

RESEARCH ARTICLE

Functional morphology and kinematics of terrestrial feeding in the largescale foureyes (*Anableps anableps*)

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ABSTRACT

A major challenge for aquatic vertebrates that invade land is feeding in the terrestrial realm. The capacity of the gape to become parallel with the ground has been shown to be a key factor to allow fishes to feed on prey lying on a terrestrial surface. To do so, two strategies have been identified that involve a nose-down tilting of the head: (1) by pivoting on the pectoral fins as observed in mudskippers, and (2) curling of the anterior part of the body supported by a long and flexible eel-like body as shown in eel-catfish. Although *Anableps anableps* successfully feeds on land, it does not possess an eel-like body or pectoral fins to support or lift the anterior part of the body. We identified the mechanism of terrestrial prey capture in *A. anableps* by studying kinematics and functional morphology of the cranial structures, using high-speed video and graphical 3D reconstructions from computed tomography scans. In contrast to the previously described mechanisms, *A. anableps* relies solely on upper and lower jaw movement for re-orientation of the gape towards the ground. The premaxilla is protruded anteroventrally, and the lower jaw is depressed to a right angle with the substrate. Both the lower and upper jaws are selectively positioned onto the prey. *Anableps anableps* thereby uses the jaw protrusion mechanism previously described for other cyprinodontiforms to allow a continued protrusion of the premaxilla even while closing the jaws. Several structural adaptations appear to allow more controlled movements and increased amplitude of anterior and ventral protrusion of the upper jaw compared with other cyprinodontiforms.

KEY WORDS: Cyprinodontiformes, Functional morphology, Premaxilla

INTRODUCTION

The ray-finned fishes (Actinopterygii) are a morphologically diverse group that contain about half of all described vertebrate species (Near et al., 2012). The success of these fishes is partly due to the highly kinetic musculoskeletal system of the cranium, which has allowed ray-finned fishes to exploit nearly all types of aquatic food (Alexander, 1967a; Sibbing and Nagelkerke, 2000). In some cases, the morphological variation in this kinetic feeding apparatus has even allowed species of ray-finned fishes to capture prey on land. Records of terrestrial feeding exist for the reedfish, *Erpetoichthys calabaricus* (Polypteridae) (Sacca and Burggren, 1982), the largescale foureyes (*Anablepsidae*) (Zahl et al., 1977;

Brenner and Krumme, 2007), the eel-catfish, *Channallabes apus* (Clariidae) (Van Wassenbergh et al., 2006), blennies (Blennioidei) (Rao and Hora, 1938; Nieder, 2001) and mudskippers (Oxurcinae) (Stebbins and Kalk, 1961; Sponder and Lauder, 1981; Michel et al., 2014). These primarily aquatic animals have adapted to the functional demands of feeding in the terrestrial environment. As such, studying these animals could clarify our understanding of the major evolutionary challenges of moving onto land.

To date, the functional morphology and kinematics of terrestrial feeding in fish have been reported for eel-catfish, *Channallabes apus* (Van Wassenbergh et al., 2006) and mudskippers, *Periophthalmus barbarus* (Stebbins and Kalk, 1961; Sponder and Lauder, 1981; Michel et al., 2014, 2015). These fishes each use a different strategy to position the head and oral jaws in an orientation that allows them to capture terrestrial prey. The eel-catfish uses its flexible vertebral column to position and angle the head so that the oral jaws can be placed over the prey (Van Wassenbergh, 2013). The mudskipper pivots the head down, aided by the support of the strong pectoral fins, to allow the oral jaws to capture terrestrial prey (Michel et al., 2014), often aided by forward and backward motion of the water retained in the buccal cavity (Michel et al., 2015). One species within Cyprinodontiformes, the largescale foureyes (*Anableps anableps*) is also known to move and feed in the water column and to jump and feed on mudbanks, (Zahl et al., 1977; Brenner and Krumme, 2007). However, individuals of *Anableps anableps* lack an axial or pectoral mechanism to reorient the mouth ventrally, toward terrestrial prey. Consequently, it is unclear how *A. anableps* is able to feed in the terrestrial environment.

Cyprinodontiformes are a group of fishes characterized by species that are able to ‘pick’ individual prey items from the water column, surface or bottom (Weisberg, 1986; Taylor, 1992; Mansfield and Mcardle, 1998; Ferry-Graham et al., 2008). ‘Picking’ is defined as a subcategory of biting, where individual nutritive prey items are selected from among non-nutritive items (Motta, 1982, 1985; Horn and Ferry-Graham, 2006; Ferry-Graham et al., 2008). Effective picking requires oral jaws with fine control by synchronized movement of both upper and lower jaws; this concentrates the application of force toward the anterior end of the jaws (Hernandez et al., 2008). More-derived species within this order are specialized herbivores that nip and scrape encrusted algae material from the substrate. Thus, cyprinodontiform fishes have developed a means of selectively moving the upper and lower jaws to interact with prey. However, it remains to be shown how *A. anableps* uses its cyprinodontiform morphology and kinematics to feed on land.

Here, we investigate how *A. anableps* is able to feed in the terrestrial environment. We combine three approaches to assess the underlying mechanism of feeding in the aquatic and terrestrial environment in *A. anableps*: (1) we describe the musculoskeletal and ligamentous architecture of the oral jaws; (2) we describe the kinematics of the oral jaws during feeding in both the terrestrial and aquatic environments; and (3) we perform mathematical simulations

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of jaw movement to identify the specific elements of the feeding system that allow terrestrial prey capture.

RESULTS

Morphology

The description and terminology of the morphology is based on previous work on *A. anableps* (Gregory, 1933) and other cyprinodontiforms from Winterbottom (1974), Motta (1984), Ghedotti (2000) and Hernandez et al. (2009). We focus on the morphology of the feeding apparatus through description of the bony skeletal elements that are used during both aquatic and terrestrial feeding: premaxilla, maxilla, anguloarticular, dentary and the bones constituting the suspensorium. Previous work has reviewed the origin and role of the adductor mandibulae muscle complex in jaw closing (Alexander, 1967b; Ballintijn et al., 1972; Motta, 1984; Hernandez et al., 2009). Muscles, ligaments or other connective tissues are also described and illustrated where possible. We will describe the morphology and inter-connections of the elements involved in feeding by considering them in the context of the following functional groups: neurocranium, suspensorium, upper jaw and lower jaw.

The neurocranium

The neurocranium of *A. anableps* is characterized by large, dorsally oriented orbits. In addition, the neurocranium posterior of orbits is rostrocaudally elongated, providing a broad area for the articulation with the hyomandibular (see Fig. 1A,B). Two articulations between the neurocranium and suspensorium are present: posteriorly, the neurocranium provides articulation support for the suspensorium at the hyomandibular and anteriorly behind the palatinum.

The suspensorium

The suspensorium primarily consists of the palatinum, the ectopterygoideum, the quadratum, the metapterygoideum, the preoperculum and the hyomandibulum. The palatinum, pterygoideum and quadratum are fused and illustrated here as a single unit (Figs 1 and 2). The *A. anableps* suspensorium is wide posteriorly (where the hyomandibulum connects to the neurocranium) and tapers toward the anterior of the fish (Figs 1 and 2). There is a large surface on the hyomandibulum and preoperculum that serves as the origin of adductor mandibula A1 and A2 muscles. Anteriorly, the quadratum, ectopterygoideum and palatinum together form the anterior region of the suspensorium; this supports both the lower jaw at the quadrato-mandibular joint and the upper jaw at the maxillo-palatine ligament, which connects the palatinum with the anterodorsal region of the maxilla (Fig. 1B).

