Anableps, Eye morphology, Morphometry, Neurocranium, Mugiliformes

In fishes the development of skeletal elements, such as the skull, is largely influenced by functional demands. These can differ significantly during their ontogeny as for example larval, juvenile and adult life stages exhibit different feeding modes¹. Even though such demands can lead to different shape, function and modes of growth, a surprisingly large degree of evolutionary conservation at the level of developmental mechanisms in skull formation for vertebrates has been uncovered^{2,3}. While formation of the cartilaginous neurocranium generally starts early during embryogenesis followed by ossification during the early larval stages^{4–6}, it is not uncommon that shape changes occur in later developmental phases, such as the migration of the eye in planktonic flatfish larvae⁷ or the development of enlarged jaws in belonid species⁸. Furthermore, the developmental sequence of neurocranial elements appears to be rather conserved, at least between closely related species, however, developmental timing of single elements may exhibit large variation⁹.

Special adaptations of the skull often correspond to unique modes of living like in the four-eyed fishes of the genus *Anableps*¹⁰⁻¹⁷. Their skull is adapted to accommodate the dorsolaterally positioned eyes which are peaking above the waterline and along with the horizontal division of the eyes allow *Anableps* spp. to simultaneous perceive light coming from water and air¹³. *Anableps* spp. are live-bearing species and their ontogeny including embryonic, larval, and juvenile phases takes place within the womb of pregnant females and results in the birth of subadult specimens^{13,18,19}. Perez, et al.¹³ showed that the formation of the neurocranium in *Anableps* starts quite late during the late larval phase, in contrast to other teleosts^{4–6}, and that the eyes initially are positioned laterally. Furthermore, changes of the head shape, i.e., dorsoventral flattening and repositioning of the eyes, are

¹MARBEC, Université de Montpellier, cc093, Place E. Bataillon, Montpellier Cedex 05, 34095 Montpellier, France. ²Ocean Museum Germany, Katherinenberg 14-20, 18439 Stralsund, Germany. ³Aquatic Ecology, Institute of Biosciences, University of Rostock, Universitätsplatz 1, 18055 Rostock, Germany. ⁴Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research (HCMR), P.O. Box 2214, 71003 Iraklion, Crete, Greece. [⊠]email: phil.thieme2016@gmail.com only initiated during the juvenile phase (indicated by onset of squamation)^{18,20} and proceed more rapidly at the transition to the subadult stage¹³.

During fish evolution several taxa secondarily adopted a lifestyle that requires adjustments to amphibious vision, i.e., the ability to see in water and air. The evolution of amphibious vision allows certain taxa for instance mudskippers to thrive in special habitats such as intertidal zones²¹, enables unusual feeding tactics seen for example in archerfish^{22,23}, or particular predator avoidance strategies known from flying fish²⁴. Amphibious vision also came with several adaptations in eye morphology and structure. In the flying fish *Cypselurus heterurus*, which spends short times gliding outside the water, the cornea is shaped like a three-sided pyramid reducing corneal refraction in air and therefore allowing emmetropic aerial vision^{24,25}. Amphibious mudskippers on the other hand have non-spherical lenses to enhance aerial vision and use blinking for wetting the eyes among other functions²⁶. While amphibious vision and corresponding changes in the eye structure can be observed in several fish taxa, most species do not have the ability of simultaneously aquatic and aerial vision²⁵ and thus often do not exhibit similar adaptations in eye placement and skull morphology as observed in *Anableps*¹³.

For a better insight into the evolution of simultaneous amphibious vision and related morphological adaptations in fish, additional data from species similar to *Anableps*is necessary for comparative analyses. Schwab, et al.²⁷ compared the amphibious labrisomids *Dialomnus fuscus* and *D. macrocephalus*, which also exhibit unique morphological adaptations related to aquatic and aerial vision, with *Anableps*. However, there is only limited information available on labrisomid development and even ontogenetic data on other rock-and mudskippers is scarce^{28–31}. Furthermore, their mode of living differs drastically from that of *Anableps* as they inhabit shallow coastal areas and reside in the benthic zones rather than swimming at the water surface²⁷. Yet, there exists another species much more similar in morphology and behaviour to *Anableps*, the mugilid *Rhinomugil corsula*³².

