CRANIAL ONTOGENY OF THE SOLE, *DAGETICHTHYS MARGINATUS* (SOLEIDAE), WITH CONSIDERATIONS ON THE FEEDING ABILITY OF LARVAE AND EARLY JUVENILES

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ABSTRACT

The overall aim of this study was to gain a better understanding of the feeding mechanisms of the larvae and early juveniles of the sole, *Dagetichthys marginatus* (Soleidae), with which to assess the suitability of current feeding protocols and to facilitate the development of an appropriate pelleted feed. This was achieved by examining the ontogeny of the cranium of laboratory reared sole, with particular emphasis on those elements associated with feeding and by comparing the cranium of juvenile fish with that of adult fish. At 4 dah (days after hatch) *Dagetichthys marginatus* larvae develop the first rudimentary branchial arches that facilitates the capture and ingestion of food items. Subsequent development of cranial structures, such as the oral jaws, suspensorium, neurocranium, hyoid and branchial arches and the opercular apparatus enables the larvae, at 16 dah, to switch from ram feeding to suction feeding on live prey. The use of live *Artemia* nauplii from 4 to 16 dah is therefore appropriate. The first morphological asymmetries developed at 16 dah in the dentaries and at 22 dah the maxillae and the premaxillae began to show asymmetries. Teeth were present only on the blind side of the oral jaw elements and during this period (16 to 22 dah) the existing elements began to ossify. From 16 to 35 dah the standard feeding protocol consists of a combination of pelagic (*Artemia* metanauplii) and benthic prey (dead, frozen *Artemia* nauplii) and from 25 dah onwards a sinking pellet is provided. The time (dah) at which frozen *Artemia* and sinking pellets were provided, appropriately corresponded to the initiation of benthic feeding behaviour. However at this stage the use of pelagic *Artemia* metanauplii is inappropriate and unnecessary. At 31 dah the cranial morphology resembled that of adult fish. Adult *D. marginatus* display extreme asymmetries among the elements of the oral jaws, the suspensorium and certain elements of the neurocranium. Elements on the blind side are larger and more robust than those on the ocular side and are adapted for feeding, while those on the ocular side appear to have a respiratory function. From 31 dah the cranial elements are identical to those of adult fish, suggesting that no further feeding behavioural changes occurred and that a sinking pellet, of which the nutrient composition meets the requirements of the fish, would be appropriate for ongrowing.

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CHAPTER 1

GENERAL INTRODUCTION

Recent interest in the culture potential of marine finfish species in South Africa has been stimulated by increasing consumer demand, a sharp increase in fish price in recent years and declining wild stocks (Hecht & Britz 1990, Hecht 2000). In particular, attention has been focused on the culture potential of the dusky kob *Argyrosomus japonicus* and the silver kob *A. inodorus* (Sciaenidae) and more recently on the shallow water sole *Dagetichthys marginatus** (Thompson *et al*. in prep.).[1]

Flatfishes have consistently attracted high market prices on the European market (Howell 1997, Brown 2002) and are considered to be well suited for intensive culture (Slaski 1999 a and b). Currently, South African sole fisheries cannot meet consumer demand and a shortfall of around 715 tonnes has to be imported from Namibia, Pakistan and India (Anon 2002). Only two sole species, *Austroglossus pectoralis* and *A. microlepis,* are targeted by trawlers along the South African coast. It appears that the Total Allowable Catch of 800 tonnes per annum for *A. pectoralis,* set on the basis of historical catch per unit effort data and the precautionary principle is a sustainable level of exploitation, although the average size of sole is decreasing (Badenhorst 1987, Britz *et al.* 2001). Industry reports also suggest that catch rates of *A. microlepis* are declining (Anon. 2002). It seems reasonable to conclude that the future catch of soles is more likely to decrease than increase (Thompson 2004). Consequently the culture potential of flatfishes in South Africa is being investigated.

In the selection of the most suitable species Thompson (2004) applied a number of exclusion criteria such as maximum size, depth distribution and abundance for a total of 56 flatfish species. The shallow water sole *Dagetichthys marginatus* appeared to be the most suitable candidate. By describing several life history characteristics Thompson (2004) assessed the biological suitability and the culture potential of this species. *D. marginatus* is a relatively fast growing species with a phi prime growth

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[1] The shallow water sole, *Dagetichys marginatus* was until recently recognised as *Synaptura marginata* (see Vachon *et al*. in press.)

performance index of 2.65, reaching a maximum length of 410 mm Total Length (~ 1kg) at a maximum age of about seven years. Growth rates are fast until the onset of sexual maturity at two years when energy is mainly utilized for reproduction instead of somatic growth (Booth & Walmsley-Hart 2000). As a result of the onset of sexual maturity at an earlier age in males (Roff 1981) female *D. marginatus* reach a larger size than males (Thompson 2004). Females reach 50% sexual maturity at 235 mm TL, (57% of maximum length) at an approximate proportion of 22% of maximum age.

D. marginatus has a sex ratio of 1 male : 5.1 females, although recent sampling programmes undertaken by colleagues of the Department of Ichthyology and Fisheries Science suggest that the sex ratio may be even more skewed towards females (Thompson 2004). The left ovary is much smaller than the right ovary and is situated between the epipleural ribs and muscles of the ventral body wall (Thompson 2004). The testes (maximum recorded weight during the peak of the breeding season = 0.5g) are relatively small compared to the ovaries (50 g maximum weight) (Thompson 2004). The presence of non-functional testicular tissue tissue in mature females suggests that *D. marginatus* might be a rudimentary hermaphrodite or ‘late’ gonochorist and not a true gonochorist (Thompson 2004). During the protracted spawning season from September/October to March/April there are three cohorts of vitellogenic oocytes and a high percentage of maturing, ripe and spent ovaries. Based on these reproductive characteristics Thompson (2004) suggested that *D. marginatus* is a batch pair spawner. The relative fecundity of *D. marginatus* is 34 ± 6 eggs / g and an egg size of 1.38 ± 0.18 mm (Thompson 2004).

D. marginatus feeds mainly on polychaetes with *Morphysa sanguine* being the dominant prey item, comprising 95.5% of the Index of Relative Importance (Thompson 2004), which corroborates the findings of de Groot (1971) who, on the basis of its anatomy, suggested that *D. marginatus* is a “polychaete-mollusc” feeder.

Based on his studies Thompson (2004) concluded that *D. marginatus* appears to be biologically suitable for aquaculture. The first milestone towards the development of aquaculture technologies of this species was reached during the 2004/2005 spawning season when eggs, obtained from wild females, where successfully fertilised. Despite following standard protocols for the early rearing of flatfish species, this initial success, as well as subsequent attempts, were thwarted by the total mortality of the early juveniles shortly after metamorphosis between 35 – 45 dah (days-after-hatching). To overcome this problem an examination to identify the possible reasons for the total mortality was undertaken.

Spawning was induced by hormone injection (see Chapter 2). Although hormone induced spawning provided good results in other flatfish species, e.g. turbot, this technique appears to be less suitable for sole as it is generally associated with poor ovulation and poor egg quality (Dinis 1986). This may have affected larval quality and could have contributed to post-metamorphosis mortality.

Cylindro-conical tanks were used as incubation and larval rearing tanks (see Chapter 2). Nearly 100% of fertilised eggs hatched after 72 h and no mortalities were recorded during the pelagic stage (Thompson *et al*. in. prep.). Subsequently larvae settled on the circular mesh insert. It was concluded that the incubation and rearing system used met the physical requirements of the larvae and post-larvae. Poor water quality can cause reduced growth or mortalities (Person-Le Ruyet *et al*. 1997a, Imsland *et al*. 2003). In all rearing trials water quality parameters were monitored twice a week and results were constantly at optimum levels. In addition stocking densities were kept at less than 5 fish. L^{-1}, significantly lower than standard rearing densities in sole culture (Imsland *et al*., 2003). Diseases caused by bacteria, such as black patch necrosis (BPN) often associated with poor water quality (Imsland *et al*. 2003) were not observed. It was concluded that neither poor water quality nor any of the chemical water parameters had a negative effect on larval or juvenile survival.

The feeding protocol used in rearing trials were similar to those described for other sole species (Dinis *et al.* 1999, Imsland *et al*. 2003). *Artemia* nauplii and *Artemia* metanauplii were used as first feed until 35 dah when live food was withdrawn and the larvae were weaned onto an extruded pellet diet (Chapter 2). First mortalities were recorded shortly after live food was withdrawn around 35 dah and by 45 dah all fish had died. This leads to the suggestion that the formulated feed did not meet the specific requirements of the fish. Four possible avenues were then explored.

First, that the nutritional properties of the pelleted feed did not meet the nutritional requirements of early juvenile sole. Generally, flatfish have a higher crude protein requirement in comparison to other finfish species. For example, the Dover sole, *Solea solea* performed best when fed a dietary crude protein level of 57 – 58 % (Guillaume *et al.* 1991). The protein requirements of larval and juvenile *D. marginatus* have yet to be determined. Quantitative and qualitative protein requirements, particularly amino acids (AA) change during larval ontogeny (Dabrowski 1986). For example, the total protein requirement of the Senegal sole, *S. senegalensis* varied between 56 – 51% during early larval development (Vazquez *et al*. 1994). Because the protein content of the formulated feed used in the rearing trials of *D.marginatus* was 52 % it seemed reasonable to conclude that the protein requirements of the early juveniles were met. (Thompson, pers. comm). However, during larval ontogeny lipids play an important role (Vazquez *et al*. 1994) and the lipid requirements of *D. marginatus* still need to be defined. Moreover, nutrients and chemical attractants of small pellets may be lost due to leaching (Day *et al*. 1997). However, the stability of the extruded formulated diet used in our studies was considered comparable to commercially available pelleted diets (Thompson, pers. comm.).

Second, the juvenile sole may not have been able to properly digest the pelleted feed. Efficient digestion of certain foodstuffs is believed to be impaired by the absence of detectable pepsin activity in early juvenile Dover sole (Clark *et al*. 1986). In contrast to a formulated diet, the natural food of post-larval juvenile sole consists mostly of small polychaetes (De Groot 1971, Molinero & Flos 1992), which maintain high levels of dissolved solutes providing a readily assimilated source of nutrients (Fyhn 1989). Polychaetes also possess an important complement of digestive enzymes, which increases their digestibility through autolysis (Day *et al*. 1997). The use of easily digestible hydrolysed fish protein concentrate (HFPC) had a marked effect on survival during weaning of Dover sole, *S. solea* (Day *et al*. 1997). The digestive capabilities of larval and early juveniles of *D. marginatus* are currently being investigated (E. Thompson, unpl.).

Third, pellet acceptability may have been low due to unsuitable chemical characteristics of the formulated diet. With the beginning of metamorphosis juvenile sole mainly feed on polychaetes, molluscs and occasionally small crustaceans (De

Groot 1971) and detect and select food mainly by way of chemoreception (Norman 1934, de Groot 1971, Appelbaum 1983, Appelbaum & Schemmel 1983, Harvey *et al*. 1992, Batty & Hoyt 1995). Natural food organisms contain relatively high levels of glycine betaine (Konosu *et al*. 1966, Konosu & Hayashi 1975), which is known to stimulate feeding in sole (Mackie *et al*. 1980). The formulated feed used for *D.marginatus,* as with other commercially available feeds, largely consisted of fish meal (53%), which does not contain high glycine betaine levels. This may be one explanation for the poor acceptability of the feed.

Fourth, pellet acceptability may have been low due to unsuitable physical characteristics. Feed uptake in sole is stimulated by a soft feed texture, which is achieved by high water content. The pelleted diet used by Day *et al*. (1997) had a moisture content of approximately 20%. The formulated pellets used in the *D.marginatus* rearing trials, however, only contained approximately 4% moisture (Chapter 2). In addition, size and shape characteristics of pelleted feeds may or may not stimulate the fish to eat, as the food item may or may not be perceived as desirable (Smith *et al*. 1995). The size (diameter) of the formulated pellets used in the *D.marginatus* rearing trials ranged from 100-400 µ, with an average size of 250 µ. The onset of weaning began at 25 dah (see Chapter 2) when the sole were approximately 10 mm in length. Day *et al.* (1997) weaned 30mm Dover sole, *S. solea* onto a pelleted diet of approximately 700 µ in diameter, which corresponded to the mouth diameter of the fish at that stage. The mouth diameter of early juvenile *D. marginatus* has not been studied yet, however, when compared to *S. solea*, *D. marginatus* were weaned onto a considerably smaller pellet at an earlier stage of development. From this it may be concluded that the physical properties of the feed could have been such that it could not be handled by early juvenile *D. marginatus*.

To summarise, the nutritional properties of the formulated feed may not have fulfilled the nutritional requirements of early juvenile *D. marginatus*, and/or certain nutrients could not be efficiently utilised by the fish. This question can be addressed by using hydrolysed fish protein concentrate (HFPC) (for example, Day *et al*. 1997) and the nutritional requirements and digestive capabilities of larvae and early juvenile *D. marginatus* are currently being investigated (E Thompson, Department of Ichthyology and Fisheries Science, Rhodes University, pers.comm.). Moreover, pellet acceptability may have been reduced due to low levels of chemical attractants.

This study focuses on the physical ability of the larvae and early juveniles to handle feed particles. We consider this field of investigation as pivotal in larval rearing studies, which in most instances is unfortunately neglected in favour of nutritional biochemistry. Hence this study is based on the hypothesis that an understanding of the behavioural processes involved in the ingestion of food will contribute towards the design of appropriate artificial feeds. Behavioural selectivity during feeding is partly assumed to be a function of the mechanics of capture and ingestion, and consequently of morphometric limitations imposed by the feeding apparatus (Wankowski 1979). For example, maximum prey size is limited by the maximum mouth diameter or the handling capacities of a particular size of fish. The minimum prey size on the other hand may be governed by the fish's retentive capacities (Wankowski 1979).

The thesis comprises five chapters. Chapter 2 describes the general method of larval procurement and the rearing methods and systems. The cranial ontogeny of *D. marginatus,* until after metamorphosis, is described in Chapter 3 and provides an understanding of the mechanics of prey capture and morphometric limitations imposed by the elements involved in feeding. For example, knowledge of the first appearance of the branchial arches will help to determine when external food organisms can successfully be retained by the fish, and suction feeding, which greatly improves the fish's prey capturing abilities, depends on the presence and functional capacity of several cranial elements. By following this developmental process it may ultimately be possible to develop a precise day-to-day feeding protocol. The cranial morphology of adult *D. marginatus* is described in Chapter 4. The purpose of this investigation was firstly to assure that no changes in feeding morphology take place from post-metamorphosis up to a size when the fish can be sold. Secondly, it was undertaken on the assumption that such knowledge will improve our understanding of the feeding behaviour and feeding adaptations of benthic feeding flatfish species. In addition, such knowledge may contribute the fundamental basis upon which to custom design appropriate pellets for the grow-out phase of sole production. General conclusions and suggestions for further research are presented in Chapter 5.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Origin of the fish and sampling methods

Sole were caught during the extended breeding season between September 2004 and April 2005 and between September 2005 and April 2006 along the south-east coast of South Africa between Port Elizabeth (33º57'S; 25º38'E) and the Great Fish River Point (33º31'S; 27º06'E). Sole were caught by wading on shallow sandbanks while spearing a four-pronged "sole" fork into the sand. This method is the most efficient and is also the common method used by recreational fishers along the coast. The diameter of the barb-less prongs was 2.5 mm. Other methods, such as seine nets, feike nets, traps and diving were tried but proved to be completely ineffective (Thompson 2004). Fish were transported to the facilities of the marine hatchery of the Department of Ichthyology and Fisheries Science in Grahamstown using plastic boxes containing about 40 L of aerated seawater and sand. Fish that were speared through vital organs, i.e. heart, head, vertebral column or abdomen, were killed and preserved for morphological studies (Chapter 4). The wounds of the fish that had been speared through the musculature were treated with an antiseptic tincture (Mercurichrome, 1%) before directly transferring them to the broodstock holding systems. Spear wounds healed within two days.