The upper jaw

The upper jaw is anteroposteriorly compact in comparison to other cyprinodontiforms (Ferry-Graham et al., 2008), giving the appearance of a short 'stubby' snout located directly anterior to the orbits. The premaxilla has a small ascending process, a posteriorly descending arm and a dentigerous, tooth-bearing region (Figs 1 and 2). The descending arm of the premaxilla and maxilla are connected to each other at the ventral-most tip by the premaxillo-maxillary ligament (Fig. 1B and Fig. 3). When the jaws are closed, the maxillae cover most of the lateral sides of the premaxillae (Fig. 1). The adductor mandibular A1 muscles insert onto the posterior aspect of the descending arm of the maxilla

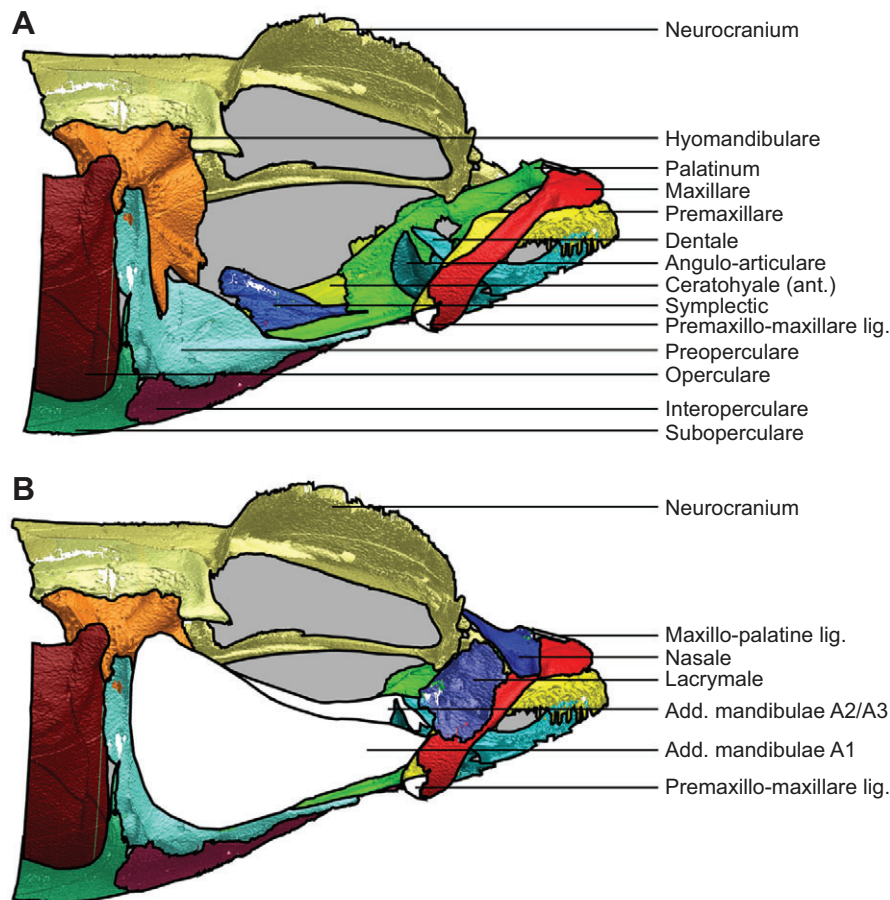


Fig. 1. Body plans of the head of the largescale foureyes (*Anableps anableps*). (A) Overall skeletal body plan in lateral view, without nasal or lacrymal bones or muscles. Each colour represents a separate ossified unit, with exception of part of the suspensorium; the quadratum, ectopterygoideum and palatine form one solid unit (light green). (B) Body plan with position with adductor mandibulae muscles, lacrymal bone and nasal bone.

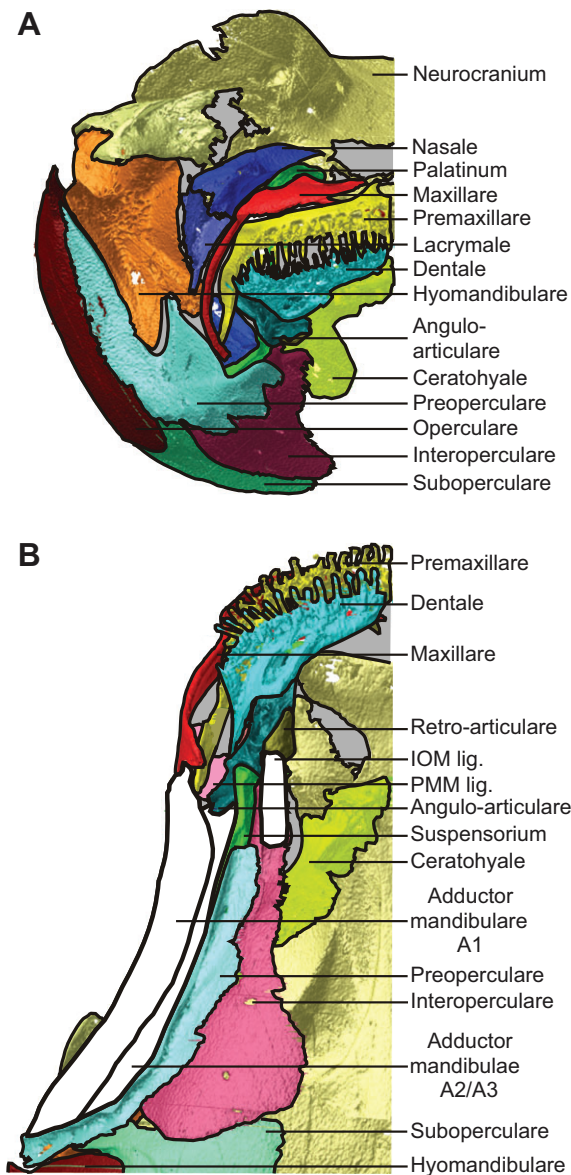


Fig. 2. Frontal and ventral views of the body plan of *A. anableps*. (A) Frontal view of the right side of the skull, excluding ligaments and muscles. (B) Ventral view of the right side of the skull, including the ligaments and muscles involved in feeding. (PMM lig., premaxillo-mandibular ligament; IOM lig., interoperculo-mandibularis ligament).

(Fig. 1B and Fig. 2B). A ligament connects the lateral side on the premaxilla to the lateral side of the anguloarticular and the coronoid process of the lower jaw (Fig. 3).

The lower jaw

The dentary has a prominent dorsal process and a smaller ventral process that converge in a V shape that interdigitates with the anguloarticular bone. The anguloarticular has a process on the anterodorsal aspect that inserts between the two processes of the dentary. As a result, the dentary and anguloarticular are tightly articulated and the anguloarticular and the dentary comprise the lower jaw (Fig. 1). The adductor mandibulae A2/A3 muscles connect through tendons onto the medial side of ascending process of the anguloarticular and medio-posteriorly onto the coronoid process of the dentary (Fig. 3B).