The corsula mullet or false-four eye, commonly present in rivers and estuaries in India and Bangladesh, is capable of swimming with its eyes below, at the threshold of and above the water surface. Until now, only two studies have examined aspects of the eye morphology of *Rhinomugil* showing the composition of their retina and the structure of their cornea^{32,33}. While *Rhinomugil* evidently inhabits a similar niche as *Anaplebs* and has developed similar adaptations, it differs in its reproduction mode as *Rhinomugil* is oviparous and its larvae hatch from pelagic eggs³⁴. To date nothing is known about its ontogeny and thus the skull and eye development.

In this study we examine the ontogeny of *Rhinomugil corsula* in order to analyse the transformation of the head and neurocranium and the development of the eye as well as the timing when significant changes occur. We use both linear and geometric morphometric approaches and closely examine the remodelling of the neurocranium. We proceed to compare the ontogeny of *Rhinomugil* and *Anableps* to determine if late ontogenetic changes in the neurocranium are caused by early life strategies. Lastly, we identify adaptations in the eye morphology of *Rhinomugil* related to their ability of simultaneous amphibious vision.

Results

Juvenile ontogeny

Larval stages of *Rhinomugil corsula* did not show any distinct differences in their external morphology to other mugilid larvae while kept in aquaria. Even after transition into the juvenile phase, characterized by the beginning of squamation, no significant differences, especially in the head morphology, were observed (Fig. 1A, B). The eyes of the small juveniles are positioned laterally and the mouth is superior (~22 mm SL). The upper jaw ends at the level of the midline of the eyes. From a dorsal perspective, the eyes laterally protrude the outline of the skull (Fig. 1B). In following juvenile stages, the eyes are visibly positioned more dorsally and in juveniles of about 30 mm are dorsally in line with the dorsal outline of the skull (Fig. 1C). The mouth is now positioned terminal, but the upper jaw is still on the level of the skull (Fig. 1E, G). The mouth remains in a terminal position but the relative position of the anterodorsal tip of the upper jaw towards the midline of the eyes is shifted ventrally. Just like in the early juvenile stage, the eyes still protrude the outline of the skull from a dorsal perspective (Fig. 1D, F, H).

Morphometric changes

Linear morphometric analyses showed that head length changes almost isometrically (k=0.986) with body growth (Supplementary Fig. 1). Yet, many head portions grow allometrically in relation to head length: The height of the head shows significant negative allometric growth, which illustrates the dorso-ventral compression of the head (Fig. 2A). While the posterior portion of the head (measured at the end of the opercle) is less impacted (k=0.828), the anterior and especially the middle part of the head (k=0.805 and k=0.703, respectively) get more compressed during juvenile ontogeny. The posterior portion of the head, from the posterior margin of the eye to the posterior margin of the opercle, experiences positive allometric growth (k = 1.115; Fig. 3A), while the anterior portion, from the tip of the snout towards the anterior margin of the eye, is growing almost isometrically (k=1.012). Therefore, during lengthening of the head, the posterior portion gets enlarged, while the anterior portion is staying relatively the same. As a result, the orbita which is positioned in between these two areas, is shortened in relative terms. This is reflected by negative allometric growth of the eye (Fig. 3B). The horizontal growth of the eye is highly reduced during juvenile ontogeny (k=0.763) and it occupies less space at its end compared to the beginning. Shape of the eye, however, does not change during juvenile ontogeny as eye height shows a similar growth trajectory (k=0.789). Dorsally, most pronounced are the changes of the interorbital distance as well as head width anterior and posterior to the eye (Fig. 2B). The anterior and middle portion of the head get narrower as shown by negative allometric relationships of head width (anterior and posterior to the eye) and head length (k=0.865 and k=0.883, respectively). The interorbital area is the region of the head that



Fig. 1. Preserved juvenile specimens of *Rhinomugil corsula* in lateral (A, C, E, G) and dorsal view (B, D, F, H). (A) & (B) SL=21.9 mm; (C) & (D) SL=30.2 mm; (E) & (F) SL=39.3 mm; (G) & (H) 75.9 mm. Scale bars = 2 mm.

shows the most negative allometric correlation with head length and therefore least growth of all head regions (k=0.543).