Broodstock

Each of the two broodstock systems consisted of a rectangular tank (3 m x 1.90 m x 1.50 m) that formed part of a recirculation system. The total volume of water was 12 m^3 for each system. Each filter system included one mechanical sand filter and one biological trickle filter. The biological trickle filter consisted of a plastic container (1.2 m x 0.95 m x 0.8 m), which was filled half with oyster shells and shreddered plastic. The systems were vigorously aerated and fish were kept at stocking densities of approximately up to 50% bottom coverage at maximum. This is considered to be a medium stocking density for other Soleidae such as *Solea solea* (Schram *et al*. 2006). The fish were fed on shelled sand mussel (*Donax serra*) that was cut into strips on an ad libitum basis and uneaten mussel meat was removed daily. The fish were kept under constant temperature and salinity (19℃ ± 1℃ and 35 ppt ± 1) and natural light conditions, varying between 12 and 16 h of daylight depending on the season. Water quality was monitored twice a week (Table 1) and about 10% of the total water volume of the entire system was replaced weekly with fresh, mechanically filtered (10 µm) seawater.

Spawning and egg incubation

Females with oocytes in the final stages of maturation were injected intramuscularly with a gonadotropin hormone releasing analogue combined with domperidone (Aquaspawn®, Spawnrite, Ltd.) at 0.5 ml. kg^{-1} body mass. Eggs were released stripped 24 - 48 h after hormone injection, whereupon the fish were manually strip-spawned by applying pressure to the ovaries in a posterior to anterior manner. Eggs were collected into a previously sterilised 2 L bucket. Testes were removed surgically and gently squashed using a disinfected glass mortar and pestle and distributed over the eggs. The bucket containing both eggs and the testis homogenate was gently swirled for several minutes, while a saline solution, approximately 10 ml in total was added drop by drop. After 20 min about 1 L of filtered seawater (10 µm) was added to the fertilised eggs and gentle aeration was applied using disinfected (chlorine) air stones and silicone pipes. After an additional 20 min the fertilised eggs were slowly poured into the incubation tanks. Eggs were incubated in the dark for 72 h because light had a negative effect on the hatching rate in previous studies (E. Thompson, pers.comm.).

Egg incubation and larval rearing tanks

Cylindro-conical, black, glass fibre tanks (60 L) were used as incubation and larval rearing containers (Fig 1). The incubation tanks were connected to a recirculation system served by a sand filter as well as biological trickle and submerged filters. Water inflow was at the bottom of the tank and outflow at water surface level. A 500 µm mesh filter covered the outflow-pipe to prevent fish from escaping.

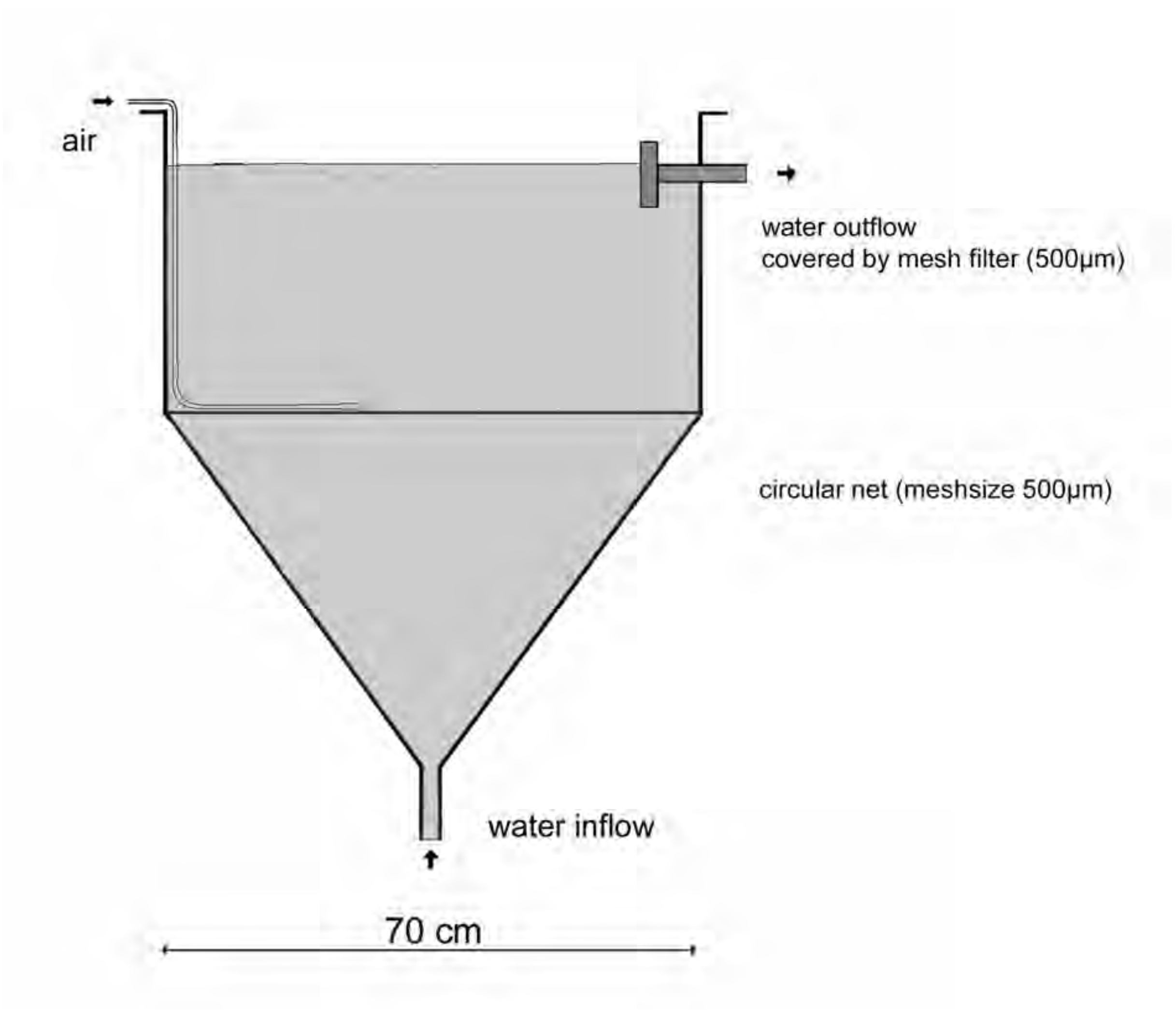


Figure 1: Egg incubation and larval rearing tank with mesh insert.

The cylindrical top section of the tank was separated from the conical bottom part by a circular insert made of a 600 mm diameter plastic ring covered with 1000 µm mesh, to prevent eggs and larvae from getting trapped in the conical centre of the tank and to provide a flat surface for settling larvae. Flow rates were kept at 5 Lh^{-1} during incubation and at 30 Lh^{-1} during the larval rearing period. Water temperature and salinity were kept constant at 19℃ ± 1℃ and 35 ppt ± 1 during incubation and larval rearing (Table 1). Gentle aeration was applied to ensure water circulation and sufficient oxygen supply. Water quality was measured twice a week (Table 1). About 10% of the total water volume of the incubation and rearing system was replaced weekly with fresh, mechanically filtered seawater (10 µm). Tanks were cleaned once a day, uneaten food was siphoned out and dead fish were removed.

Water quality

Water quality parameters (temperature, salinity and O_2 saturation) were measured twice a week. Total ammonia (NH_3^-N) and nitrite (NO_2^-N) were measured (mg L^{-1}) twice a week using a spectrophotometer (HACH, DRI 2000). Total ammonia was measured using the Nessler ammonia method (method Nr. 8038) for wastewater analysis. Un-ionised ammonia (NH_3) was then calculated according to pH, temperature and salinity (Emerson *et al*. 1975). Nitrite (NO_2^-N) was measured using the diazotisation method (method Nr. 8507) for wastewater analysis and the percentage of nitrite (NO_2^-) was then calculated.

Table 1: Water quality parameters

System	Temperature ℃	Salinity ppt	O_2 saturation ppm	Ammonia (NH_3) mg L^{-1}	Nitrite (NO_2^-) mg L^{-1}
Broodstock	19 ± 1	35 ± 1	≥ 7.4	≤ 0.014	≤ 0.026
Larval	19 ± 1	35 ± 1	≥ 7.4	≤ 0.011	≤0.023

Artemia production

Artemia cysts (INVE, Belgium) were prepared and incubated according to the method described by Hoff & Snell (1987). They were dehydrated in conical plastic flasks (1 L) containing regular tap water for 1 h under strong aeration at 25℃. The dehydrated *Artemia* cysts were than decapsulated in commercially available, unflavoured liquid bleach (approximately 10 ml of liquid bleach per gram of cysts) until cysts reached a bright orange colour (2-4 min) and thoroughly rinsed with fresh water. Decapsulated cysts were transferred into conical plastic flasks (1 L) containing filtered seawater (10 µm) kept at constant water temperature and salinity (25℃ and 35 ppt) under strong aeration. Constant illumination at light intensities of 2000 lux was applied by using light tubes (Osram L18/W72). Cysts hatched after 24 h and were then either fed to sole larvae or reared to the metanauplii stage and enriched with SuperSelco® according to the method described by the producer (Artemia Systems, INVE). Approximately 0.6 g of dry SuperSelco® were homogenised in 25 mL of seawater and were added continuously drop by drop to the *Artemia* metanauplii culture over a period of 24 h. The enriched metanauplii were thoroughly rinsed with fresh water and either fed to sole larvae or frozen for later use.

Pelleted diet composition

The formulated pellets were prepared from dry ingredients that were stored at –20℃. Diet formulation is shown in Table 2. Squid and white mussel meal used in the diet were made by freeze-drying fresh material to prevent protein denaturation and to preserve enzyme activity.

Table 2: Diet formulation (E. Thompson, Dept. Ichthyology & Fisheries Science, Rhodes University, unpublished data)

Ingredients	**%**
Squid meal (*Loligo reynaudi vulgaris*)	8 (dry matter)
Sandmussel meal (*Donax serra*)	8 (dry matter)
Fishmeal (AAA Danish low temperature)	53 (dry matter)
Cod liver oil	5
Vitamin / mineral mix	1
Starch binding agent	20
Protein	49.6
Moisture	4

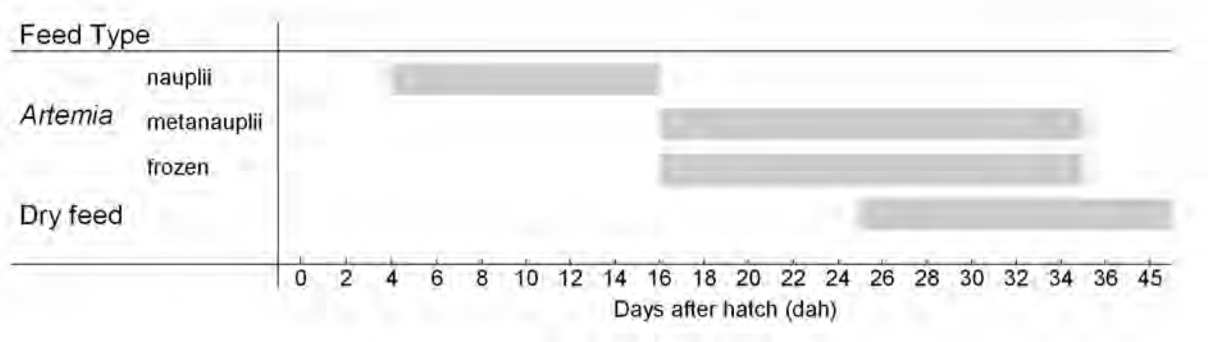
The ingredients were weighed and mixed thoroughly with water to form a paste. The paste was put into a pasta extruder (ICME Bologna) and extruded to form 2mm diameter strands. These strands were oven dried at 38℃ for 12 h. The dried strands were crumbled and sieved to provide particles ranging from 100 to 400 µ (average = 250 µ).

Feeding regimes

An overview of the currently used feeding protocol is given in Table 3. Larvae were fed on live *Artemia* nauplii from 4 dah until 16 dah. From 16 dah until 35 dah the larvae and early juveniles were fed *Artemia* metanauplii and frozen *Artemia* nauplii.

The onset of weaning to an extruded pellet feed began at 25 dah. Larvae were then co-fed on live *Artemia* metanauplii, frozen *Artemia* nauplii and the manufactured diet until 35 dah when live *Artemia* metanauplii and frozen *Artemia* nauplii were withdrawn. Larvae and early juveniles were fed twice each day at 09:00 and 17:00. *Artemia* concentrations were kept at >5 ind. ml^{-1}. The daily amount of the pelleted diet fed was not quantified. However, due to the high percentage of uneaten pellets 9 hours after the feed was administered, it was assumed that pellets where given ad libitum. Uneaten pellets and dead *Artemia* where siphoned out each day between 17:00 and 18:00.

Table 3: Feeding protocol



CHAPTER 3

CRANIAL ONTOGENY AND FEEDING

Introduction

The embryonic and larval development of the teleostean skull has been elaborated on in numerous studies (de Beer 1927, 1937, Srinivasachar 1957 a, b, 1958 a, b, 1959, Bertmar 1959, Hoedeman 1960, Weisel 1967, Paine & Balon 1984, Arratia 1990, 1992, Mabee 1993, Mabee & Trendler 1996, Patterson & Johnson 1996, Wagemans et al. 1998, Kováč 2000, Faustino & Power 2001). However, the ontogenetic development of bony elements specifically in relation to feeding or feeding modes has only been described for a limited number of teleost fishes (Otten 1982, 1983, Dilling, 1989, Liem 1991, Vandewalle *et al*. 1992, 1995, 1997, Kohno *et al*., 1996 a, b, Hunt von Herbing *et al*. 1996a, Adriens & Verraes 1997 a, b, 1998) including two pleuronectiform fishes namely turbot, *Scophthalmus maximus* (Wagemans *et al*. 1998) and the common sole, *Solea solea* (Wagemans & Vandewalle 2001). Lagardere *et al*. (1993) described abnormalities within the cephalic area of laboratory-reared larvae of *S. solea* where a large proportion of the larvae showed shape-modified and dentigerous premaxillae and dentaries on the ocular side.

The head morphology, hydrodynamic regime and diet of larval fish differ from that of their adult counterparts suggesting that larval fish function differently (Sanderson & Kupferberg 1999). Functional linkages of the jaws, feeding mechanisms and mechanisms of gas exchange change during larval ontogeny (Hunt von Herbing *et al*. 1996 a, b, Cook 1996). Furthermore, the development of musculo-skeletal couplings or linkages is expected to have consequences for feeding and respiration. The embryonic and larval periods can be seen as a constant struggle between the functional demands that have to be met and the presence of those functional structures required for performance (Galis *et al*. 1994, Hunt von Herbing *et al*. 1996, Ristovska *et al*. 2006). For example, in larvae of common sole, *S. solea,* or Atlantic cod, *Gadus morhua,* branchial arches form during the yolk sac stage at 2 – 3 dah or 2 dah, respectively (Hunt von Herbing 1996 a, Wagemans & Vandewalle 2001). The early appearance of the branchial arches is considered to primarily fulfil the requirements of exogenous feeding (Kjorsvik *et al*. 1991, Van der Meeran 1991, cited in Hunt von Herbing 1996 a). In addition, food ingested by the larvae must be transported from the oro-branchial cavity to the oesophagus passing the overlying brain (Adriens & Verraes, 1998). In order to protect the brain from damage a parasphenoid is formed prior to the onset of exogenous feeding in larvae of *S. solea* (Wagemans & Vandewalle 2001). In other words, the timing of the development of feeding and respiratory systems may be crucial for survival of fish larvae (Hunt von Herbing 1996 a).