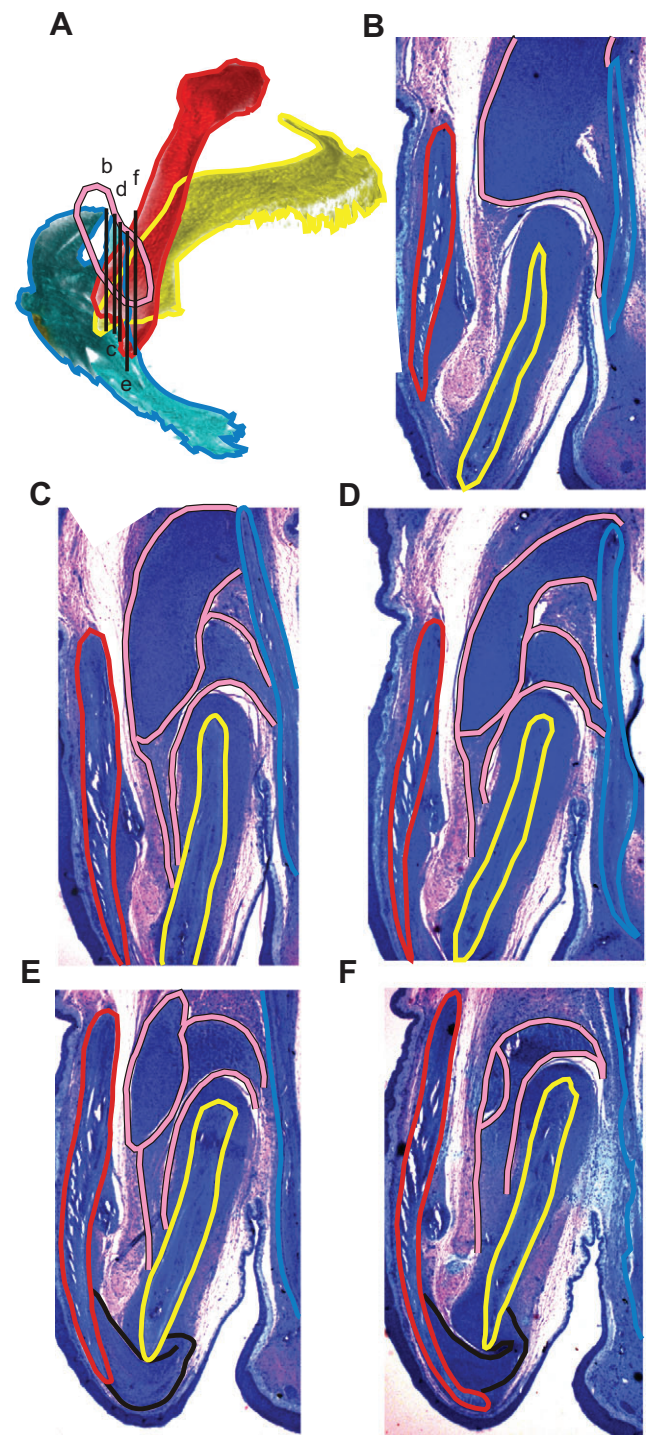


Fig. 3. Transverse histological sections of interaction of premaxilla, maxilla and lower jaw. (A) Overview of the orientation of the jaw elements from a lateral view with the position of the sections B-F from posterior to anterior (left to right). Each element is highlighted with a colour in each section: maxilla (red), premaxilla (yellow), lower jaw (blue), premaxillo-mandibular ligament (pink), premaxillo-maxillar ligament (black). Note the two divisions of the premaxillo-mandibular ligament.

In freshly killed specimens, we were able to elevate the lower jaw by pulling on the adductor mandibula A2/A3 muscle. When the lower jaw was manually depressed, the upper jaw protruded anteriorly. When pulled, the adductor mandibula A1 muscle retracted the maxilla. When the adductor mandibula A1 muscle

was pulled posteriorly while the lower jaw was depressed, the tip of the premaxilla rotated ventrally. Elastic, transparent membrane tissue connects the premaxilla to the maxilla, bridging the gap between the protruded premaxilla and the rostrum.

Kinematics

First, we will describe the aspects of the prey-capture behaviour of *Anableps anableps* that apply to feeding in an aquatic environment and then for the terrestrial environment. In the aquatic environment, *A. anableps* approached the prey and used a combination of biting and suction feeding to capture prey (Fig. 4). Upon approaching the prey, the lower jaw was depressed and the upper jaw was protruded. The prey item was then transported into the mouth, most probably as a result of suction (Fig. 4A). In cases where the prey was not transported sufficiently far into the mouth cavity, closing of the jaws typically allowed the fish to bite the prey. The protruded upper jaws are then retracted while the prey item is held between the jaws. If the prey was attached and the initial bite was insufficient to dislodge the prey item, the fish rapidly moved the body from side to side with the prey item held between the jaws until the prey was removed from the substrate. Cycles of jaw protrusion and biting were then repeated until the prey item entered the mouth.

When feeding in the terrestrial environment, the fish rapidly propelled itself from the water onto land, effectively ‘beaching’ itself (Fig. 4B) (see supplementary material Movie 1). At the same time, the lower jaw was depressed and the upper jaw protruded anteriorly. While on land, the fish aimed its body to position its head above the prey. Once the head of the fish was positioned above the prey, the jaws were closed by elevating the lower jaw and depressing (ventral movement) the protruded premaxilla (Fig. 4B,C). The jaws continued to move toward one another until contact was made with the prey. In cases where the jaws did not contact the prey, the lower jaw and upper jaw continued to elevate and depress, respectively, until the dentary and premaxilla made contact. The jaws were then retracted and the jaw opening cycle repeated until contact with the prey was made.

General aquatic–terrestrial comparison

In order to determine whether prey captures in the aquatic and terrestrial environment were kinematically distinct, the two

environments were compared using a principal component analysis (PCA). PCA uses the kinematic variables of maximum gape distance, maximum protrusion distance, maximum lower jaw angle, maximum upper jaw angle, duration of the gape, duration of the upper jaw protrusion, duration of the lower jaw rotation and duration of the upper jaw rotation. It yielded two significant PCs: PC1 accounting for 66% of the variance and PC2 for 14%. The computed 95% prediction interval ellipses showed no overlap in a plot of the PC1 scores against PC2 scores divided into the two environments (see supplementary material Fig. S1). All variable loadings with PC1 exceeded 0.76, with exception of maximum upper jaw protrusion distance (loading=0.61). The two environments differed significantly from each other along the PC1 (ANOVA, $F_{4,6}=114.7$, $P<0.01$) but not for PC2. Considering that the variable loading and the PC1 scores for terrestrial feeding are all positive whereas those for aquatic feeding are all negative, these statistics indicate significantly larger and longer excursions of the skeletal elements when feeding on land.

The kinematic profile of the mouth gape (distance between upper and lower jaw tip) and the way the upper jaw moved during prey capture constituted the primary difference between the environments. In the aquatic environment, the gape distance reached a maximum of 0.31 ± 0.03 cm. The upper jaw was protruded, but the lower jaw was not fully depressed (Fig. 5A). In the terrestrial environment, the gape distance was considerably larger. The upper jaw was protruded anteriorly, while the dentary was fully depressed to produce a maximum gape of about 0.5 ± 0.03 cm. In the terrestrial environment, the gape was open longer than in the aquatic environment (from $t=-50\pm 5$ ms until $t=37\pm 4$ ms and from $t=-31\pm 4$ ms until $t=26\pm 3$ ms, respectively) and there was more variation in the duration of mouth opening (Fig. 5A) on land.

The maximum protrusion of the premaxilla was significantly larger in the terrestrial environment (0.42 ± 0.06 cm) than in the aquatic environment (0.32 ± 0.05 cm) (two-sample t -test: $t=2.93$, $P<0.01$). In the aquatic environment, the time between the minimum and the maximum protrusion distance is less than in the terrestrial environment (20 ± 2 ms for aquatic and 30 ± 3 ms terrestrial, Fig. 5B). In both environments, the maximum protrusion distance is reached at the moment of maximum gape distance ($t=0$).