Geometric morphometric analyses were done using sets of landmarks and semi-landmarks to describe both the lateral and dorsal shape of the head. The linear model fitting shape (represented by the sets of landmarks) against size significantly describes shape variation in both analyses (Supplementary Table 1). These results imply a statistically significant relationship between shape or landmark configuration and a specific size, which therefore corroborates the allometric growth of the head. Plots of landmark configurations and landmark displacement from smallest to largest specimen (Fig. 2C &D) illustrate the reshaping of the head. In lateral view (Fig. 2C), dorso-ventral compression of the head is depicted by dorsal displacement of the ventral opercle line, the ventral margin of the eye as well as the posterior corner of the mouth. Further, the eye is clearly displaced antero-dorsally. The posterior area of the head is lengthening, which is visible in both, the dorsal and the ventral, configurations



Fig. 2. (A) & (B) Regressions of linear head distances against head length. (A) Squares – horizontal distances from snout tip to anterior margin of eye (dark grey) and posterior margin of eye to posterior margin of opercle (light grey); Triangles – head height at the level of the anterior eye margin (yellow), posterior eye margin (orange), and posterior margin of the opercle (red). (B) Squares – horizontal (light grey) and vertical (dark grey) eye diameters; Triangles – head width at anterior margin of eye (orange), posterior margin of eye (red), and interorbital width (yellow). (C) & (D) Shape differences illustrated by a vector displacement between the smallest (SL = 21.9 mm) and largest (SL = 75.9 mm) specimens analysed in lateral (C) and dorsal (D) view.

(Fig. 2C &D). The dorsal vector grid also illustrates the negative allometric growth of the interorbital area as well as relative shortening of the orbita length.

Osteological development

Entering the juvenile phase, the neurocranium is characterized by a compressed ethmoidal region and a higher orbital, otical and occipital region (Fig. 3A). The largest vertical extension of the neurocranium is at the level of the ascending processes of the parasphenoid ventrally reaching below the ventral margin of the supracleithrum. Enlarged and trapezoid nasal bones cover the ethmoidal region (Fig. 3A, B). In between both nasal bones, an almost rectangular gap remains. The frontals dorsally cover the orbital region and prominently feature the supraorbital canal that runs along its dorsal surface from the dermosphenotic towards the nasal. It separates the lateral portion of the frontal covering the eye dorsally from the medial portion (Fig. 3A, B). Ventrally the neurocranium is bordered by the parasphenoid. From its ascending processes towards the vomer the parasphenoid is angled upwards while it is slightly curved upwards towards the basioccipital posteriorly (Fig. 3A, B).

During juvenile ontogeny, distinct changes occur in the neurocranium: The posterior portion of the skull gets dorso-ventrally compressed (Fig. 3B-E). While the skull in lateral view has a rather oval shape entering juvenile phase (Fig. 3A, B), it changes to trapezoid in early juvenile phase (Fig. 3C) and later can be described as almost rectangular (Fig. 3D, E). Associated with the compression of the neurocranium, the shape of the parasphenoid changes drastically. At first the angle towards the vomer is flattened (Fig. 3B, C) which later results in the parasphenoid connecting basioccipital and vomer in a straight line (Fig. 3D). In subadult specimens, the anterior portion of the parasphenoid is even concave (Fig. 3E). Consequently, the space between parasphenoid and frontal is narrowed down and therefore the height of the orbita reduced (Fig. 3B-E). Dorsally, both the nasal and the frontal bones are significantly transformed. The anterior margins of the nasal bones are rounded, and the bones are shortened antero-posteriorly (Fig. 3B-E). Further, the antero-medial corner of each nasal gets more pronounced and pointed. As a consequence, the gap between both nasals is round in the subadult specimen and anteriorly closed due to the pointed corners touching. In early juvenile stages the anterior portion of the eye, gets reduced (Fig. 3A, B, E).