The larvae of pleuronectiform fishes display the same principal external morphological features as other teleost larvae with one eye on either side of the head and a terminal mouth position suited to feed on zooplankton (De Beer 1937, Brewster 1987). With the beginning of metamorphosis, flatfishes adapt to a benthic life-style with striking behavioural and morphological changes, including the migration of one eye across the skull to the opposite side of the head (Sumida *et al.* 1979, Ahlstrom *et al*. 1984, Rose & Reiss 1993). As a result of eye migration all flatfishes possess some degree of cephalic, skeletal and muscular, bilateral asymmetry (Cole & Johnstone 1902, Chabanaud 1936, 1952, Wimpenny 1953, Yazdani, 1969, Bürgin, 1986, Lagardére *et al.* 1993). Generally the bones and muscles on the blind side are larger than those on the ocular side (Yazdani 1969). However, derived groups of Pleuronectiformes, i.e. Soleidae (e.g. *S. solea*) and Cynoglossidae possess additional bilateral asymmetries as a consequence of further adaptation to benthic feeding (Yazdani 1969).

The specific objective of this study was to describe the cranial development of the shallow water sole, *Dagetichthys marginatus,* during its early ontogeny to the post-metamorphosis stage. The overall aim of the work was to gain a better understanding of these fundamental changes, and their chronological sequence, that would facilitate the development of a suitable feeding protocol during the larval and early juvenile rearing phase of this species and other soleids.

Material and methods

Larvae were sampled on 0, 4, 6, 9, 13, 16, 22, 31 and 45 days after hatch (dah). Each sample consisted of 10 specimens. Elements were identified by determining their topographical position and by comparing the structures in adult fish with those in juveniles and those of the juveniles with those in the larvae.

Clearing and staining of samples followed the procedure described by Taylor & Van Dyke (1985). Samples were fixed in 10% buffered formalin for 24h and subsequently dehydrated in a 50:50 solution of 95% ethanol and distilled water for 24h. Samples were then transferred into absolute ethanol for 24h and then placed in an acidified alcohol solution of alcian blue for 12h (70 ml 95% ethanol, 30 ml acetic acid, 20mg alcian blue) and neutralised in a saturated sodium borate solution for 24h afterwards. Samples were then cleared using trypsin enzyme solution (35ml saturated sodium borate, 65 ml distilled H_2O, about 0.2 g trypsin powder). Temperature was kept at 20℃ during the clearing process. The clearing progress was monitored every hour and lasted between 12h and 24h for larvae and early juveniles, respectively. Bones were then stained in 1% KOH solution with alizarin red and preserved in a 30% glycerine/ 70% KOH (1%) for one week and subsequently in 60% glycerine/ 40% KOH (1%). 100% glycerine was used as the final preservative. An example of the final product is illustrated in Figure 2.

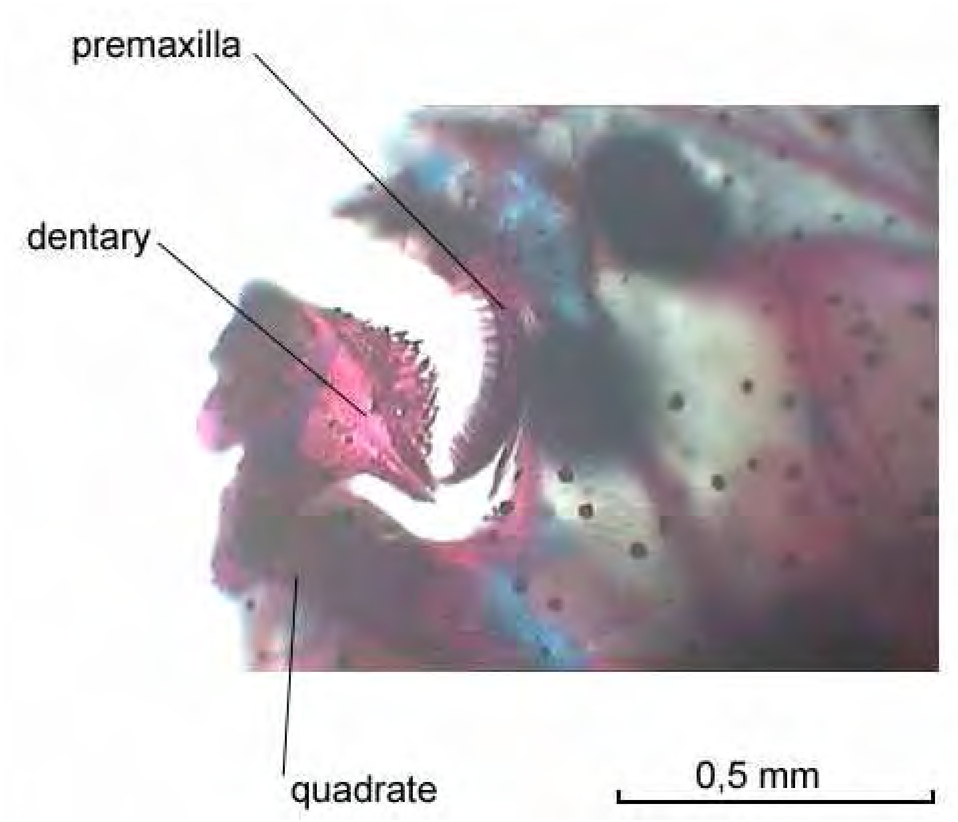


Figure 2: Cleared and stained 45 day old early juvenile *D. marginatus*. Blue stain indicates cartilaginous elements while the red stain indicates ossified elements.

Reference samples were deposited in the fish collection of the South African Institute for Aquatic Biodiversity, Rhodes University, Grahamstown (formerly the J.L.B. Smith Institute of Ichthyology). The osteological nomenclature followed is that of De Beer (1937).

Anatomical illustrations were made using a Camera Lucida attached to a binocular microscope (NICON SMZ-10). Scanned images (LEXMARK X7170; settings; black and white scan, 300 dpi) were then edited with ADOBE PHOTOSHOP® VERSION 7.0 and finally converted to the appropriate size.

Results

The morphological development of the cranial structures is illustrated in Figures 3 to 7, while Figure 8 provides a chronological overview of the development of the cranium. Elements of the branchial apparatus referred to in the text are not indicated in Fig 3 – 6, while the complete branchial apparatus is shown in Fig. 7. Larvae between 4 – 13 dah showed no signs of bilateral asymmetry therefore Fig. 3a – d illustrate only one side (blind side) of the osteocranium. At 16 dah and subsequent stages larvae and early juveniles developed bilateral asymmetries and the blind and ocular side are shown. Fish were staged as premetamorphic, i.e. yolk sac, preflexion, notochord flexion or post-flexion (Thompson *et al*. in press.), metamorphic or postmetamorphic. Metamorphic larvae/early juvenile were defined as larvae or early juveniles where the left eye had begun to migrate dorsally.

YOLK SAC (0 – 4 dah)

In newly hatched yolk sac larvae the eyes were unpigmented and no cartilaginous elements were developed (Fig. 3a). The first cartilaginous elements to appear were the maxilla and two paired branchial arches on day 4 (Fig. 3b).

PREFLEXION (4 – 11 dah)

No further development took place between day 4 and day 6. On day 9, the Meckel's cartilage appeared, and together with the extended maxilla, comprised the first elements of the buccal region. A small parasphenoid developed between the orbits and was the first neurocranial element to appear. An opercle and two branchiostegal rays were developed (Fig. 3c).

FLEXION (11 – 30 dah)

The precise chronological sequences during this period are illustrated in Figure 7.

Several new cartilaginous elements appeared on day 13 (Fig. 3d). A small and thin edentulous premaxilla became visible anterior to the maxilla and elements of the suspensorium, including the ectopterygoid, quadrate, and the hyomandibulo-symplectic were now present. The symplectic was not yet distinct from the hyomandibula. The ceratohyal appeared ventrally to the quadrate extending antero-ventrally towards the Meckel's cartilage. Six branchiostegal rays were developed postero-ventrally to the ceratohyal.

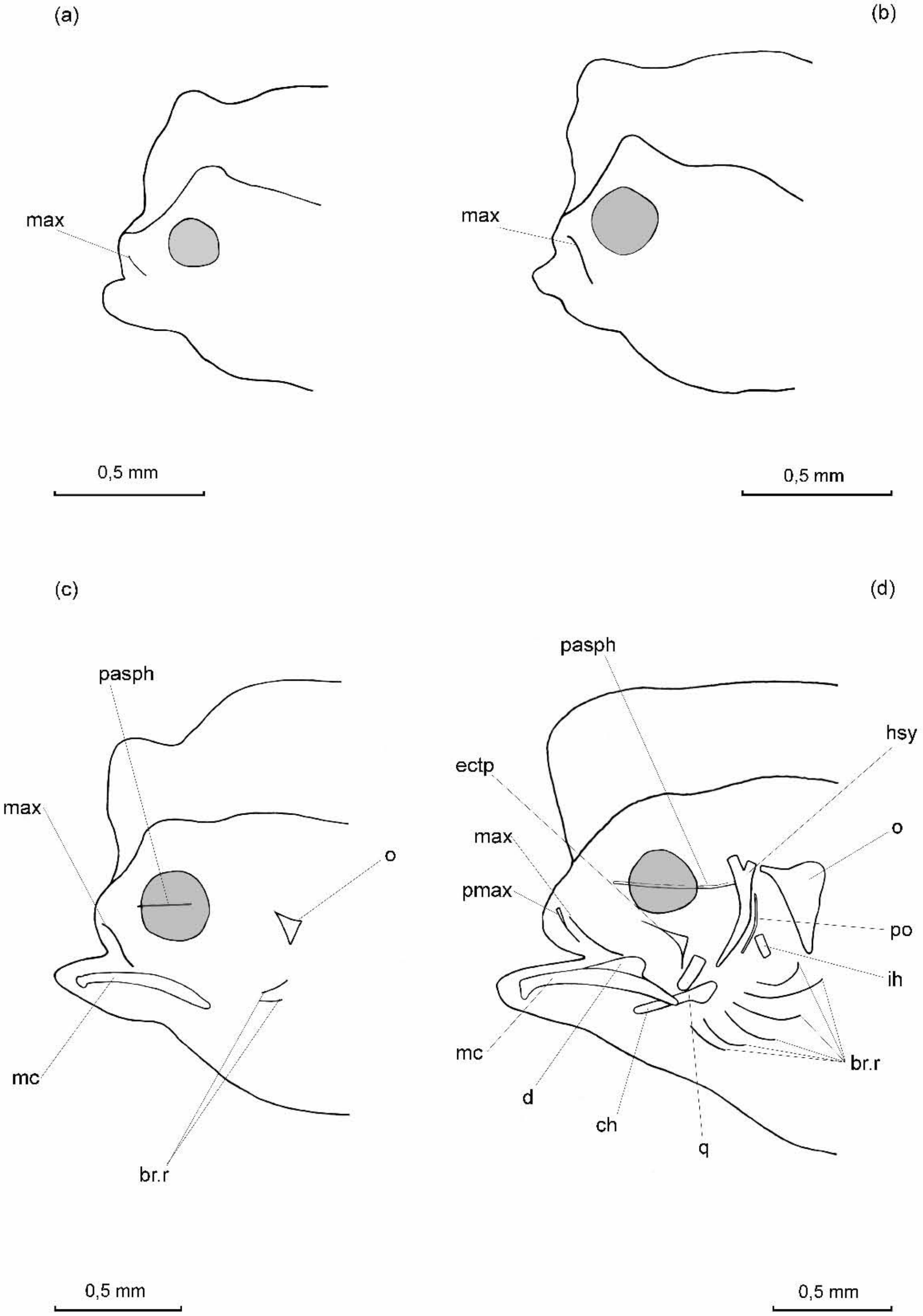


Figure 3: *Dagetichthys marginatus*: lateral view of osteocranium. (a) 4 day old larva, (b) 6 day old larva, (c) 9 day old larva and (d) 13 day old larva. br.r, branchiostegal rays; ch, ceratohyal; dent, dentary; ectp, ectopterygoid; hsy, hyomandibulo-symplectic; ih, interhyal; max, maxilla; mc, Meckel’s cartilage; o, opercle; pasph, parasphenoid; pmax, premaxilla; po, preopercle; q, quadrate.

The parasphenoid extended anteriorly over the orbit and posteriorly to the level of the hyomandibular. The dentary appeared at the dorsal arch of the Meckel's cartilage. The opercle was enlarged forming a triangle and a small preopercle appeared posterior to the hyomandibulo-symplectic.

On day 16 (Fig 4), between two and four larval teeth were developed on either side of the dentaries, which appeared postero-laterally to the Meckel's cartilage. The dentary on the right side appeared small in structure, while the one on the left side was well pronounced and triangular in structure. These differences between the right and left dentaries were the first morphological asymmetries to be observed. No asymmetries were observed between the maxillae and the premaxillae of the right and the left side. At this stage no teeth were developed on either side of the premaxilla. The opercular apparatus was complete with the appearance of the subopercle and the interopercle.

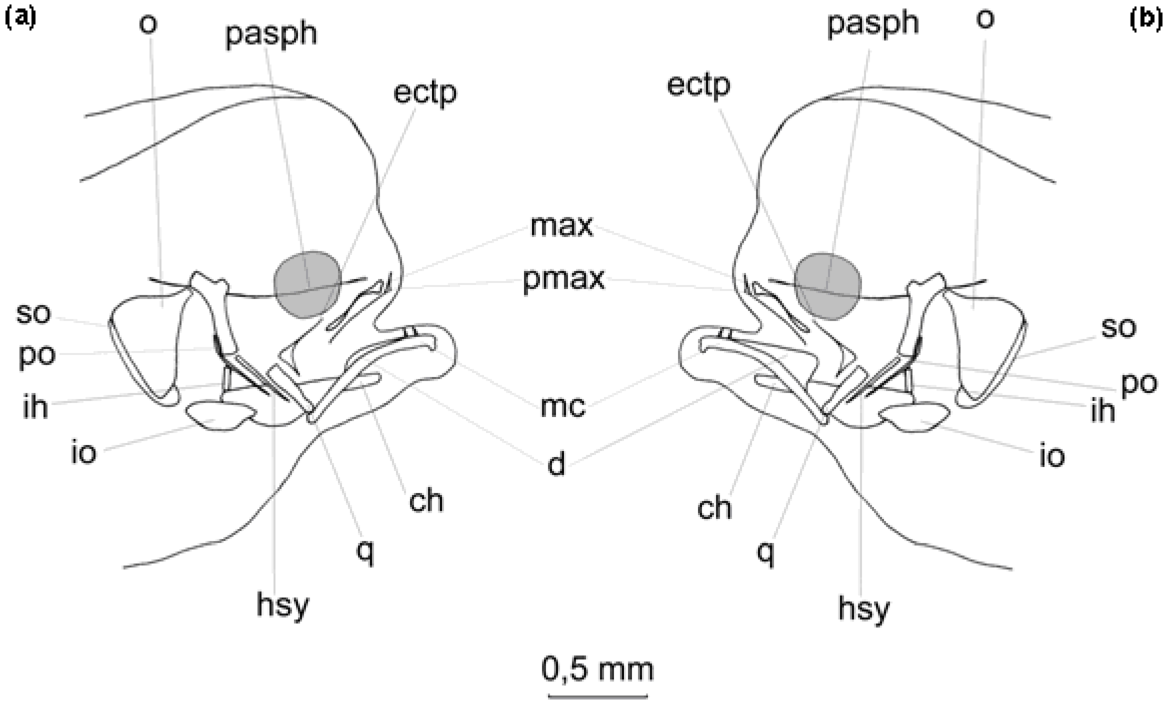


Figure 4. *Dagetichthys marginatus*: right lateral (a) and left lateral (b) view of the osteocranium of a 16 day old larva. ch, ceratohyal; dent, dentary; ectp, ectopterygoid; hsy, hyomandibulo-symplectic; ih, interhyal; io, interopercle; max, maxilla; mc,

Meckel’s cartilage; o, opercle; pasph, parasphenoid; pmax, premaxilla; po, preopercle; q, quadrate; so, subopercle.

All existing cranial structures increased in length and diameter leading to two major articulations, the first between the Meckel’s cartilage and the quadrate and the second between the hyomandibulo-symplectic and the cranium (ootic capsule). The interhyal linked the ceratohyal with the dorsal suspensorium, whose elements the hyomandibular and symplectic appeared as single bones. No asymmetries were observed between the elements of the suspensorium and the opercular bones between the future blind and ocular side. The left/prospective migrating eye remained in its initial position, slightly more dorsal than the future ocular eye. All elements were cartilaginous at this stage.