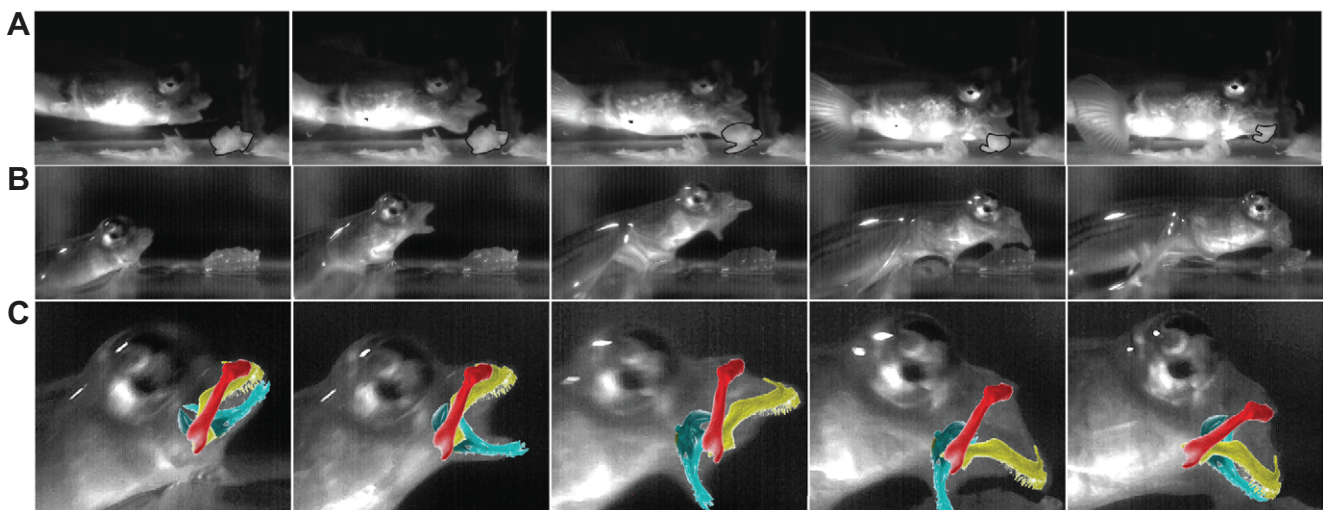


Fig. 4. High-speed video images of typical feeding events. Each frame illustrates a representative stage within the feeding event. (A) Suction feeding in aquatic environment. Food source is outlined. (B) Terrestrial feeding. (C) Schematic overlay of the positions of the jaw elements during terrestrial feeding (see B). Red, maxilla; blue, lower jaw; yellow, premaxilla.

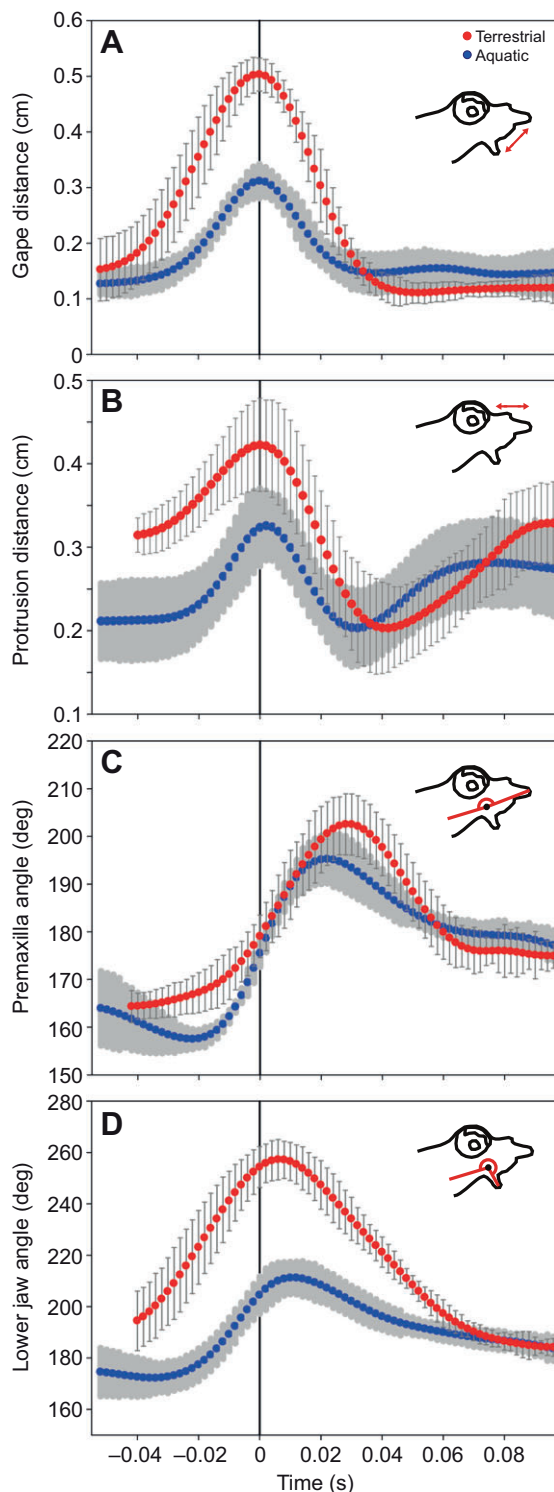


Fig. 5. Kinematic variables of *A. anableps* feeding in the terrestrial and aquatic environment over time. (A) The oral jaw gape distance. (B) The relative protrusion of the premaxillae. (C) The angle of the premaxillae relative to the neurocranium. (D) The angle of the lower jaw relative to the neurocranium. The grey bars denote the s.e.m. between individuals ($N=5$ for terrestrial, $N=4$ for aquatic).

Premaxilla rotation

After protruding, the premaxilla was rotated ventrally, toward the prey. The average maximum angle of rotation of the premaxilla during aquatic feeding was 195 ± 5 deg (see Fig. 5C for definition),

compared with 203 ± 6 deg when measured for terrestrial feeding (Fig. 5C). This maximum angle of the premaxilla was significantly higher in the individuals feeding in the terrestrial environment than for the individuals that were feeding in the aquatic environment (two-sample t -test: $t=3.10$, $P<0.02$). Maximum premaxilla angle in the aquatic environment was reached at 17 ± 3 ms after the onset of mouth closure. In the terrestrial environment, this maximum was reached 28 ± 3 ms after onset of mouth closure.

Lower jaw rotation

Depression of the lower jaw was the main contributor to the gape of the mouth. In the terrestrial environment, the lower jaw is depressed (257 ± 8 deg) significantly more than in the aquatic environment (211 ± 6 deg) (Fig. 5D) ($t=7.75$, $P<0.01$). The maximum lower jaw angle in the terrestrial environment is reached at 4 ± 1 ms and at 8 ± 2 ms in the aquatic environment, after the maximum gape distance is achieved (Fig. 5D).

Four-bar simulations

A four-bar linkage system in *A. anableps* is constituted of a moveable system comprised of the following four elements: suspensorium, lower jaw, maxilla and premaxilla. Using a μ CT scan, the 2D lengths between the points of rotation were measured. The suspensorium was 2.7 mm long, as measured from the centre of the quadratomandibular joint to the articulation face of the palatine and the maxilla. The lower jaw was measured from the quadratomandibular joint to the middle of the premaxillo-mandibular ligament (Fig. 6) and constitutes a 1.1 mm crank element. Measured from the middle of the premaxillo-mandibular ligament to the middle of the premaxillo-maxillary ligament, the descending process of the premaxilla was 1.3 mm long. Finally, the length of the maxilla was measured from the articulation point with the palatine to the middle of the premaxillo-maxillary ligament at 2.8 mm long. The position of the four-bar elements was aligned with resting state as observed in the μ CT scan. The crank element of the model was then rotated by altering the angle of the lower jaw depression, based on movements observed during feeding (Fig. 7). When we rotated the lower jaw in this setup, we found that depression of the lower jaw failed to bring about sufficient protrusion, rotation or opening of the jaws. We found that our four-bar model fails to produce protrusion of the premaxilla, and the premaxilla is immediately depressed along with the lower jaw (Fig. 7B). In order to account for potential measurement error, we adjusted the lengths of the individual bars, but this had no effect on the outcome of the simulation. The model cannot fully open the jaws and is unable to depress the lower jaw beyond the point shown in Fig. 7B. A four-bar linkage system defined above is therefore unable to accurately describe the movement of the elements (supplementary material Movie 2).