Eye morphology

Histological sections of the eye of *Rhinomugil corsula* provide an overview of the structure of cornea, iris, lens, and retina (Fig. 4A &B). The cornea is clearly divided into three distinct layers, i.e., the epithelium, the stratum,



Fig. 3. Juvenile ontogeny of the neurocranium of *Rhinomugil corsula*; isolated neurocrania of cleared and double-stained specimens in dorsal, lateral and ventral view (from left to right). (**A**) SL = 22.3 mm; (**B**) SL = 32.7 mm; (**C**) SL = 42.9 mm; (**D**) SL = 49.9 mm; (**E**) SL = 82.1 mm. Abbreviations: bas – basioccipital; der – dermosphenotic; epi – epioccipital; exo – exoccipital; ext – extrascapular; fro – frontal; lat – latero-ethmoid; mes – mesethmoid; nas – nasal; par – parietal; pos – posttemporal; psp – parasphenoid; scl – supracleithrum; sph – sphenotic; sup – supraoccipital; vom – vomer. Scale bars = 1 mm.

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and the endothelium. Further, Descemet's layer, a thin layer separating stratum and endothelium, is observable. A Bowman's layer is not observable in the histological sections. The dorsal portion of the cornea is much thicker than the ventral portion, which is mainly caused by an expanded stratum layer dorsally (Fig. 4A &B). While the stratum is about equal in thickness to the epithelium ventrally, it gets thicker dorsally, reaching more than twice the width of the epithelium. The epithelium also differs from ventral to dorsal. Ventrally it contains few cell layers which changes in the middle of the cornea, where there are fewer cells and a distinct area of extracellular matrix. Reaching the dorsal half of the cornea, the epithelium expands as well and more cell layers with less extracellular matrix are present. While there are differences in size between the examined larval specimens, the overall structure of the cornea and the relative thickness of the cell layers are the same in all of them. The lens



Fig. 4. (A) Schematic overview of the eye morphology of *Rhinomugil corsula*. (B) – (E) Histological sections of the eye of *R. corsula* stained with Methylene Blue/Azure II/Basic Fuchsin showing (B) a longitudinal section of the whole eye of F3 (areas of sections (C) and (D) marked with rectangles), scale bar = 100 μ m; (C) a longitudinal section of the dorsal retina of F3, scale bar = 50 μ m; (D) a longitudinal section of the ventral retina of F3, scale bar = 50 μ m; (D) a longitudinal section of the ventral retina of F3, scale bar = 50 μ m; and (E) a widthways section of retina surface displaying the square mosaic arrangement of single (black circle) and double cones (grey ellipsoid) of F2, scale bar = 25 μ m. Abbreviations: CH – choroid; CEN – corneal endothelium; CEP – corneal epithelium; CS – corneal stroma; GCL – ganglia cell layer; INL – inner nuclear layer; IPL – inner plexiform layer; IR – iris; LC – lens capsule; LE – lens epithelium; LF – lens fibers; ONL – outer nuclear layer; OPL – outer plexiform layer; PE – pigment epithelium; PL – photoreceptor layer; RE – retina; SC – sclera.

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STAGE	R _D	R _V	CD	Cv
F1	76.06	86.22	23.09	27.59
F2	77.44	98.11	20.08	24.17
F3	92.12	136.50	12.97	20.79

Table 1. Number of Rods (R) and Cones (C) in the dorsal (D) and ventral (V) section of the retina per $100\mu m^2$ in three different Juvenile Stages (F1: SL = 33 mm; F2: SL = 52 mm; F3: SL = 87 mm).

of *R. corsula* seems to be slightly non-spherical with the shorter axis facing the dorsal part of the cornea and the longer axis the ventral part of the cornea (Fig. 4A &B).

The retina of *Rhinomugil corsula* appears as a hemispherical sheet of the differentiated neural epithelium enclosing the lens (Fig. 4B). The differentiation of the particular layers of the retina is entirely visible (Fig. 4C &D). In the photoreceptor layer and the outer nuclear layer, the cones and the more dark-skinned nucleus of rod cells are visible (Fig. 4C &D). The arrangements of cones in the retina follow the square pattern mosaic with single cones to be placed in the centre and double cones with equal members to be placed around it (Fig. 4E). The pair of opposite double cones are arranged vertically with the axon of the second pair of double cones (Fig. 4D).

Comparison of the eyes of the three different juvenile stages showed that there is a positive relationship between the number of rods and juvenile stage (Table 1; Supplementary Fig. 2). On the other hand, there is a negative relationship between the number of cones and juvenile stage (Table 1). For both rods and cones, there

appears to be a clear disparity between the dorsal and the ventral region as there are more rods and cones present in the ventral region (Fig. 4C &D, Table 1).