At 22 dah the left/migrating eye still remained in its initial position. Except for the mesopterygoid all elements of the splachnocranium were developed and were completely or partially ossified (Fig. 5). Pronounced asymmetries were observed among elements of the oral jaws which prefigured the structures observed in the adult. The premaxillae and maxillae appeared ossified on either side of the head. The concave shaped premaxilla of the left side was covered with one to two rows of villiform teeth, while its counterpart on the right side was smaller and edentulous. The maxilla on the left side appeared concavely curved, robust and almost entirely excluded from the mouth gape by a large premaxilla. The maxilla of the right side appeared curved with a roughly “Y-shaped” anterior end, bearing the socket of the premaxilla. The dentaries and the anguloarticulars appeared well ossified with the left side lower jaw being quadrangular in shape and its dentary bearing numerous inwardly curved conical teeth, while the lower jaw on the right side was an edentulous bar-like structure.

The hyomandibula and the symplectic appeared as single structures on either side of the head, separated from each other at the level of the preopercular arch. The “y-shaped” dorsal edge of the hyomandibula showed some degree of ossification at the level of the neurocranial articulation. The symplectic appeared lightly ossified at the anterior and posterior ends fitting into the posterior slit of the quadrate. No differences in the degree of ossification were observed among the elements of the hyomandibula and the symplectic between the left and right side. The ectopterygoids were ossified on either side of the head and palatines had formed at their dorso-anterior arm. The palatine on the left side appeared ossified, while the one on the right side remained cartilaginous. Both quadrates appeared to be well ossified at the level of the joints with the degree of ossification decreasing posteriorly. The quadrate of the left side was larger than its counterpart on the right side. The metapterygoid appeared postero-dorsally to the quadrate on either side of the head. These rhombic-like, at this stage translucent, structures were connected to the bordering suspensorial structures by a cartilaginous complex, which extended towards the palatine anteriorly on the left side of the head.

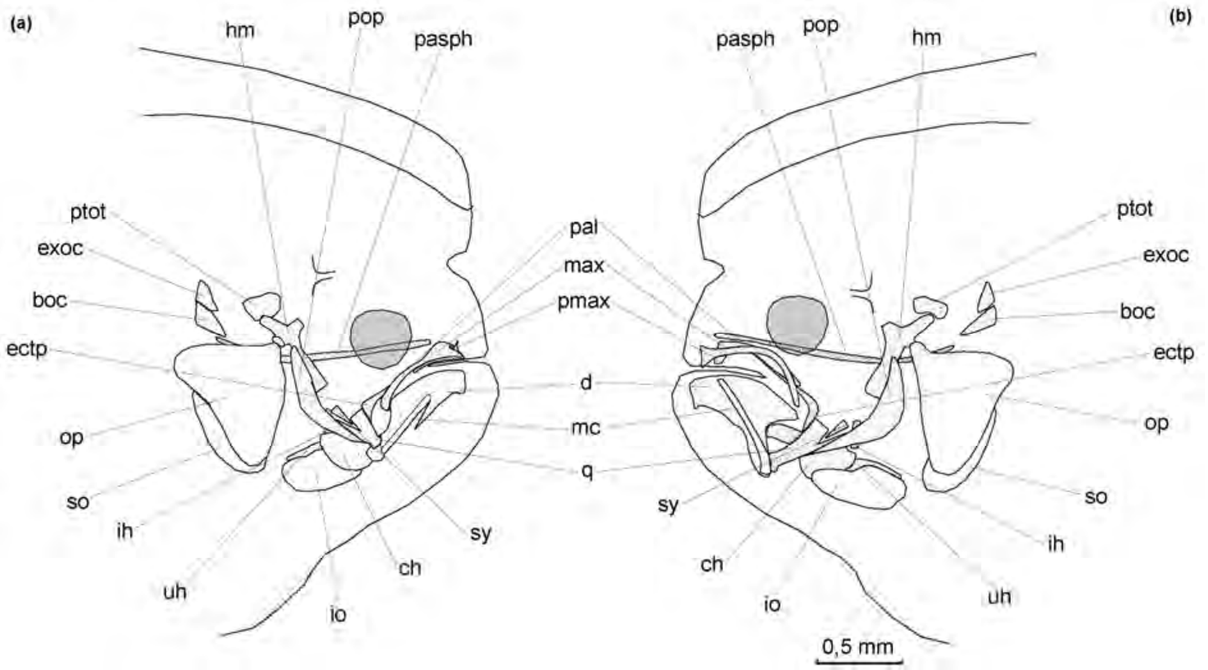


Figure 5. *Dagetichthys marginatus*: right lateral view (a) and left lateral view (b) of the osteocranium of a 22 day old larva. br.r, branchiostegal rays; boc, basioccipital; ch, ceratohyal; dent, dentary; ectp, ectopterygoid; exoc, exoccipital; hm, hyomandibula; ih, interhyal; io, interopercle; max, maxilla; mc, Meckel’s cartilage; op, opercle; pal, palatine; pasph, parasphenoid; pmax, premaxilla; pop, preopercle; prot, prootic; ptot, pterotic; q, quadrate; so, subopercle; sy, symplectic; uh, urohyal. Dotted structures indicate calcified bones.

Two halves of the basioccipital appeared along with paired exoccipitals and pterotics comprising the first parts of the cranial vault. These elements were lightly ossified on either side of the head. The parasphenoid appeared well ossified and extended towards the basioccipital posteriorly and towards the ethmoidal region antero-dorsally.

The opercles and subopercles were similar in shape and size on either side of the head, while the preopercle and interopercle were larger on the future blind side. The preopercles appeared well ossified on either side of the head. The opercle were only ossified on their anterior margin. The subopercle and interopercle remained unossified at this stage. No significant differences in the degree of ossification were observed between the blind and ocular side opercle, subopercle and interopercle.

The ceratohyals were similar in shape and size on both sides of the head. The anterior extended process and the posterior triangular parts were ossified, while the middle section, best described as y-shaped, was cartilaginous. Four ossified branchiostegal rays were articulated to the middle section and three ossified branchiostegal rays were connected to the anterior arm. The interhyal was situated vertically between the posterior part of the suspensorium and the dorso-posterior end of the ceratohyal. The middle section appeared ossified, while the articulating dorsal and ventral ends were cartilaginous. The posterior bar-like extension of the urohyal was slightly curved downwards and connected to the ceratohyal anteriorly.

POSTFLEXION (30 – 45 dah)

At 31 dah the left/migrating eye still remained in its initial position (Fig 6). Most existing structures of the splanchnocranium had advanced in size and degree of ossification. Only the subopercle and interopercle remained unossified. The mesopterygoids were still absent on either side of the head. The cranial vault was almost completely enclosed by cartilaginous structures, though only the basioccipital, the exoccipital, the prootic and pterotic appeared ossified at this stage. Due to the light ossification it remained difficult to distinguish between the single elements of the cranial vault.

POSTMETAMORPHIC (after 45 dah)

At 45 dah the transforming stage was completed (Fig. 7). Both eyes were now located on the right side of the head. The neurocranium was almost completely ossified, though cartilage remained along the borders of some elements. The least degree of ossification was observed among the elements of the sphenotic, parietal and the posterior parts of the frontals. The supraoccipital and the epioccipital were clearly recognizable. Pronounced asymmetries were evident among the anterior elements of the neurocranium. The frontals on the eyed side appeared ventrally curved making contact with the eyed side lateral ethmoid.

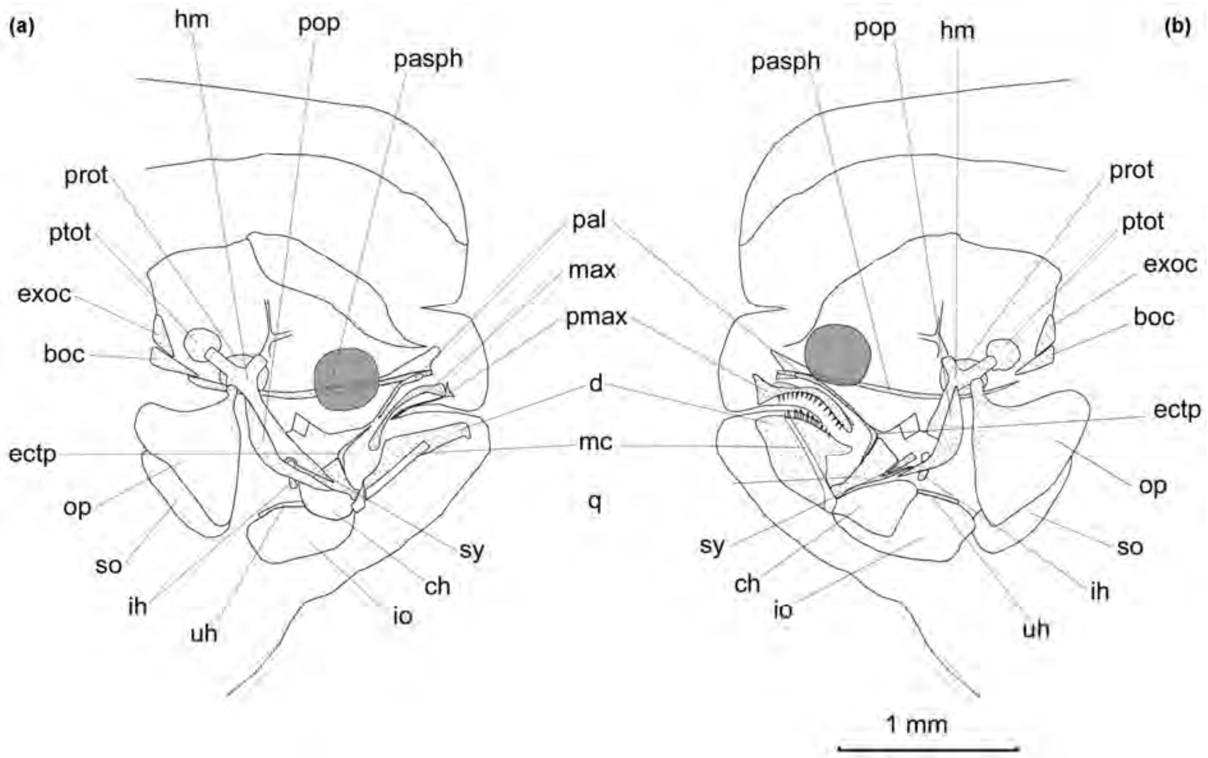


Figure 6: *Dagetichthys marginatus*: right lateral view (a) and left lateral view (b) of the osteocranium of a 31 day old larva. br.r, branchiostegal rays; boc, basioccipital; ch, ceratohyal; dent, dentary; ectp,ectopterygoid; exoc, exoccipital; hm, hyomandibular; ih, interhyal; io, interopercle; max, maxillary; mc, Meckel's cartilage; op, opercle; pal, palatine; pasph, parasphenoid; pmax, premaxilla; pop, preopercle; prot, prootic; ptot, pterotic; q, quadrate; so, subopercle; sy, symplectic; uh, urohyal. Dotted structures indicate calcified bones.

The lateral ethmoid of the blind side extended dorso-posteriorly and fused with the frontals to form a wall for the migrated eye's orbit. The ethmoid was curved towards the blind side while the vomer was curved towards the eyed side. Asymmetries between suspensorial and oral elements became very distinct with the elements of the blind side generally being broader than those on the eyed side. Differences in length and shape were observed between the oral jaw elements of the blind side and eyed side. The metapterygoids on either side remained unossified and similar in size and shape. The mesopterygoids were absent on either side of the head.

The subopercle and interopercle remained unossified. All elements of the branchial arch (basibranchial, hypobranchials, ceratobranchials, epibranchials, and infrapharyngobranchials) were ossified, with cartilaginous elements between articulation points. The fifth pair of ceratobranchials was dentulous and together with the dermal toothed plates on infrapharyngobranchials 2, 3 and 4 formed the pharyngeal jaws.

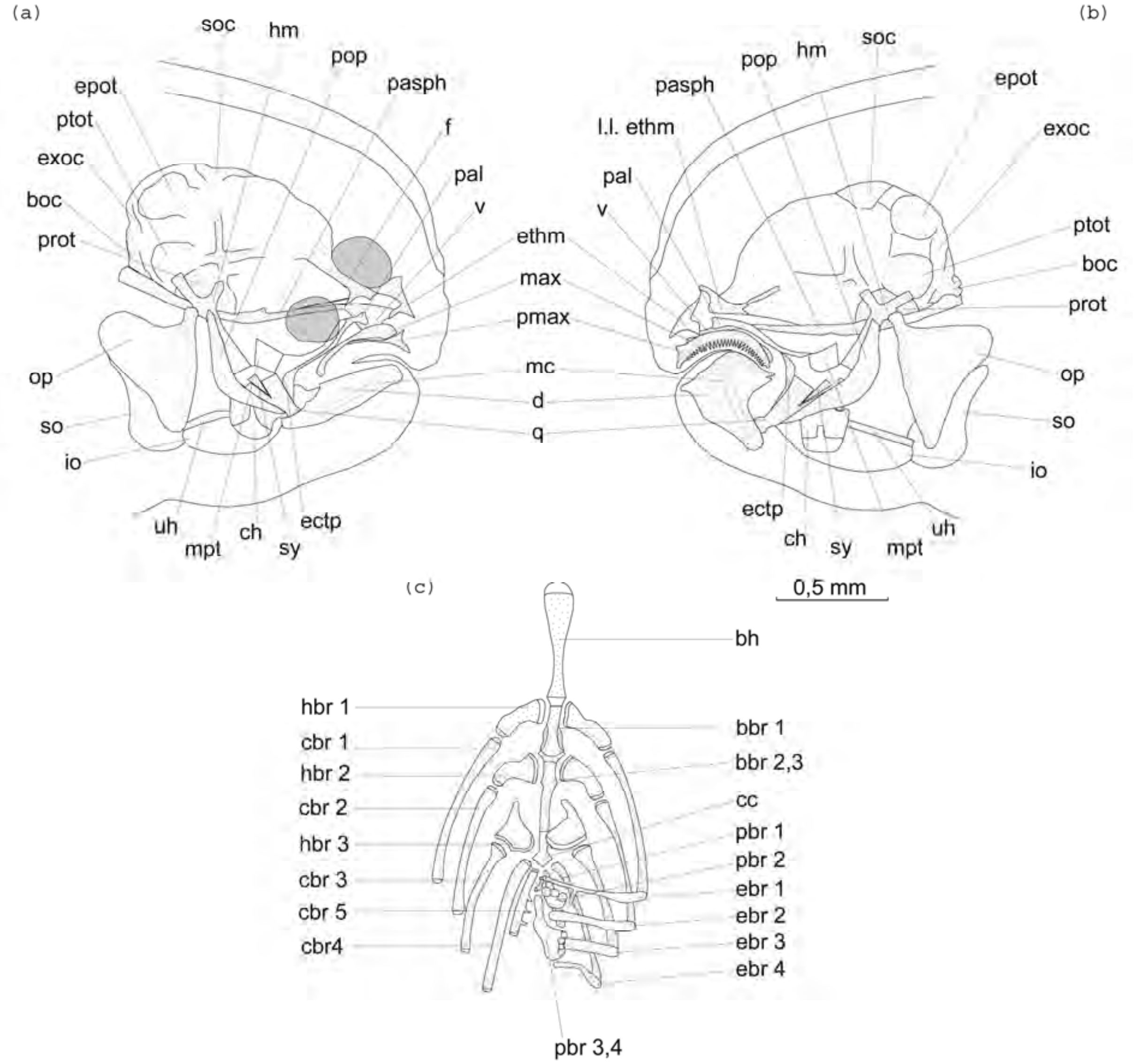


Figure 7: *Dagetichthys marginatus*: right lateral view (a) and left lateral view (b) of the osteocranium of a 45 day old larva and (c) dorsal view of the branchial apparatus of a 45 day old larva with the left half of the upper elements removed. bbr, branchiostegal rays; boc, basioccipital; br.r, branchiostegal rays; cbr, ceratobranchial; cc, central cartilage; ch, ceratohyal; dent, dentary; ebr, epibranchial; ectp,ectopterygoid; epot, epioccipital; ethm, ethmoid; exoc, exoccipital; f, frontal; hbr, hypobranchial; hm, hyomandibula; ih, interhyal; io, interopercle; l.l. ethm, left lateral ethmoid; max, maxillary; mc, Meckel's cartilage; mpt, metapterygoid; op, opercle; pal, palatine; pasph, parasphenoid; pbr, infrapharyngobranchial; pmax, premaxilla; pop, preopercle; prot, prootic; ptot, pterotic; q, quadrate; so, subopercle; soc, supraoccipital; sy, symplectic; uh, urohyal; v, vomer. Dotted structures indicate calcified bones. Branchiostegal rays are not indicated.