Instead of using a point joint at the premaxillo-mandibular ligament, a more flexible connection between the crank and coupler element could allow for a more realistic simulation of the feeding kinematics. We therefore introduced a stiff spring as a flexible fifth bar between the lower jaw and premaxilla. The spring was restricted to stretch and compress along the length of crank element bar only. For this model, the motion was solved as a result of torque actuation in a forward dynamics mode of simulation. The actuation of the crank in the four-bar model was set to depress the lower jaw until the maximum angle was reached, based on measurements from the kinematics. The inertial properties of the four-bar elements and torques used were set to match the kinematic data using arbitrary numbers. In this manner, the stiff spring would allow for some

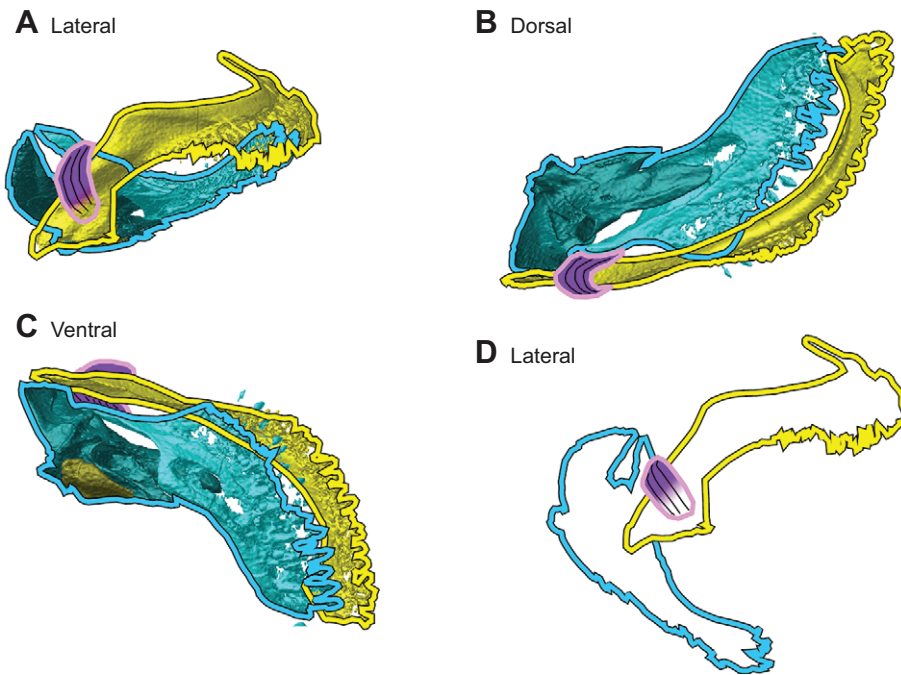


Fig. 6. Schematic representation of the position of the medial division of the premaxillo-mandibular ligament between the premaxillae and the lower jaw. (A) Lateral view when the jaws are closed. (B) Dorsal view when the jaws are closed. (C) Ventral view when the jaws are closed. (D) Schematic lateral view while protruding the premaxillae. Protrusion of the premaxillae occurs by pulling forward the premaxillo-mandibular ligament through the lever arm of the lower jaw around the quadrato-mandibular joint. Pink/purple, premaxillo-mandibular ligament; yellow, premaxilla; blue, lower jaw.

freedom of movement similar to that of a ligamentous connection. The resulting four-bar linkage with spring successfully opened the oral jaws and approximated the measured kinematics (Fig. 8) (supplementary material Movie 3). The four-bar spring model was

also able to enact a full closure of the jaws, as well as approximate the kinematics of feeding in the aquatic environment.

DISCUSSION

When feeding on land, a fish must position the head and oral jaws directly above the prey. *Anableps anableps* is part of a group of cyprinodontiform fishes known for being ‘picky’ eaters – fish that are able to control the movement of both upper and lower jaw – allowing them to select prey items from non-nutritive items (Ferry-Graham et al., 2008). How does *A. anableps* use its cyprinodontiform jaw morphology to feed in the terrestrial environment? What are the adaptations to the cyprinodontiform morphology that allow *A. anableps* to feed in the terrestrial environment?

When we compare the overall morphology of the skull of *A. anableps* with other cyprinodontiforms (Hernandez et al., 2009), we find a suite of distinct features in this species. One such feature is the ascending process of the premaxillae in *A. anableps*. The upper jaw of cyprinodontiform fishes such as *Gambusia affinis* and *Kyptolebias marmoratus* has been described as beak-like, because the premaxillae are narrow mediolaterally and have a long ascending process (Ferry-Graham et al., 2008). In *A. anableps*, the ascending process has been greatly reduced, but can still be found just above the anterior tip of the premaxillae (Fig. 2A) and a reduction of the ascending process appears to allow space for the large dorsal facing eyes. However, we hypothesize that the reduction of the ascending process also allows for increased freedom of movement. Rather than a long ascending process that protrudes by sliding along a single axis, a short ascending process attached to the head by elastic skin would allow protrusion and further rotation (similar to *Poecilia sphenops* in Gibb et al., 2008).

In contrast to the ascending process, the descending arm at the posterior end of the premaxilla is well developed. The descending arm of the premaxilla is where both the ligaments to the lower jaw (premaxillo-mandibular ligament) and the maxilla (premaxillo-maxillary ligament) attach. The premaxillo-mandibular ligament inserts high on the lateral aspect of the descending arm of premaxilla and the connection with the maxilla inserts at the ventral-most tip (Fig. 1A,B). In *A. anableps*, the premaxillo-mandibular ligament

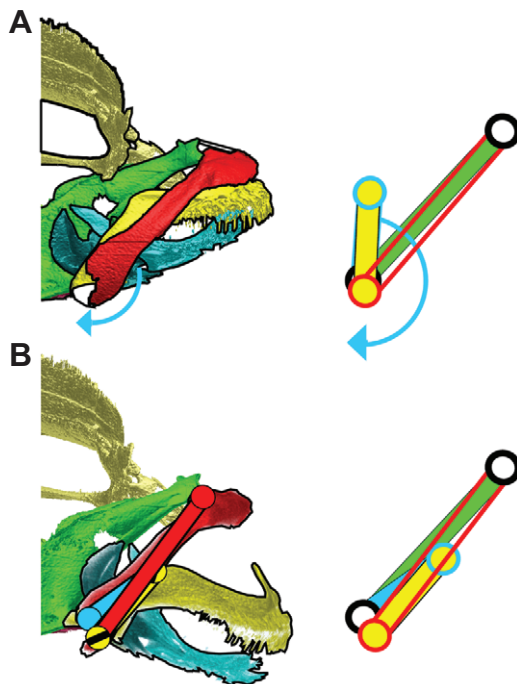


Fig. 7. Simplified schematical representation of elements involved in the four-bar linkage system of the feeding apparatus of *A. anableps*. The coloured bars represent the lever arms and the circles represent the joints between the elements. Black circles illustrate the fixed joints with the unmoving green bar (suspensorium). Red, yellow and blue bars represent the lever arms of the maxillae, premaxillae and lower jaw, respectively. In A, the blue coloured arrow represents how muscle force will act on the same colored element (lower jaw) to move to the maximum possible situation shown in B.