Discussion

During embryonic and larval phases, the head region of *Rhinomugil* is characterized by a superior mouth as well as laterally located eyes similar to other mugilid larvae⁶. However, adult specimens of *Rhinomugil* have a terminal mouth and dorsolaterally placed eyes³⁵. During the transition between these two phenotypes, the orbital area as well as the neurocranium in total is restructured and the eyes protrude the dorsal headline. The frontal and parasphenoid seemingly are the bones mostly affected by these changes, but the dorsoventral flattening of the neurocranium also includes changes in all other neurocranial bones and these processes are mainly driven by allometric growth.

These anatomical changes occur only at an advanced juvenile stage, identified by the progressed state of squamation⁶, which coincides with a change in behaviour. Larvae of *Rhinomugil* are limited to an underwater lifestyle possibly caused by water surface tension which either constitutes an unbreakable barrier or a source of mortality for smaller life stages³⁶. The pelagic lifestyle is reflected by the superior position of the mouth and the lateral position of the eyes (Fig. 1). These character states match the larval feeding behaviour as they tend to feed on small particles on or just below the water surface. As the larvae of *Rhinomugil* can only briefly live of their yolk reservoirs and have to quickly learn how to hunt for food and ingest external food particles, a functional skull is necessary to allow this feeding mode. Juveniles then start breaking through the water surface and swimming with the dorsal portion of the eyes or even the anterior part of the head outside the water. Consequently, a terminal mouth is necessary to allow them to feed on plant derivates or insects available on the water surface³⁷ and the flattened head reduces the overall area that is protruding out of the water.

Similar changes in the position of the eyes can be found in other species with amphibious vision. In mudskippers and some amphibious blenniiforms the free-swimming larvae have laterally positioned eyes which shift dorsally during juvenile development^{28–31}. Conspicuously, larvae of the four-eyed fishes *Anableps* follow almost the same developmental trajectory as *Rhinomugil*. In embryonic and larval stages, their eyes are positioned laterally before shifting to a dorsolateral position during the juvenile phase¹³. *Rhinomugil* spends their late embryonic, larval, and juvenile phases in a free-swimming state and needs to hunt autonomously and ingest prey which requires the existence of solid skeletal elements in the skull. On the other hand, *Anableps* spp. are live-bearing species and these developmental phases are spent almost exclusively within the womb of the mother¹⁸. Therefore, reproduction mode seemingly has an influence on the chondrification and ossification of the neurocranium, which in *Anableps* spp. occurs only during late larval and juvenile stages. *Anableps* spp. offspring are supplied with a persisting yolk-sac and are shielded from external constraints which permits a delayed development^{13,18}. However, in other viviparous species neurocranial development proceeds more similar to that of oviparous species⁵, which in these cases can be attributed to early yolk-sac depletion and other modes of subsequent feeding within the womb^{38,39}.

In both taxa, *Anableps* and *Rhinomugil*, the shape of the neurocranium changes within the juvenile phase, mainly caused by allometric growth of neurocranial elements. While *Anableps* offspring is shielded from external factors in the mother's womb, *Rhinomugil* larvae and juveniles are affected by interactions with their environment. An explanation for changes to the head of *Rhinomugil corsula* occurring only during juvenile life stages may be found in physical restrictions. Before eye migration, *Rhinomugil* larvae remain below the water surface and have not been observed breaking through it. Possibly, embryonic and larval life stages are unable to penetrate the water surface due its surface tension. Experimental data has shown that larvae deaths in rearing tanks is connected to water surface tension⁴⁰, hinting at physical restrictions acting on early life stages. An early change in head morphology which is accompanied by a change in feeding behaviour therefore would negatively affect their early life strategies. Furthermore, *Anableps* spp. did not encounter similar physical restrictions in their evolution, due to viviparity evolving before changes in their head morphology occurred⁴¹.