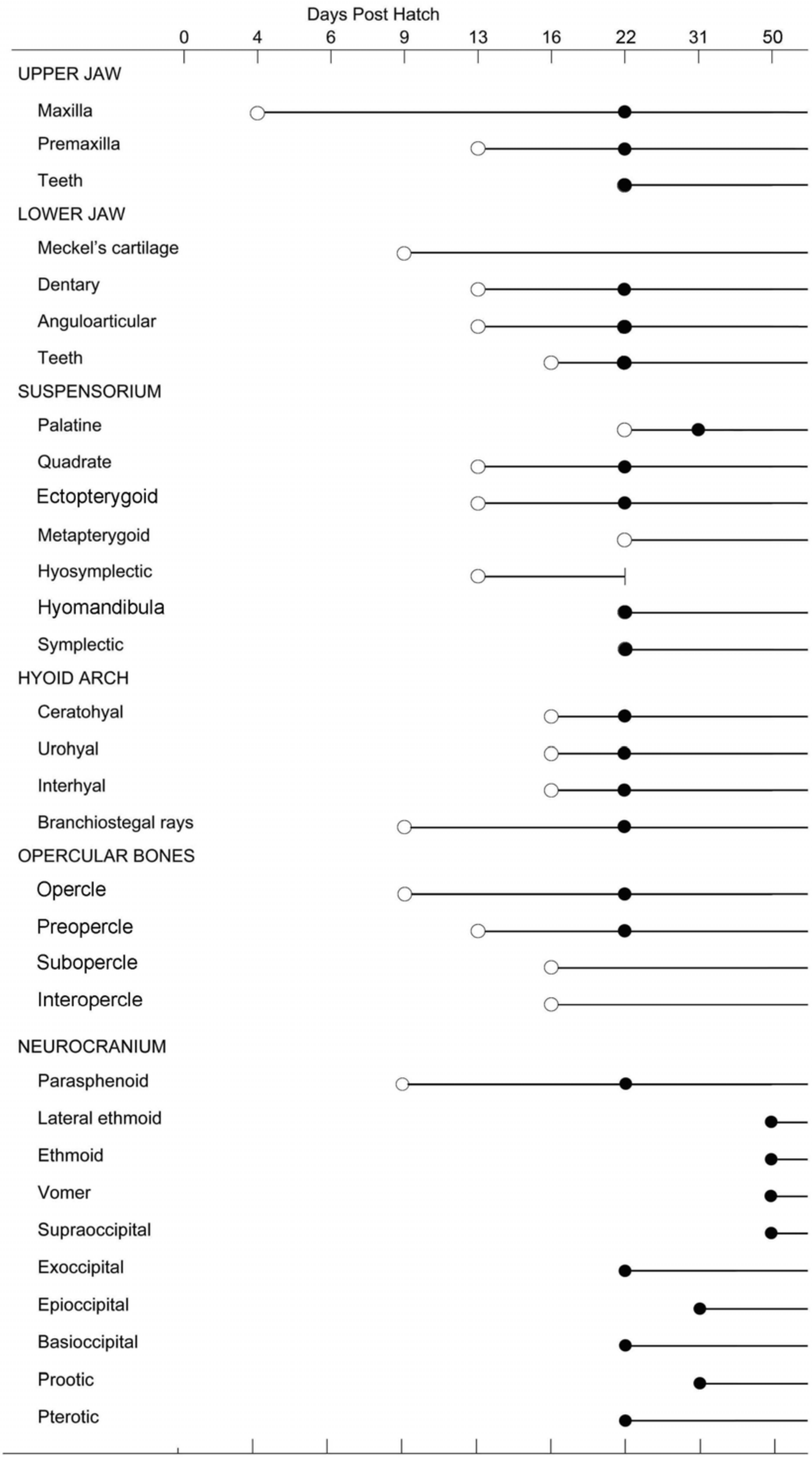

Days Post Hatch
0
4
6
9
13
16
22
31
50
UPPER JAW
Maxilla
Premaxilla
Teeth
LOWER JAW
Meckel's cartilage
Dentary
Anguloarticular
Teeth
SUSPENSORIUM
Palatine
Quadrate
Ectopterygoid
Metapterygoid
Hyosymplectic
Hyomandibula
Symplectic
HYOID ARCH
Ceratohyal
Urohyal
Interhyal
Branchiostegal rays
OPERCULAR BONES
Opercle
Preopercle
Subopercle
Interopercle
NEUROCRANIUM
Parasphenoid
Lateral ethmoid
Ethmoid
Vomer
Supraoccipital
Exoccipital
Epioccipital
Basioccipital
Prootic
Pterotic

Figure 8: Schematic representation of appearance of cranial elements. ○: appearance of cartilaginous elements; ●: appearance of ossified bones or beginning of ossification of cartilaginous elements.

The branchial apparatus showed no significant asymmetries and no deflection to either side of the head.

The chronological appearance of the cartilaginous elements and their subsequent ossification is shown in Fig. 8. Most elements of the splanchnocranium appeared ossified at 22 dah, while the majority of the neurocranial elements only became ossified 45 dah, after eye migration was completed.

Discussion

The sequential development of cranial bony elements is variable in teleosts (De Beer, 1937; Jollie, 1983; Vandewalle *et al*. 1995; Adriaens & Verraes, 1998). As observed for other teleosts including *Solea solea* (De Beer 1937, Vandewalle *et al*. 1992, 1995, Wagemans & Vandewalle 2001), elements of the splanchnocranium generally developed earlier than those of the neurocranium in *D. marginatus* larvae. Moreover no cartilaginous elements, i.e. maxillae or Meckel's cartilage, were present in the larvae of *D. marginatus* at hatching as has been reported for some pleuronectiform species (Wagemans *et al.*, 1998, Wagemans & Vandewalle, 2001) and other teleosts (Hubendick 1942; Elman & Balon, 1980). In contrast to *D. marginatus*, the parachordals in *Salmo trutta* L. (Salmonidae) are present 6 days before hatching (De Beer, 1937), though this is most probably related to its precocial life strategy style and may be a common feature of fish that do not have an extended larval stage (Bruton 1989).

In *D. marginatus,* the onset of exogenous feeding at 4 dah coincides with the complete absorption of the yolk sac (Thompson *et al*. in press) and the appearance of the first cartilaginous elements, the maxillae and two small branchial arches. The appearance of the gill arches may suggest that filter feeding is the first feeding mode of *D.marginatus* larvae. Similar to *D. marginatus,* the formation of branchial arches in *S. solea* begins 2 – 3 dah (Wagemans & Vandewalle 2001). Generally, branchial arches form at hatching or shortly after in many teleosts (van der Meeran 1991, Otten 1982, Hunt von Herbing *et al*. 1996, Liem, 1991; Otten, 1982; Wagemans & Vandewalle 2001) suggesting that filter feeding could be of particular importance for fish larval survival (Hunt von Herbing *et al*. 1996). Based on morphology and biomechanical measurements, Francis (2002) predicted that early premetamorphic larvae of the southern flounder, *Paralichthys lethostigma,* uses ram and/or suction feeding to acquire prey. During ram feeding the prey is stationary while the fish rapidly approaches with an open mouth to engulf the prey (Norton & Brainerd 1993). This rapid approach towards the prey could also be observed in *D.marginatus* larvae at 4 dah, suggesting that ram feeding may play an important role in feeding during early larval development.

After ingestion, food must be transported from the orobranchial cavity to the oesophagus passing the overlying brain (Adriens & Verraes, 1998). In *S. solea* and many other teleosts a parasphenoid is formed prior to, or simultaneously, with the beginning of exogenous feeding to protect the overlying brain from any damage during food ingestion (Verraes 1974, Potthoff *et al*. 1987; 1988; Vandewalle *et al*. 1992, 1995a, 1997, Wagemans & Vandewalle 2001). However, in *S.maximus* larvae the absence of a parasphenoid at the time of switching from endogenous to exogenous feeding does not appear to be an obstacle, though due to the absence of a hypophyseal fenestra it is suggested that the *trabecula cummunis* may provide sufficient protection to the braincase (Wagemans *et al*., 1998). In the present study the parasphenoid in *D. marginatus* appeared at 9 dah, shortly after the depletion of the yolk sac and it is suggested that similar to *S. solea* the parasphenoid provides sufficient protection to the braincase at this stage. As observed in many teleosts including *S. solea* and *S. maximus* the appearance of the buccal and pharyngeal jaws coincides with the beginning of exogenous feeding or shortly before (Wagemans & Vandewalle, 2001; Potthoff *et al.*, 1987, Wagemans et al. 1998). This is also the case for *D. marginatus,* where the maxilla and the Meckel's cartilage appear 4 dah and 9 dah, respectively. Generally, the early development of at least several of the upper and lower jaw elements emphasizes the importance of these structures in relation to first feeding.

Suction feeding is the most common feeding method in teleosts. This is effected by the creation of a negative pressure in the oral cavity resulting in an efficient antero-posterior water flow (Alexander 1970, Osse & Muller 1980). The mechanical mechanisms for suction feeding normally only develop some time after the start of exogenous feeding. Although the fundamental elements for suction feeding were developed in *D. marginatus* at 13 dah, interactions between the functional units could not take place since the palatoquadrate was still separated from the Meckel´s cartilage, the hyomandibulo-symplectic was not connected to the palatoquadrate and the interhyal had not yet linked the hyoid arches with the suspensorium. At 16 dah all existing cranial structures had increased in length and diameter, the Meckel's cartilage had articulated with the quadrate and the dorsal suspensorium articulated with the cranium. This now allowed for the interhyal to act as a fulcrum between the suspensorium and the hyoid apparatus. In addition, the opercular apparatus (opercle, preopercle, subopercle and interopercle) formed a functional unit and became articulated to the hyoid unit and the lower jaw. As mentioned previously, the musculoskeletal system and the consequential kinematics during feeding were not considered in this study. However, the interlinked arrangement of the functional units at 16 dah suggests a sufficient functional ability to generate negative pressure within the buccal cavity such that suction feeding could take place. Similar results are reported for 16 day old *S. solea* larvae (Wagemans & Vandewalle, 2001). At this stage *D. marginatus* larvae still approached their prey by rapidly moving forward. Hence, it is concluded that larvae of *D. marginatus* use a combination of ram and suction feeding to acquire prey.

In addition, transport and processing of ingested food is facilitated by the functionally developed branchial basket. The formation of a functional opercular apparatus together with the set of 7 branchiostegeal rays may control food loss through the gill slits as has been suggested for other teleosts (Hunt von Herbing *et al*. 1996). The terminal mouth position together with the symmetrical buccal elements is suited for feeding in the water column. However, observations on the feeding behaviour revealed that larvae spend significantly more time swimming and feeding near the bottom instead of in the water column at 16 dah. This change from pelagic to benthic feeding behaviour can be correlated to the beginning of metamorphosis shortly after day 16 at a mean total length of 6.6 mm (Thompson *et al*. in press).

The first pronounced osteocranial asymmetries were developed at 22 dah. Early juveniles had changed their swimming plane by a 90° rotation in body position. The dentaries and premaxillae of the blind side were larger and dentulous, while the premaxillae on the ocular side remained edentulous and teeth on the ocular side lower jaw had disappeared. Edentulous lower jaws on the ocular side have also been reported for metamorphosing larvae of *S. solea* (Wagemans & Vandewalle 2001) and can be considered as a functional adaptation to benthic feeding, where feeding is carried out by elements of the blind side, while respiration is accomplished by elements of the ocular side (Yazdani, 1969; Wagemans & Vandewalle 2001). Moreover, suspensorial elements of the blind side were more robust and hence better suited for feeding than those on the ocular side (Yazdani 1969). Most elements of the splanchnocranium were ossified at this stage, only the interopercle, subopercle and the metapterygoid remained cartilaginous. Ossification of cranial elements and the acquisition of teeth greatly enhances feeding efficiency (Gosline, 1971; Kohno *et al*. 1996b, Wagemans & Vandewalle, 2001). Similar to the ontogenetic timing in *Solea solea* (Wagemans & Vandewalle, 2001), the basi- and exoccipital were the first elements of the cranial vault to ossify in *D. marginatus*. Such an ossification pattern leads to a reinforcement of the attachment of the skull to the notochord (Adriens & Verraes, 1998).

In *S. solea*, an early ossification pattern was observed among the elements of the suspensorium, i.e. quadrate, symplectic, ectopterygoid and metapterygoid of the blind side (Wagemans & Vandewalle 2001). This is only partly the case in *D. marginatus* at 22 dah, where the palatine bone on the blind side of the head appeared ossified earlier than the palatine on the ocular side. It seems necessary that structures that participate essentially in the feeding mechanism ossify before those on the ocular side (Wagemans & Vandewalle 2001).

Generally, the splanchnocranium tends to develop faster than the neurocranium in many teleosts (De Beer, 1937; Vandewalle *et al*., 1992, 1995a). This is also the case in many Pleuronectiformes including *S. solea* (Soleidae). However, in *S. solea* a rapid ossification of neurocranial elements takes place once the migrating eye has reached the ocular side (Brewster 1987). Similarly, in *D. marginatus* the majority of the neurocranial elements (lateral ethmoid, ethmoid, vomer, supraoccipital, epioccipital and protic) ossify shortly after the completion of eye migration at 45 dah.

In the present study, eye migration did not begin before 31 dah and eye translocation was completed by 45 dah. In contrast to *D. marginatus*, eye migration in *S. senegalensis* started between 8 – 12 dah. The time required to complete the transformation was 4.4 up to 12.5 days, depending on the feeding regime and rearing environment (Dinis *et al.*, 1999; Fernàndez-Díaz *et al.*, 2001). In *S. solea*, migration of the left eye begins at 18 dah and is completed at 23 dah (Wagemans & Vandewalle 2001). The beginning of eye migration and the period of eye translocation appears considerably delayed in *D. marginatus* when compared to that of *S. senegalensis* and *S. solea*.

CHAPTER 4

MORPHOLOGIAL ADAPTATION AND FEEDING

Introduction

The skull of a teleost fish is structurally complex and is composed of numerous bony, muscular and ligamentous elements capable of intricate movement. Apart from providing space for the brain and protecting it, it lodges and protects sense organs and performs respiratory and feeding movements. The existing morphological structures and their functional capacities impose limitations on feeding behaviour of a fish and ultimately affect somatic growth (Wankowski 1979). Functional morphology of individual fish species is therefore an important consideration in feed development and may facilitate to improve the feeding performance of fish under culture conditions.