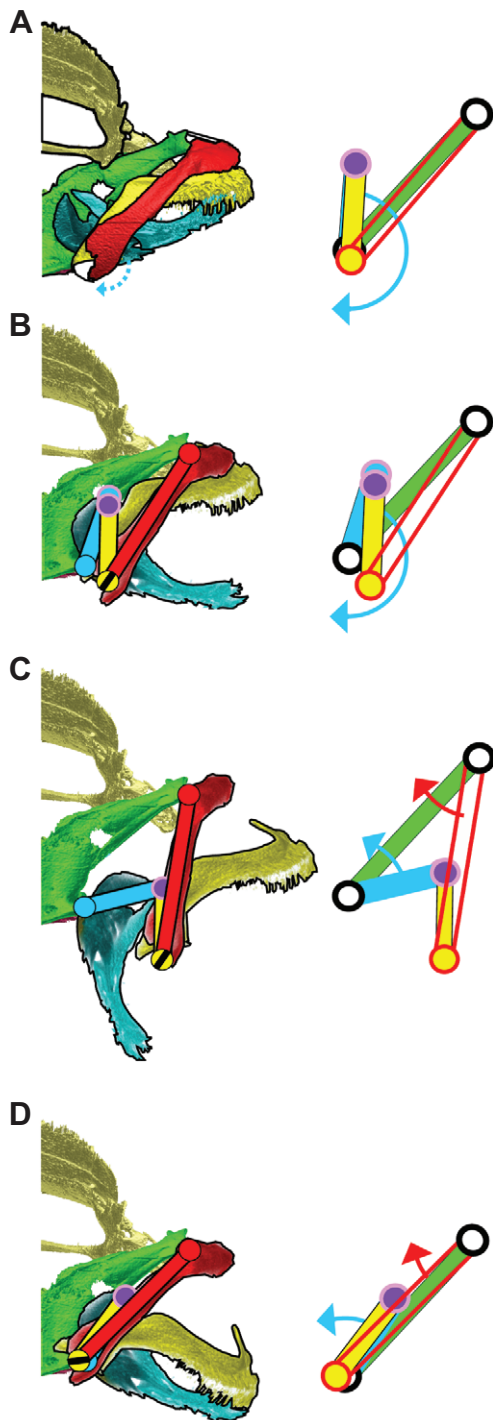


Fig. 8. Simplified schematic representation of the main joints, ligaments and muscles and four-bar system involved in feeding of *Anableps anableps* on land. The coloured bars represent the effective lever arms associated with the same coloured elements of the feeding apparatus. The coloured arrows indicate on which elements muscle forces act and how these rotate. (A) Representation of the position of the elements as the rotation starts. (B) The position as the lower jaw is rotated, leading to further protrusion of the premaxilla. (C) The maximum rotation of the lower jaw and protrusion of the premaxilla is reached. (D) The adductor mandibulae muscles act on the lower jaw and maxillae to close the jaw within the rotated and protruded state.

attaches to the relatively long ascending process of the anguloarticular and coronoid process of the dentary (Fig. 6), a characteristic trait seen in many cyprinodontiforms (Ferry-Graham et al., 2008).

Premaxillary protrusion in cyprinodontiform taxa is mediated by the unusual architecture of the premaxillo-mandibular ligament. In many cyprinodontiform species, the ligament loops around the posterior of the premaxilla and connects onto the lateral aspect of the premaxilla, instead of connecting the bones directly (Hernandez et al., 2008). Similarly, the premaxillo-mandibular ligament in *A. anableps* connects the lateral aspect of the premaxillae to the lateral aspect of the lower jaw (Figs 3 and 6). In *A. anableps*, depression of the lower jaw drives the ligament anteriorly and pulls the paired premaxillae in an anteroventral direction (Fig. 6). This allows the ventral rotation of the lower jaw to protrude the premaxillae.

In *A. anableps*, we find a difference in protrusion between feeding in the terrestrial and aquatic environment. The amount of protrusion appears to be linked to the angle of the lower jaw. Protrusion of the premaxilla (Fig. 5B) only begins after the lower jaw is rotated beyond 180 deg (Fig. 5D), as defined in Fig. 10. When the lower jaw is rotated to an angle of about 195 deg (Fig. 5D), the premaxilla are protruded to a mean of about 0.32 cm in both the terrestrial and aquatic environment. In both environments, maximum gape ($t=0$) coincides with the maximum protrusion and an angle of 180 deg at the premaxilla (Fig. 5A–C). Past this point, the lower jaw continues to depress until $t=0.01$; however, at this point the rotation of the premaxilla is closing the gape faster than the lower jaw is enlarging it (Fig. 5B,D).

The protrusion of the premaxilla in *A. anableps* appears greater than has been seen in other cyprinodontiforms, even when corrected for body length [3% of total length (TL) in *A. anableps* versus a maximum of 2% of TL in other cyprinodontiforms] (Ferry-Graham et al., 2008). The angle of the lower jaw is not tightly correlated with the angle of the upper jaw in *A. anableps*, despite the ligamentous linkage between the two elements (Fig. 5C,D). This follows reports from other cyprinodontiforms, which appear to alter the contribution of the premaxilla to mouth closure, depending on the capture event (Ferry-Graham et al., 2008). This suggests that the rotation of the premaxilla must be subject to another driving force. For *A. anableps*, the relative contribution of the premaxilla toward closing the mouth is greater in the terrestrial environment: here, the upper jaw is rotated to meet the depressed lower jaw. The rotation of premaxilla in *A. anableps* is much greater than that of other cyprinodontiforms, regardless of environment (average of 195 deg in water and 203 deg on land) (Fig. 9). Other cyprinodontiforms measured by Ferry-Graham et al. (2008) find average angles of between 160 deg and 170 deg at the time of mouth closure (Fig. 9). This large degree of protrusion and rotation of the premaxilla allows *A. anableps* to orient its jaws to catch prey below its head in the terrestrial environment.

However, these kinematic results do not explain the extent of protrusion and rotation and morphological descriptions do not reveal how these are attained. In order to find out, we require an understanding of how each element of the feeding apparatus contributes to the overall function. A 2D four-bar model of the *A. anableps* jaw system may allow us to better understand the function of each element in relation to each other.

Our initial rigid four-bar simulation did not properly incorporate the flexibility of the ligamentous connections between the upper and lower jaw. The position and angle of the premaxillo-mandibular ligament allows for some separation of the premaxillae and lower jaw (Figs 3 and 6). Because the premaxillo-mandibular ligament loops over and behind the premaxilla, it is free to slide up and down relative to the lower jaw. This movement is best incorporated into the model as a connection with the freedom to slide dorsoventrally along the length of the crank with the addition of a stiff spring to emulate the stretching ligament. Through incorporation of