Despite their vastly differing early life strategies, both taxa show striking similarities in their juvenile neurocranium development. Further, the remodelling of the neurocranium is necessary, because the embryonic and larval skull shape does not support their adult lifestyle. This points at limitations and restrictions acting on the general shape of the skull during early ontogenetic stages. We infer that skull shape during early ontogeny is highly conserved, because it is not altered even in the absence of restricting external factors. This hypothesis is additionally corroborated by the age of both genera. *Rhinomugil* presumably split from its sister taxon around 27 Ma⁴², while the genus *Anableps* evolved about 12.5 Ma⁴¹. During this time, new features and behaviours evolved which, however, did not affect the initial shape of the neurocranium¹³ (Fig. 3). The independent evolution of anatomical characters associated with simultaneous amphibious vision and the retention of similar developmental timings supports our hypothesis of the highly conserved early ontogenetic skull shape. Further data from species showing similar changes in the shape of their skull such as rock- and mudskippers may further corroborate this hypothesis.

Teleosts with aquatic and aerial vision show different adaptations in their eye morphology to accommodate the different refractive indices of water and air or to avoid desiccation²⁵. The four-eyed fishes of the genus *Anableps* are capable of simultaneous aquatic and aerial vision due to a combination of morphological adaptions such as divided pupils, distinct ventral and dorsal corneal features, a pyriform lens, and a subdivided retina^{12,13,16,17}. In *Rhinomugil*, we found similar adaptations: the cornea is subdivided into three areas characterized by unequally pronounced cell layers (Fig. 4A &B), the lens is non-spherical, and the retina is divided in a ventral and dorsal area recognizable by distribution and number of rods and cones.

While the general composition of the cornea is similar to other teleosts^{43,44}, the thickened epithelium and stroma in the dorsal corneal portion may bear importance in preventing dehydration of the cornea²⁵. Whether the refractive properties of the eye are influenced by the thickened dorsal portion of the cornea, need to be

clarified in future studies. Other amphibious fishes use a non-spherical lens, e.g., *Periophthalmus* spp.²⁵, or a pyriform lens, e.g., *Anableps*spp. ¹³, to adjust their visual acuity for aerial vision. The slightly non-spherical lens of *Rhinomugil*, where the shorter axis is directed towards the dorsal cornea and, therefore, towards the aerial light, is similar to mudskipper eyes²⁵.

The structure of the retina of *Rhinomugil* overall is similar to a generalized teleost retina⁴⁵. However, the composition of rods and cones is known to influence the photopic and scotopic abilities as well as the visual acuity of fish^{46–48}. In *Rhinomugil*, we observed a higher density of rods and cones in the ventral retina than in the dorsal retina, presumably resulting in different visual acuities in dorsal and ventral direction. This also indicates that *Rhinomugil* can more effectively perceive light from above the water surface. Further, a higher number of rods, which are related to scotopic vision, could indicate that this species is more active at twilight (or night) and its visual system adjusted to mesopic conditions. The square mosaic arrangement of the cones is known to increase visual acuity and image contrast resulting in more privileged visual characteristics^{49–51}. Additionally, it supports a more uniform optical signal⁵², thus contributing to more detailed data for chromatic analysis⁵³ and can help the perception of polarized light^{54–56}. Therefore, it seems to enhance the visual capabilities of *Rhinomugil* necessary for vision directly above and beneath the water surface^{57–59}.

It is known that the size of the retina in fish increases throughout their life^{60,61}. Much like in other teleosts^{62–65}, in *Rhinomugil*the rods spread across the retinas surface and their density increases rapidly. The density of cones slightly decreases over time, which seems to be related to the increase of cone width at early life stages. Interestingly the eyes of *Rhinomugil* show most of these adaptations already in larval stages where they have not been displaced to their dorsolateral position on the head and thus aerial vision was most likely not yet required. This indicates that the development of the eye is less conserved than the skull morphology and adaptations to behavioural changes can be implemented in early developmental phases.

Conclusion

Herein we describe anatomical alterations closely linked to the transition from an exclusively underwater lifestyle to a life at the interface between water and air of *Rhinomugil corsula*. This shift is associated with great changes in abiotic and biotic factors acting upon these fishes, which are reflected by several changes to the anatomical organization of the skull of *Rhinomugil*. A comparison to the four-eyed fishes *Anableps* spp. which are also capable of simultaneous amphibious vision revealed that late ontogenetic changes to the structure of the skull occur independent of reproduction mode and environmental factors. This suggests that the shape of the skull during early ontogeny is highly conserved and specific alterations such as dorsolateral displacement of the eyes and a dorsoventral flattening of the neurocranium are achieved by allometric growth during later life stages. Furthermore, we identified several adaptations in the morphology of the eye, such as differences in the structure of the dorsal and ventral retina and the subdivision of the cornea, which are linked to their ability of simultaneous amphibious vision.