The primary tools of contemporary functional morphology include a detailed analysis of the anatomical structure and design, high-speed imaging kinematics and electromyography (Ferry-Graham & Lauder 2001, Ashley-Ross & Gillis 2002). The cranial morphology of a vast number of teleosts has been extensively studied (e.g. Cunningham 1890, Gregory 1933, van Dobben 1935, De Beer 1937, Tchernavin 1953, Kirchhoff 1958, Schaeffer & Rosen 1961, Kayser 1962, Nelson 1969, Gosline 1971, Greenwood 1971, Kershaw 1971) including some Pleuronectiformes (e.g. Kyle 1921, Gregory 1933, Chabanaud 1936, 1938, Bürgin 1986). Adaptations and mechanisms of the jaws have been described for a variety of flatfish species (van Dobben, 1932, Flüchter 1963, Yazdani 1969). For example, Yazdani (1969) described adaptations in the jaws of derived Pleuronectiformes such as members of the Soleidae and Cynoglossidae, where feeding is exclusively performed by the elements of the blind side, while respiration is carried out by the elements of the ocular side. The functional explanations of the above mentioned authors were based mainly on the cranial morphology and manipulations of post-mortem specimens. Assessing function from anatomy and post-mortem manipulations alone has often led to wrong conclusions, which has become evident with the advent of direct measurement techniques (Tchernavin 1953, Schaeffer & Rosen 1961, Alexander 1966, 1970, Osse 1969, Lauder 1979, Lauder 1980b, 1982, Liem 1967, 1970). More recent studies used direct measurement techniques such as high-speed video imaging to describe the cranial kinematics (Gibb 1995, 1996, 1997, 2003). Nevertheless a comprehensive description of the cranial morphology remains the first step towards assessing feeding behaviour.

The aim of this chapter was to describe the cranial morphology of adult *Dagetichthys marginatus* and to compare structures with those of early juveniles and larvae. Comparisons are made with other Pleuronectiformes and possible kinematic functions of the existing structures are discussed. It is assumed that an understanding of form and function of the cranial structures related to feeding will facilitate further studies on feeding behaviour and feeding preferences and the development of a commercial diet for *D. marginatus*.

Material and Methods

The soles used for this study were caught between September 2004 and April 2005. Ten adult soles with an average total length of 340 mm ± 5mm were used. Cuts were made posterior to the cleithrum and the head was removed from the body. Boiling water was gently poured over the head, and soft tissue was removed using a scalpel. Specimens were than cleared and stained, following the method described by Taylor & Van Dyke (1985) (see Chapter 3). Reference samples were deposited in the fish collection of the South African Institute for Aquatic Biodiversity, Rhodes University, Grahamstown (formerly the J.L.B. Smith Institute of Ichthyology).

The general osteological nomenclature followed is that of Chapleau (1988). Bony structures are indicated by fine stippling, while cartilage is indicated by larger stippling.

Morphological description

D. marginatus showed asymmetries in the neurocranium, the suspensoria and the upper and lower jaws. Elements of the opercular series and the hyoid showed few asymmetries.

The ethmoidal region appeared remarkably asymmetrical (Fig. 9, 10, 11). The ethmoid extended forward beyond the vomer, curved in structure and was clearly directed towards the eyed side. The vomer on the other hand was directed towards the blind side.

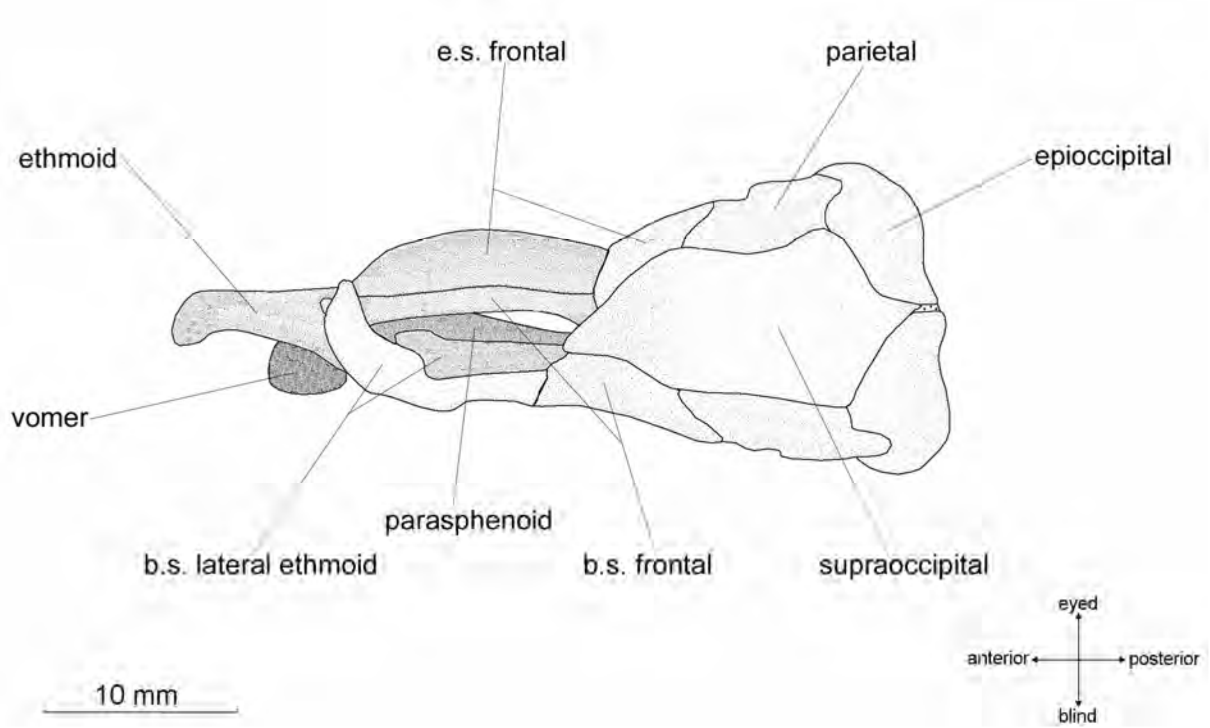


Figure 9: Camera lucida drawing of the skull of *Dagetichthys marginatus* (dorsal view).

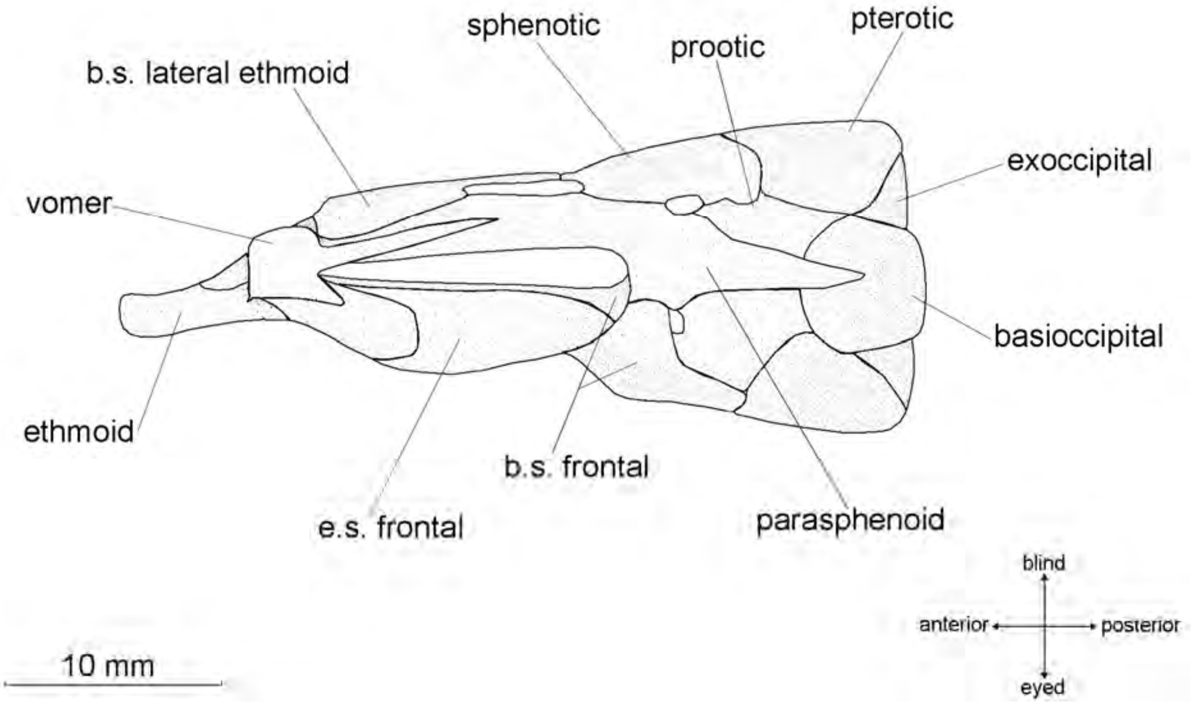


Figure 10: Camera lucida drawing of the skull of *Dagetichthys marginatus* (ventral view).

The small lateral ethmoid (Fig. 12 A) of the eyed side was articulated with the ethmoid and the vomer anteriorly. Dorsally it was articulated with the anterior arm of the eyed frontal. The blind side lateral ethmoid in contrast appeared large and formed the margin of the upper orbit (Fig. 9). Its postero-dorsal process was articulated with the posterior arm of the blind side frontal while the postero-ventral side was articulated with the dorsal margin of the parasphenoid. The anterior part was articulated with the ethmoid and the vomer. The posterior part of the eyed side frontal was articulated with the sphenoid and postero-dorsally to the parietal and the supraoccipital. The broad anterior arm of the eyed side frontal extended towards the ethmoid and formed the external margin of the interorbital complex.

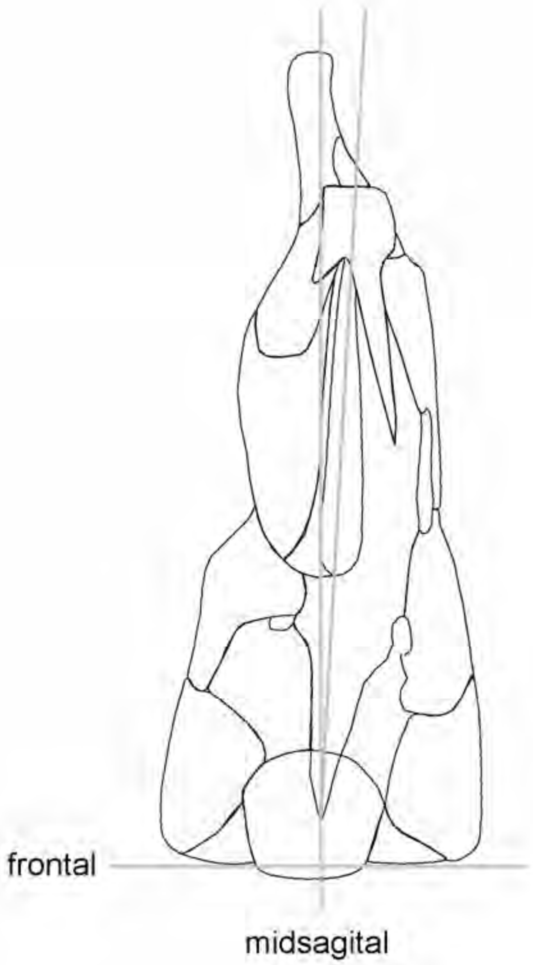


Figure 11: Ventral view of the neurocranium *Dagetichthys marginatus*. The parasphenoid and the vomer are tipped out of the sagital midplane.

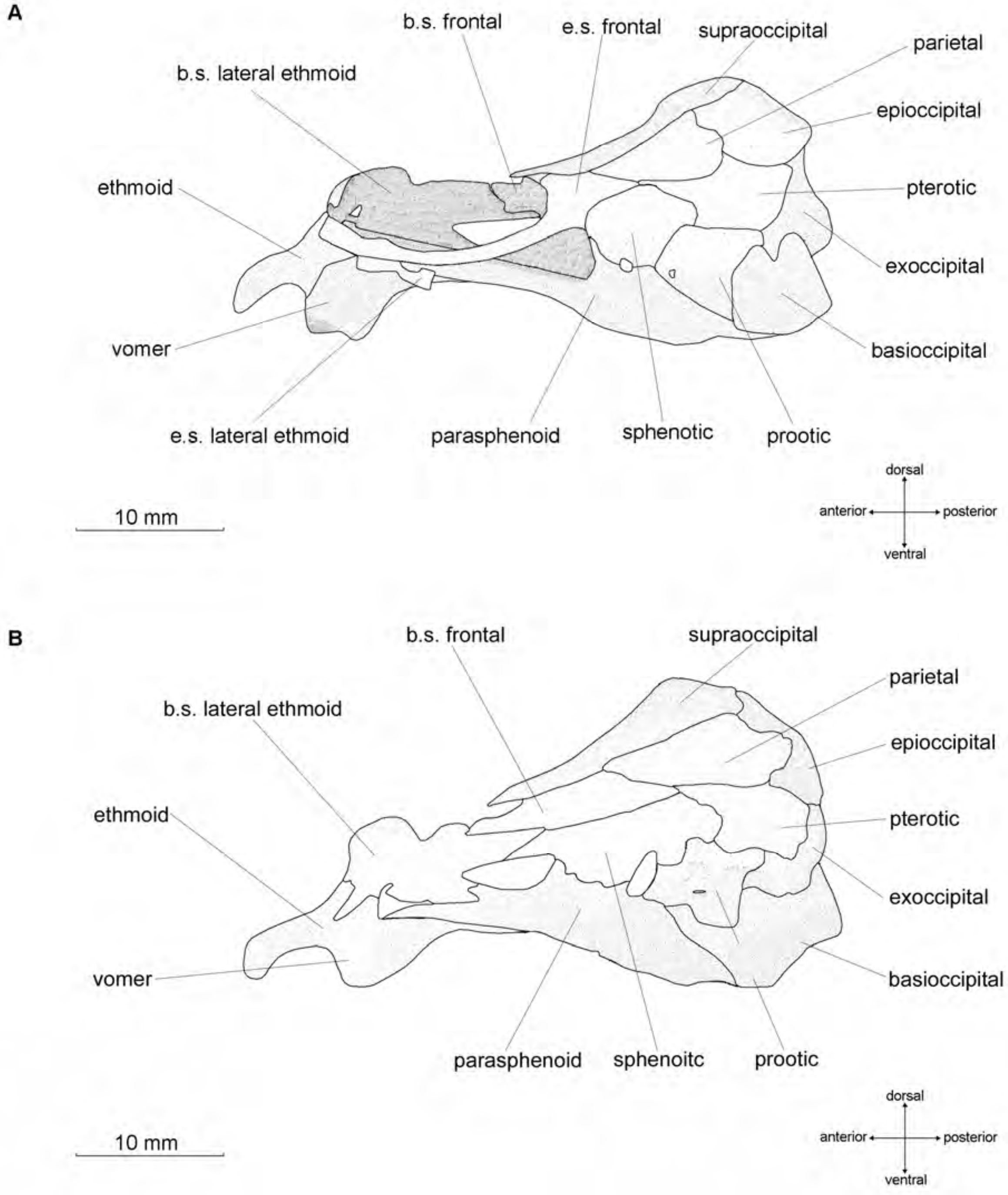


Figure 12: Camera lucida drawings of the skull of *Dagetichthys marginatus*: (A) lateral view of the eyed side and (B) lateral view of the blind side. Note that the drawing of the eyed side of the skull has been reversed horizontally to facilitate comparisons with the drawing of the blind side and ventral drawings.

The blind side frontal extended further posteriorly when compared to its counterpart on the eyed side. Posteriorly it was articulated with the parietal, postero-dorsally to the supraoccipital and postero-ventrally to the sphenotic. Its median part posterior to the orbital region was articulated the blind side lateral ethmoid. A thin and long arm of the blind side frontal extended posteriorly towards the ethmoid forming the inner margin of the interorbital complex. The parasphenoid, tilted relative to the sagital plane (Fig. 11), formed the floor of the neurocranium extending anteriorly and is articulated with the vomer, the ethmoid and the lateral ethmoids, while its posterior process was inserted medially in the basiooccipital and was bordered dorsally by the prootic and the sphenotic on both sides. The posterior part of the neurocranium (cranial vault) will not be described here, as its association with feeding is limited.

The jaw bones are extremely asymmetrical (Fig. 13). Both premaxillae were curved and about as long as the corresponding maxilla on the blind side, but considerably shorter than that on the eyed side. The premaxilla on the blind side appeared robust and curved with conical teeth in up to five rows inserted in the posterior half of the curved ventral margin of the bone. Anterior to this toothed segment the bone extended along the inner margin of the corresponding maxilla. A small ascending process was formed antero-dorsally and was loosely connected by connective tissue to the antero-lateral face of the median rostral cartilage. The anterior head was enlarged on the eyed side premaxilla (but smaller as on the blind side premaxilla) with a short ascending process which was firmly attached to the lateral face of the median rostral cartilage. Posteriorly, the eyed side premaxilla appeared feeble in structure. There are no teeth on the eyed side premaxilla.