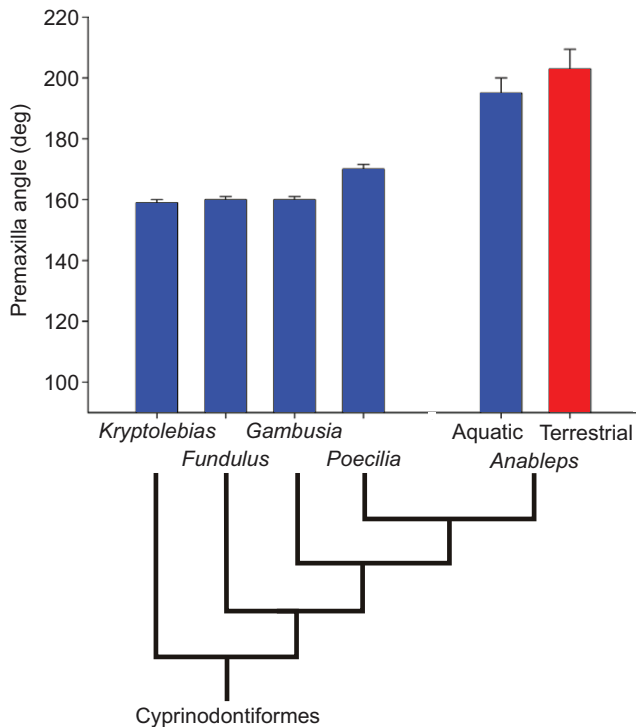


Fig. 9. Graph of the angle of the premaxillae depression during mouth closure in various cyprinodontiform species relative to that found in aquatic and terrestrial feeding *A. anableps*. Data for *Kryptolebias marmoratus*, *Fundulus rubrifrons*, *Poecilia sphenops* and *Gambusia affinis* was adapted from Ferry-Graham et al. (2008). Relationships within Cyprinodontiformes are after Parenti (1981, 2005).

the flexible premaxillo-mandibular ligament connection, the four-bar simulation behaved very similar to that observed in *A. anableps* (Fig. 8). The four-bar simulation is able to emulate the protrusion and opening of the jaws when the lower jaw is depressed (Fig. 8A,B). Through depression of the lower jaw the model can be brought to the situation displayed in Fig. 8C without any additional applied forces (see supplementary material Movie 3). The effect of a flexible connection between these two models illustrates the importance of the presence of premaxillo-mandibular ligament flexibility within the feeding system.

The flexible ligament four-bar simulation allowed us to identify how *A. anableps* manages the extent of upper jaw protrusion and rotation observed on land. The position of attachment of the premaxillo-mandibular ligament onto both the descending arm of the premaxilla and the ascending process of the lower jaw is relatively dorsal (Fig. 6A,D). This makes the moment arm that pushes the premaxillae relatively long, translating rotation of the lower jaw into a relatively large amount of upper jaw protrusion. When the upper jaw is protruded and lower jaw is in the depressed state, the maxillae are also swung anteriorly along with the upper jaw (Fig. 8C). In the protruded state, it is possible to rotate the upper jaw by retracting the maxilla in the posterior direction. Within the four-bar simulation, the premaxilla can be rotated by applying a torque to the ‘follower’ bar (Fig. 8D), retracting the maxillae. This causes the descending arm of the premaxilla to be pulled in the posterior direction, as a result of the premaxillo-maxillar ligament connection. The adductor mandibulea A1 muscles insert onto the posterior of maxillae; these allow for retraction of the maxillae and drive the rotation of the upper jaws (Fig. 1B and Fig. 8C,D). In effect, the upper jaw is rotated approximately around the premaxillo-mandibular ligament connection through the premaxillo-maxillar ligament connection (Fig. 8D).

We also simulated a similar retraction of the maxillae, in the state of the four-bar elements as observed when feeding in the aquatic environment. In the aquatic environment, the angle of lower jaw is only 210 deg when the maxillae are retracted and the upper jaw is rotated (Fig. 5C,D). Even without fully depressing the lower jaw, rotation of the maxillae through retraction by the adductor mandibulea A1 muscles caused a rotation of the premaxillae, closing the gape. This illustrates that our four-bar model is capable of emulating the kinematics of the feeding system observed in *A. anableps*. Unfortunately, the simulated protrusion is slightly less that observed from the kinematics; this may be due to the inability of the model to simulate freedom of movement allowed by all the ligamentous connections between the jaw elements, or because our simulations were calculated based on a two-dimensional abstraction of a 3D jaw system. This may be particularly true in the case of the protrusion of the upper jaw, where the premaxillo-mandibular ligament is pulled taut in the medioanterior direction, within the 3D space around the premaxillae. This might also explain why the protrusion in *A. anableps* only begins after the lower jaw angle reaches 180 deg. The premaxillo-mandibular ligament may only affect protrusion when taut, beyond the initial depression of the lower jaw.

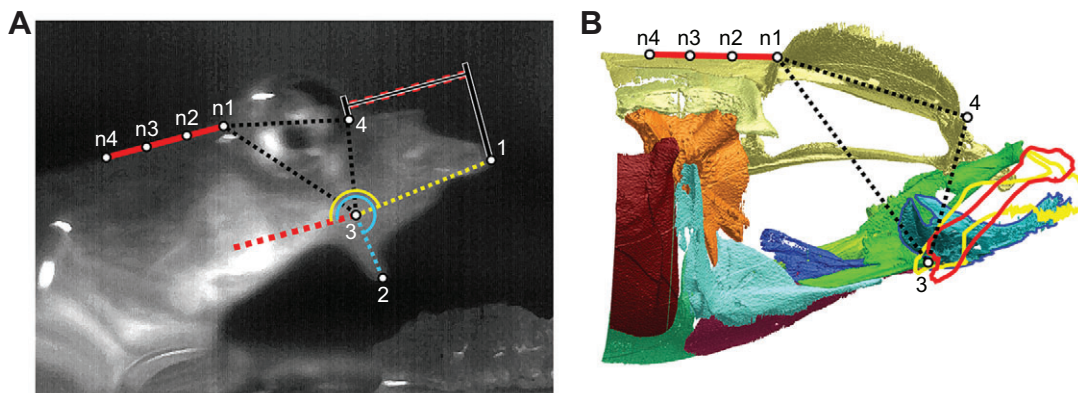


Fig. 10. Position of the anatomical landmarks on the head of *A. anableps* and their representative structures. (A) Landmarks that were followed during prey capture on the external high-speed videos (points 1 to 4 and n1 to n4). The line between points 1 and 4 shows how the dx distance between the base of the eye and the tip of the premaxillae was measured relative to the roof of the neurocranium (red line through n1 to n4). The solid half circles illustrate how the angles of the premaxillae (yellow) and lower jaw (blue) were measured relative to the roof of the neurocranium. (B) Illustration of the position of the landmarks relative to the cranial skeletal structure.

Our model also explains both the consistency and variation in jaw placement of *A. anableps*. Lower jaw depression consistently protrudes the upper jaw. However, the instant of retraction of the maxillae is variable. Using these four-bar lever mechanics, which include an additional flexible link (i.e. the premaxillo-mandibular ligament), *A. anableps* can selectively close either the lower or upper jaw. Contraction of the adductor mandibulae A2/A3 muscles will elevate the lower jaw to meet the premaxilla, whereas contraction of the adductor mandibulae A1 muscles will rotate the premaxilla to meet the lower jaw (Fig. 8). Alternatively, if both jaws are in contact with the prey, only one of these two mechanisms is sufficient to close the mouth. This allows the *A. anableps* jaws to be selectively placed, and prey items to be picked from the terrestrial substrate.

The adaptations we find in the *A. anableps* feeding apparatus follow the evolutionary changes in the jaw mechanics previously described in other cyprinodontiform fishes (Hernandez et al., 2008, 2009; Ferry-Graham et al., 2008). Several species of Cyprinodontiformes have undergone an evolutionary shift toward a more dexterous and precise feeding mechanism by modifications of the ligamentous connections to the upper and lower jaws (Hernandez et al., 2008). This has allowed more-derived cyprinodontiform fishes the ability to select individual prey items from the water column or substrate, in effect, a picking-based prey-capture mechanism (Ferry-Graham et al., 2008). In *A. anableps*, this feeding mechanism has been extended to allow more protrusion and rotation of the upper jaws (Fig. 9). This has allowed *A. anableps* the ability to orient the gape more ventrally without losing the ability to select prey items.