Material & methods Morphometric analyses

A total of 35 specimens of *Rhinomugil corsula* were available for examination. The specimens ranged from 21.9 mm standard length (SL) to 75.9 mm SL. Five cleared and double stained specimens (DMM IE/11,370, 11,400, 1587 and 15,926), as well 9 formalin fixed specimens (DMM IE/15,947, 16,034, 17,561, 17,656) were deposited in the ichthyological collection of the Ocean Museum Germany. The staging system of *Mugil cephalus* provided by Thieme et al.⁶ was adapted to *R. corsula* and specimens were staged to assess their developmental phases.

Both linear and geometric morphometric analyses were performed. For linear morphometrics, 12 linear measurements were taken of 20 specimens: standard length; head length (HL); snout length, tip of snout to anterior margin of eye; postorbital length, posterior margin of eye to posterior margin of opercle; head height at level of anterior eye margin, posterior eye margin and posterior margin of opercle; head width at level of anterior and posterior eye margin; interorbital width, between midpoints of orbita; vertical and horizontal eye diameter. We analysed these measurements to identify which areas of the head show allometric and which isometric growth. Therefore, we calculated the relationship between SL and HL as well as HL and all other measurements by utilizing the power law⁶⁶. In $Y = bX^k$, the slope k directly indicates the growth rate of a measurement in relation to the length it is compared to (herein, either SL or HL)⁶⁷. We further calculated the coefficient of determination to verify that the model fits our data.

We performed geometric morphometric analyses on the same set of 20 specimens and analysed head shape in lateral and dorsal view. Photographs of specimens were taken with a Canon EOS 80D (lenses: Canon MP-E 65 mm, Sigma EX 105 mm). Photographs were then compiled and randomly ordered using tpsUtil (version: 1.78). Landmarks (LM) and Semi-Landmarks (SLM; resampled by length) were placed using tpsDig2 (version: 2.31). A total of 10 LM and two sets of 40 and 16 SLM, respectively, were placed on photographs of specimens in lateral view. A total of 13 LM and three sets of 16 SLM each were placed on photographs of specimens in dorsal view. Landmark data was analysed using RStudio (version: 2022.07.1) with R (version: 4.2.2) and the packages geomorph^{68,69} and RRPP^{70,71}. Generalized Procrustes analyses were performed, and head shape variation was assessed by Procrustes ANOVA analyses with permutation procedures (1000 permutations). Further, landmark distributions, mean shape, and shape differences were visualized.

Osteological analysis

15 of the available specimens were cleared and double stained to examine osteological transformations of the skull. Clearing and double staining with Alcian blue (cartilage) and alizarin red (bone) principally followed the

protocol of Thieme, et al.⁷². The chosen specimens covered a size range from 22.3 mm SL to 64.9 mm SL. The skulls of six specimens were dissected to examine the neurocranium.

Histological analysis

Three individuals were chosen for histological analyses of the eye morphology (F1, SL=33 mm; F2, SL=52 mm; F3, SL=87 mm). Fish were preserved for histology in 70% ethanol. Before embedding in methacrylate resin (Technovit 7100*, Heraeus Kulzer, Germany) larvae were dehydrated in gradually increased ethanol solutions (70–96%). Serial sections of 3 μ m were obtained with a microtome (Leica, RM 2245, Germany). Sections were stained with methylene blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA), according to Bennett, et al.⁷³. To describe the eye morphology the sections were examined using a compound microscope (Nikon Eclipse 50i, Melville, NY). For each juvenile, two microphotographs were obtained at × 40 magnification from sections obtained from the dorsal and ventral areas of the eye. The number of cones and rods were counted using image-analysis software (Image J, NIH, USA).

Data availability

Raw data used in this study are available from the corresponding author upon request.

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Declaration

Competing interests

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Additional information

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