The enlarged anterior region of the blind side maxilla formed two large processes, which embraced the ascending process of the corresponding premaxilla. The maxilla overlapped the premaxilla and was excluded from the mouth gape. On the eyed side the anterior head was roughly Y- shaped overlapping the main part of the premaxilla, posterior to the ascending process. The postero-ventral end formed an articular surface with the socket of the anguloarticular.

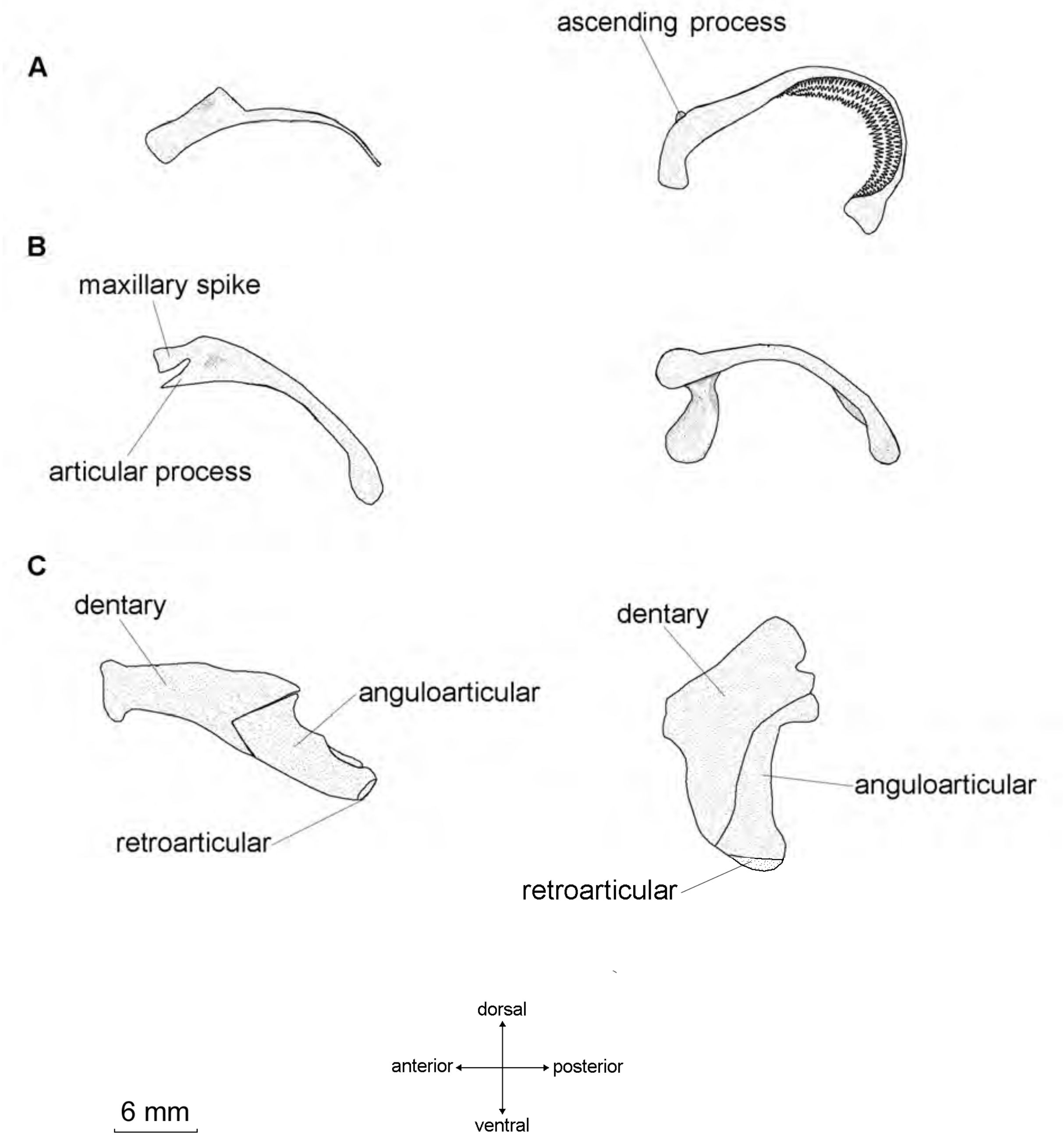


Figure 13: Camera lucida drawings of lateral views of isolated bones from *Dagetichthys marginatus*: (A) lateral view of the premaxillae, (B) lateral view of the maxillae and (C) lateral view of the lower jaw. Bones from the eyed side are on the left, bones from the blind side are on the right. Note that bones from the eyed side have been reversed horizontally to facilitate comparisons with the drawing of the blind side and ventral drawings.

In an intact specimen the premaxillae and maxillae are bound together by connective tissue and together with associated cartilage and connective tissue compose a single unit (Fig. 14). The median rostral cartilage was displaced and firmly attached to the eyed side maxillary head.

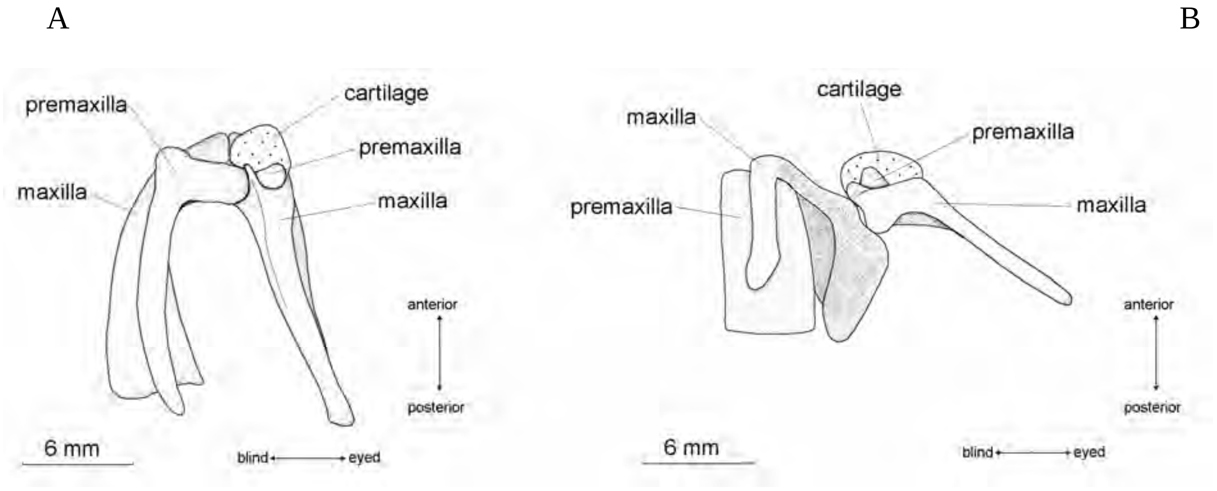


Figure 14: Camera lucida drawings of the maxilla-premaxilla complex (upper jaws) of *Dagetichthys marginatus*:: (A) dorsal view (looking down on the upper jaw) and (B) a posterior view (looking out through the mouth). The maxilla-premaxilla complex is held together by connective tissue.

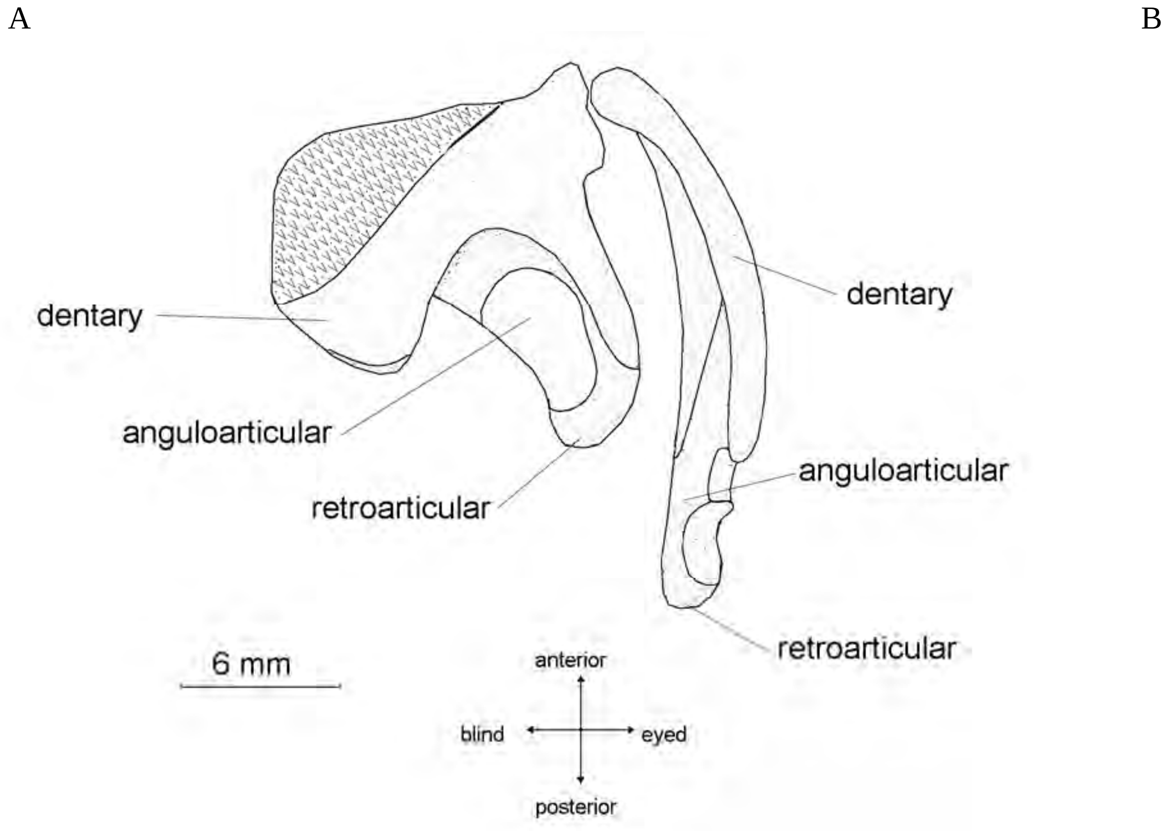


Figure 15: Camera lucida drawings of a medial view of the lower jaw (dentary, anguloarticular and retroarticular) of *Dagetichthys marginatus*.

The lower jaw bones (Fig. 13 & 15) of the blind side appeared stout and triangular, whereas the eyed side lower jaw was extended, bar like in shape. The dentary on the eyed side was edentulous, while on the blind side dentary the strongly convex curved dorsal margin bore numerous, inwardly curved, conical teeth. The convex shape fitted to the corresponding concave shaped premaxilla when the mouth was shut. The coronoid process of the dentary, together with the posterodorsal arm of the anguloarticular, were attached ligamentously to the premaxilla on the blind side. In contrast, on the eyed side the postero-ventral knob of the maxilla was attached to the coronoid process of the dentary and the antero-dorsal arm of the anguloarticular. The articular cavity was broader on the blind side, while that of the eyed side was somewhat extended. The quadrate articulation to the lower jaw of the blind side was situated antero-ventral to the articulation point of the eyed side articulation point. On the blind side, the ramus had a socket in which the corresponding knob of the quadrate fit, while on the eyed side the socket fit into the corresponding knob of the quadrate. The retroarticular enclosed the ventral region of the anguloarticular on each side.

The suspensoria of the blind side and ocular side showed pronounced asymmetries with the suspensorium on the blind side being larger than its counterpart on the eyed side (Fig. 16). The suspensorium on the eyed side consisted of seven elements, while the suspensorium of the blind side had a mesopterygoid in addition to the other existing elements.

The quadrate on both sides appeared triangular in shape with a pronounced antero-ventral articulatory condyle. A long postero-dorsal process extended between the preopercle and the symplectic. The quadrate of the blind side was larger and more robust than its counterpart on the eyed side. The ectopterygoid was angular extending towards the articulary condyle of the quadrate. Its antero-dorsal arm was articulated with the palatine. The blind side ectopterygoid was postero-dorsally covered by the metapterygoid. The blind side ectopterygoid was larger than the eyed side one. The palatine, toothless on either side, continued the anterior arm of the ectopterygoid. The palatine on the blind side was long and curved medially and articulated with the vomer. The extended antero-ventral arm abutted to the posterior surface of the head of the maxilla. The palatine on the eyed side was short and stout with two processes extending dorsally and ventrally. It articulated with the vomer, but did not abut the maxilla. The symplectics on each side of the head appeared wedgeshaped fitting into a groove on the postero-medial portion of the quadrate. The blind side mesopterygoid was situated dorso-posterior to the ectopterygoid and dorsal to the quadrate, while its counterpart on the eyed side was absent.

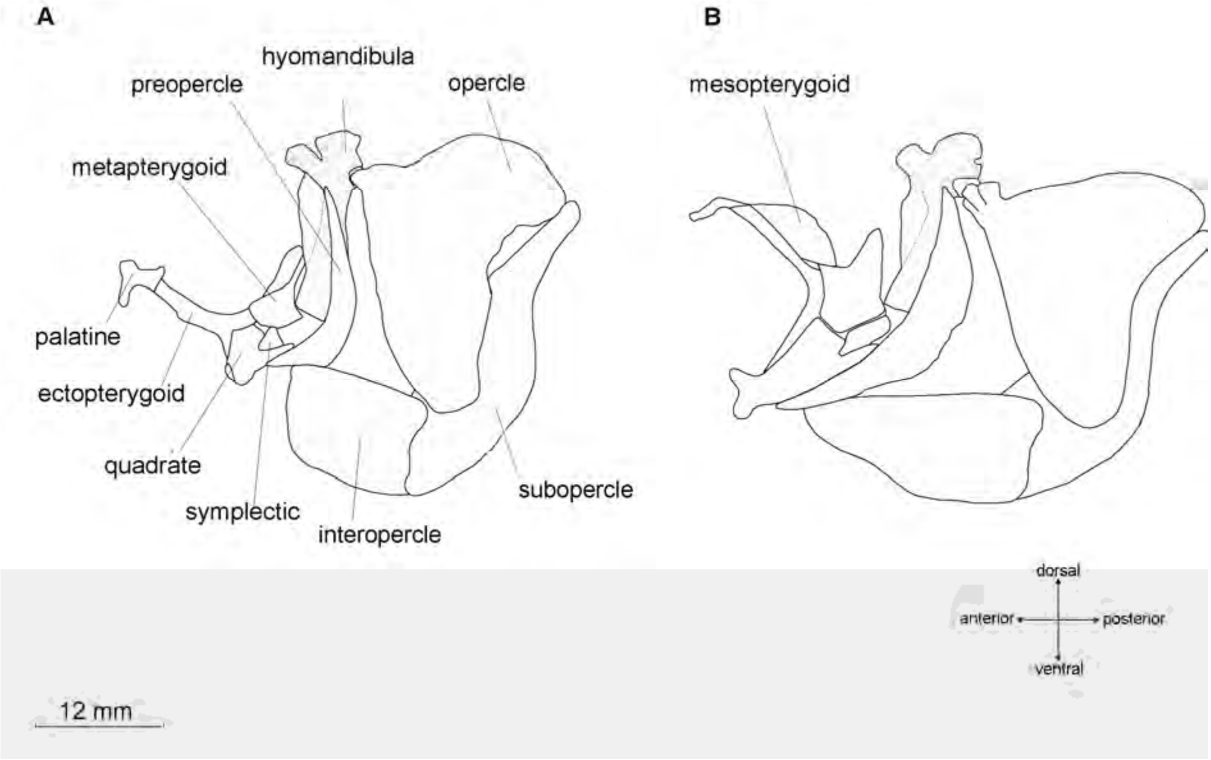


Figure 16: Camera lucida drawings of a lateral view of the suspensorium and opercular bones of *Dagetichthys marginatus*: (A) lateral view of the eyed side and (B) lateral view of the blind side. Note that bones from the eyed side have been reversed horizontally to facilitate comparisons with the drawing of the blind side and ventral drawings.