Although this is the first time a cyprinodontiform feeding mechanism has been described by means of a flexible four-bar system, this system may not be unique to *A. anableps*. The extent of the rotations and protrusion in this system depend on the position of the joints and ligaments, but this mechanism could also be used by other cyprinodontiform species. In the case of *A. anableps*, this mechanism has been extended to allow selective grasping of prey from the ground without requiring a long flexible spine similar to eel-catfish (Van Wassenbergh, 2013) or the capacity to pivot on the pectoral fins as observed in mudskippers (Sponder and Lauder, 1981; Michel et al., 2014).

MATERIALS AND METHODS

Animals

Nine individuals of adult *Anableps anableps* (Linnaeus 1758) were acquired from commercial fish trade. The fish were kept together in a large (60×60×90 cm) aquarium and were fed pieces of brown sea shrimp and red mosquito larvae. One individual was killed using an overdose of MS-222 (Sigma Chemical) for computed tomography (CT) scanning and another individual for histological sectioning after high-speed video recording. The animals were transferred to a separate aquarium (35×18×30 cm) for high-speed video recording sessions. This aquarium was equipped with a terrestrial excursion ramp with a transparent floor and sides. A constant temperature of 27°C was maintained, with a 12 h:12 h light:dark cycle. All of the specimens used in this study were handled according to University of Antwerp Animal Care protocols.

High-speed videos from the lateral view (500 frames per second using either a Redlake Motionscope M3 or a Redlake MotionPro HS1000 camera; IDT, Tallahassee, USA) were recorded of *A. anableps* during aquatic feeding on the bottom (four individuals of 7.1±0.6 mm; TL±s.d.), 5 sequences analysed for each) and terrestrial feeding (five individuals; 6.0±1.2 mm TL, 6 sequences analysed for each). The lateral view allowed us to follow the main elements of the feeding system. Attempts to study feeding kinematics using X-ray video proved unsuccessful since the animals were too small to implant radio-opaque markers. Only those sequences in which the sagittal plane of the fish was approximately perpendicular to the lens centreline axis were used for analysis; this allowed us to retain two recordings per individual.

Pieces of brown shrimp of about 0.5 cm³ were used as prey in the experiments. Prey were always presented at the bottom of the aquarium for aquatic feeding and lying on a terrestrial ramp during terrestrial feeding.

Morphology

CT scans

A single *A. anableps* specimen with a total length of 6.3 cm was stained using a 1% potassium iodide solution in water (as per Buytaert et al., 2014) and scanned using the modular micro-computed tomography (CT) setup at Brussels University. Micro-CT scanning was performed with a Skyscan 1172 high resolution CT scanner (Bruker microCT, Kontich, Belgium). The specimen was scanned in air. The X-ray source was operated at 70 kV and 141 mA. Over a range of 180 deg, 5 shadow images were taken every 0.2 deg, with an exposure time of 440 ms. These 5 replications were averaged, to reduce noise. The scanning time was 6.42 h. The shadow images were back projected, which resulted in 2548 reconstructed images of 2720×2720 pixels, with a voxel size of 5.98 mm. For further processing, this was downsampled to 11.96 mm in order to facilitate computer manipulations. The raw data were rendered and segmented using Amira (Mercury Systems).

Histological sections

The preparation and sectioning of the histological sectioning were done by B. De Kegel and J. Christiaens at the Evolutionary Morphology of Vertebrates lab in Ghent. One specimen (SL, 6.4 cm) was used to produce serial histological sections. The head was processed by decalcification using Osteosoft (Merck) and embedded in Technovit 7100 (Kulzer Heraeus), before it was sectioned using a Leica Polycut SM2500 microtome equipped with tungsten carbide blades. The 7 µm sections were stained with Toluidine Blue, placed on glass slides, and sealed with ethyl acetate-coated coverslips. These sections were used to determine the precise attachment positions of the tendons and ligaments, and to identify the tissue types.

Kinematics

Kinematic analysis

The anatomical landmark coordinates were determined for two prey-capture sequences per individual using NIH ImageJ (v1.43) to quantify movement of pertinent cranial elements during the prey-capture event. Time zero (t_0) was defined by the onset of mouth closing. To assess upper jaw movements and their contribution to jaw closure, we obtained eight 2D (sagittal plane projected) coordinates frame by frame on the head and jaws over the course of each prey-capture sequence (Fig. 10A): (1) the anterior tip of the premaxilla; (2) the anterior tip of the lower jaw and (4) the anterior end of the orbit. An additional four, approximately equally distributed points from the posterior of the orbit to back along the neurocranium (Fig. 10, n1 to n4), were used as reference axes to measure the angle of the neurocranium. The distance and angle between the lower jaw joint and points 4 and n1 on the CT scan were used to measure the position of point 3, the articulation between the lower jaw and the suspensorium (at the quadrate bone) (black dashed lines, Fig. 10).

The following five kinematic profiles were determined from the x, y coordinates of these landmarks: (1) The angle of earth frame of reference with the roof of the neurocranium (neurocranium angle), where the roof line is a least-squares regression line of points n1, n2, n3 and n4. (2) The distance between jaw tips (points 1 and 2; gape distance). (3) The distance from premaxillae tip to eye (upper jaw protrusion) through the difference in x coordinates along the neurocranium angle between points 1 and 4 (Fig. 10A). (4) The angle of the premaxillae relative to the head angle (upper jaw angle) through the angle between the line connecting points 3 and 1 and the neurocranium angle (Fig. 10A). (5) The angle of the lower jaw relative to the head angle (lower jaw angle) through the angle of the line between points 2 and 3, relative to the neurocranium angle (Fig. 10A).

To test whether feeding kinematics differs between the two environments, first a principal component analysis (PCA) was performed on the eight kinematic variables described above in the Results section. Because the individuals measured in the aquatic environment are different from those in the terrestrial environment, it is not possible to account for variance between individuals. Therefore, a one-way ANOVA was run to test whether the principal component scores differed between environments. Additionally, based on the outcome of the PCA and subsequent ANOVA, the maximum

values of each kinematic variable were compared between the terrestrial and aquatic environment, using a two-sample *t*-test assuming unequal variances. None of the variables showed a significant deviation from a normal distribution.

Four-bar simulations

A four-bar system is used to model the interaction of the lower jaw with the upper jaw using Matlab R2013b (MathWorks, MA, USA) 'second generation' SimMechanics (v8.2) software. In mechanical engineering, the fixed bar is referred to as the 'frame' and the two bars connecting to the frame are the 'crank' and the 'follower'. Any force input into the system is generally applied to the crank. The crank and follower are connected to opposite ends of the 'coupler'. A given four-bar linked chain only has one degree of freedom, and the angle between crank and frame determines the entire configuration. To simulate the rotation of the jaw system, we require the position of the joints of the four-bar system, the initial angle of the crank, and the rotation of the crank over time (see Aerts and Verraes, 1984).

In *A. anableps*, the fixed frame bar is set as the part of the suspensorium between the follower joint and the crank joint: the point of articulation of the maxilla with the palatine and the quadratomandibular joint, respectively. The crank is the distance between the quadratomandibular joint and the premaxillo-mandibular ligament. The coupler sits between the premaxillo-mandibular ligament and the premaxillo-maxillary ligament. The follower completes the chain by connecting from the premaxillo-maxillary ligament to the frame bar at the point of articulation of the maxilla with the palatine. The length and position of the frame, crank and follower bar were measured based on the distance between their respective rotation joints in the μ CT scan.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.C.G. and K.B.M. collected the data; K.B.M. analysed the data; K.B.M. and S.V.W. wrote the manuscript; all authors interpreted and discussed the results and commented on the manuscript.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.124644/-/DC1>

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