The hyomandibula appeared similar in shape and size on both sides of the head and carried two well-defined cranial articulary processes at its dorsal end. The broader postero-dorsal process and the smaller antero-dorsal process mainly articulated with the prootic. An articulatory facet to which the opercle articulates was situated at the postero-ventral edge of the postero-dorsal process. The hyomandibula extended ventrally as a large arm bordering along the preopercle posteriorly.

The opercle appeared similar in shape and size on both sides of the head and articulated antero-dorsally with the hyomandibula. The postero-ventral margin overlapped the subopercle. The subopercle appeared similar in size and shape on either side of the head. Its anterior margin was overlain by the posterior end of the interopercle while its bar-like extension was overlain by the opercle posteriorly. The interopercle was situated ventrally and attached to the preopercle, the blind side interopercle was about one third larger compared to the eyed side counterpart. The preopercle on the blind side was larger compared to the one on the eyed side. This bone was curved anteriorly overlapping the postero-ventral arm of the hyomandibula and the postero-ventral arm of the quadrate.

The hyoid arch of the blind side and the eyed side showed no signs of asymmetry in size or shape (Fig. 17). The dorsal hypohyal was articulated with the ventral hypohyal, both of which abutted anteriorly with the curved ceratohyal. The long antero-ventral arm of the anterior ceratohyal abuts the postero-ventral margin of the ventral hypohyal .

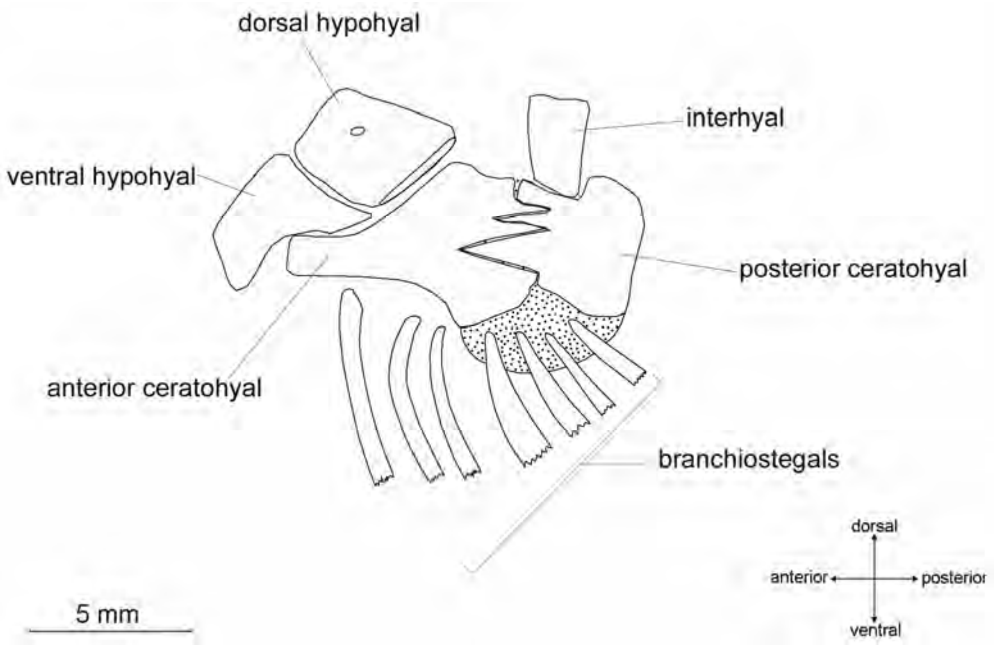


Figure 17: Camera lucida drawing of a lateral view of the hyoid arch.

The posterior end of the anterior ceratohyal interdigitated deeply with the posterior ceratohyal. The dorsal surface of the posterior ceratohyal provided the articulary facet where the ventral end of the interhyal articulated. This bone was trapezoidal and hollow with its postero-ventral end of the shorter side articulating with the posterior ceratohyal and the interopercle laterally. Dorsally the interhyal was linked to the median face of the cartilaginous structure found between the hyomandibula arm, the symplectic and the metapterygoid. Seven branchiostegal rays were situated posteriorly. Four (the posteriormost) articulated with the lower ceratohyal cartilage, while the three anteriormost were ligamentously linked to the antero-ventral arm of the anterior ceratohyal.

Discussion

The degree and type of cephalic morphological asymmetry varies considerably among flatfish taxa according to their feeding behaviour. Benthic feeding flatfish can generally be distinguished from mid-water feeders by the degree of jaw asymmetry (Yazdani 1969, de Groot 1971, Tsurata & Omori 1976). Mid-water feeders such as *Lepidorhombus whiffiagonis* (Scophtalmidae) or *Psettodes belcheri* (Psettodidae, Chapleau 1993), only exhibit morphological asymmetry directly related to eye migration (Yazdani 1969). *Psettodes belcheri* has symmetrical jaws with relatively large teeth and a big mouth well suited for feeding on free swimming prey (Yazdani 1969). In contrast, *Cynoglossus* sp., a member of the most derived family, the Cynoglossidae (Chapleau 1993), exhibits pronounced cephalic morphological asymmetry, which suggests to represent further modification of the head and jaws for benthic feeding behaviour (Yazdani 1969). Similarly, *D. marginatus* showed an extreme degree of cephalic morphological asymmetry among the elements of the anterior neurocranial region, the oral jaws and the suspensorium. Elements of the blind side were generally broader than those on the eyed side. The oral jaws on the blind side were covered with numerous inwardly curved teeth and seem well suited to capture polychaetes, as has been suggested for *S. solea* (Yazdani 1969). In contrast, elements of the oral jaws on the eyed side are edentulous. This has also been observed among other members of the Soleidae, including *S. solea*, and is suggested to be a further adaptation to benthic feeding where feeding is carried out exclusively by the jaw elements of the blind side, while respiration is performed by the edentulous elements of the eyed side (Yazdani 1969). Such a mechanism requires independent movement of the jaws which is brought about by the contraction of the levator operculi muscle. Consequently, the mandibulo-interopercular ligament would tighten and pull the ventral edge of the lower jaw ventrally and medially. If one half of the jaw is kept adducted, only medial movement would be possible. By rotation of the jaw about an axis through the quadrate articulation and the symphysis, the dorsal margin of the jaw would move laterally, rather like a proboscis. The convex shape of the dorsal margin of the blind side, the lower jaw allows the mouth to open much wider on the blind side (Van Dobben 1935, Yazdani 1969). The musculoskeletal system and the consequential jaw movements have not been considered in the present study. However, the absence of teeth on the eyed side oral jaws in *D. marginatus* suggests that respiration is conducted by the elements of the eyed side, while feeding is exclusively performed by elements of the blind side as has been suggested for other members of the Soleidae. Based on the morphology of the alimentary tract, De Groot (1971) concluded that *D. marginatus* (as *Synaptura marginata*) is a polychaete-mollusc feeder. This was confirmed by stomach content analyses (Thompson 2004) of adult *D. marginatus* in which the polychaete *Morphysa sanguine* is the dominant prey item (95.5%) (Thompson 2004). The present study strongly supports the above mentioned results.

Morphological asymmetry, i.e. the deflection of the parasphenoid, ethmoid and vomer, has the potential to create asymmetrical jaw movements of the upper and lower jaw as has been suggested for *Pleuronichthys verticalis* (Gibb 1995, 1996). Further, asymmetrical QM-joints (quadrate-mandible-joint) can create asymmetrical movement of the lower jaw. As a consequence, *P. verticalis* bends its jaws out of the midline down toward the blind side and produces a larger gape on the blind side during prey capture. It is hypothesised that this bending of the jaws towards the substratum allows individuals of this species to direct suction towards the prey item (Gibb 1997). Similar to *P. verticalis*, the parasphenoid and the vomer are deflected toward the blind side in *D. marginatus* and the QM-joint on the blind side is situated antero-ventral to the QM-joint on the eyed side. Based on this morphology, it could be suggested that similar asymmetrical kinematics take place during prey capture in *D. marginatus.*

Inertial suction feeding is the most widespread prey capture technique in fishes (e.g. Alexander 1969, Liem 1979, Lauder 1980b, 1980c, 1985, Lauder & Shaffer 1985, Lauder & Reilly 1988, Lauder & Prendergast 1992) and thought to be the basal feeding mechanism for benthic Pleuronectiformes such as *P. verticalis* (Gibb 1995). A rapid strike caused by suction may facilitate the prevention of tube-dwelling prey to retreat (Gibb 1997). Like *P. verticalis* or *S. solea*, *D. marginatus* mainly feeds on tube dwelling polychaetes, which suggests suction feeding to be the dominant prey capture method. However, further studies on prey capture kinematics are needed in order to validate this hypothesis.

CHAPTER 5

GENERAL DISCUSSION

Dagetichthys marginatus was identified as well suited for marine aquaculture in South Africa (Thompson 2004). First rearing trials were conducted during January and April 2005 with larvae obtained from hormone-induced spawning. Larvae could successfully be reared to the onset of metamorphosis with negligible mortalities and acceptable growth rates when fed on live food. Shortly after metamorphosis, when the early juveniles were weaned onto a formulated pelleted diet, total mortalities were recorded. Rearing technologies and methods were debated and a number of critical areas were identified that may have caused or contributed to the high mortalities. Firstly, nutritional properties of the pelleted diet may have been inadequate and/or the pellet was indigestible. Secondly, acceptability of the formulated weaning diet may have been reduced due to unsuitable chemical characteristics. Thirdly, physical characteristics of the formulated pelleted diet did not meet the requirements of the early juveniles. The present study on the cranial ontogeny of *D. marginatus* from larvae to post-metamorphic early juveniles was conducted to answer the question whether the feeding apparatus of the early juveniles is adequately developed to cope with a pelleted feed and to gain a better understanding of the feeding behaviour of this species. This in turn could facilitate the development of a suitable feed and feeding protocol and to facilitate further studies on feeding behaviour and feed development for this species.

During our rearing trial the larval *D. marginata* were first fed on live *Artemia* nauplii at 4 dah. Feeding of *Artemia* nauplii at first feeding is a standard rearing procedure for marine fish and flatfish larvae such as *S. solea* and *S. senegalensis* (Cañavate & Fernández-Díaz, 1999; Appelbaum, 1985; Fernández-Díaz *et al.*, 2001; Day *et al.*, 1996). This timing coincides with the depletion of the yolk sac, the mouth opening of the larvae and the onset of exogenous feeding (Thompson *et al*., in press). Larvae of *D. marginatus* rapidly approached the nauplii suggesting that ram feeding may have served as the first feeding mode. Observations revealed that the larvae successfully approached, captured and ingested the prey and *Artemia* nauplii were found in the digestive tract of sole larvae at 4 dah. This shows that nauplii were small enough to pass through the open mouth of the larvae and that the retention ability of the small branchial arches at 4 dah fulfilled the requirements of exogenous feeding. Although the parasphenoid only began to form 9 dah, larvae seemed capable of ingesting the prey without suffering damage to the brain from 4 dah. *Artemia* nauplii were fed to the larvae until 16 dah when larvae started to settle. During this period (4 – 16 dah) feeding attempts by the larvae became more successful and the time required to capture nauplii decreased. This improvement in prey capture ability could be explained by three concepts: 1) learning ability improvement; 2) increased swimming speed due to flexion of the notochord at 11 dah (Thompson *et al*., in press) (Blaxter 1988); 3) the integration of existing cranial elements allowing the larvae to switch to a suction feeding mode.

With the beginning of settlement at 16 dah the larvae also changed from pelagic to benthic feeding. At this stage the first morphological asymmetries began to develop among the elements of the oral jaws and at 22 dah early juveniles resembled the early juvenile stage. For example, teeth never appeared on the ocular side of the oral jaw elements, while those elements of the blind side were dentigerous. This morphological change suggests that feeding at this stage is already exclusively carried out by the elements of the blind side and fish then feed exclusively benthonicly. The development of teeth together with the onset of ossification of existing cranial elements facilitates grasping, which improves the fish’s ability to seize prey. Preliminary observations on the feeding behaviour of metamorphic sole have shown that the quantities of live (pelagic) *Artemia* metanauplii consumed decreased during this transition stage. Therefore, live *Artemia* nauplii where withdrawn at 16 dah and replaced by *Artemia* metanauplii and dead frozen *Artemia* nauplii from 16 dah until 35 dah. It was assumed that live *Artemia* metanauplii may constitute an alternative mobile prey to the settling larvae as they may not readily accept an inert food source such as dead frozen *Artemia* nauplii. Dead frozen *Artemia* nauplii on the other hand readily sink to the bottom and may therefore be preferred by the benthic feeding early juveniles. In addition, dead frozen *Artemia* nauplii provide an inert feed which may pre-condition the larvae for subsequent weaning onto a pelleted diet (Dinis *et al*. 1999). However, observations showed that once larvae settled, pelagic *Artemia* metanauplii were hardly consumed. Stomach content analyses of metamorphic sole may help to determine whether or not dead frozen *Artemia* and *Artemia* metanauplii are consumed by settled juveniles and at what quantities.

Reports on the successful onset of weaning in sole differ among authors. Appelbaum (1985) reared *S. solea* from first feeding to metamorphosis exclusively on inert diet. Gatesoupe & Luquet (1982) began weaning *S. vulgaris* at 10 dah, Fuchs (1979) at 25 dah, Person Le-Ruyet et al. (1980) at 30 – 40 dah and Bromley (1977) at 25 – 40 dah. Dinis (1992) started weaning *S. senegalensis* at 30 dah. Day *et al*. (1997) and Howell (1998) began to wean *S. solea* at a size of about 30 mm SL (50 – 60 dah). Flos *et al*. (1995) only started weaning *S. senegalensis* onto inert feed at 6 months. During the present study weaning onto an extruded pellet began at 25 dah. This timing was chosen for two reasons. Firstly, early juveniles had already settled, which allowed the use of a sinking pellet. Secondly, at 25 dah the cranial elements already resemble the sub-adult shape, the braincase was well separated from the buccal cavity and the splanchnocranium had begun to ossify. Whether or not the state of development of cranial structures involved in feeding at that stage limits the fish's ability to ingest and process a pelleted diet could not be answered, although clearly the ossification of these structures will be advantageous when having to feed on a pellet. However, studies on *S.solea* have shown that larvae could be successfully weaned onto a pelleted diet before metamorphosis and even from first feeding (Appelbaum 1985). According to Wagemans & Vandewalle (2001) cranial elements of *S. solea* only begin to ossify at 18 dah. This means that jaw elements may not necessarily have to be ossified before the larvae can feed on pellets.

The use of sinking pellets at the onset of weaning (25 dah) and thereafter was based on the assumption that metamorphic juvenile and subsequent stages display the same benthic feeding behaviour. The present study provides support for this assumption. The cranial morphology of early juveniles (22 dah) closely resembles that of adult fish, suggesting that once larvae start metamorphosis benthic feeding behaviour becomes established. This suggests that the use of a sinking pellet as applied in the current feeding protocol is adequate for *D. marginatus* at juvenile and adult stages.

To summarise, the use of *Artemia* nauplii as a first feed for *D.marginatus* up to 16 dah seemed adequate and resulted in good growth and survival rates. However, the use of *Artemia* metanauplii from 16 to 35 dah is probably inappropriate because at 16 dah the larvae begin to switch to a benthic feeding mode. The use of dead, frozen *Artemia* nauplii with the onset of benthic feeding behaviour seems suitable, however this needs to be tested. Finally, based on the cranial ontogeny and ossification of the cranial elements at 25 dah it can be suggested that the timing at which weaning was initiated and the use of a sinking pellet from 25 dah is appropriate.

To validate the functional kinematics predicted in this study, future studies need to be conducted using methods such as high-speed imaging and/or electromyography (Ferry-Graham & Lauder 2001, Ashley-Ross & Gillis 2002). In addition, and as mentioned previously, (see Chapter 1) the next step towards further improvement of the feeding protocol should include investigations of the behavioural responses towards food pellets of different size classes.

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