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“Ontogenetic shape change of the seahorse  
*Hippocampus reidi* with respect to dietary differences”

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Marius Koppler, BSc

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## Abstract

Habitat destruction and increasing fishing pressure have become the main threats to seahorses (genus *Hippocampus*). Consequently, these fishes were listed in CITES Appendix II in 2004, and increasing efforts for their aquacultural rearing have been undertaken.

The aim of this master's thesis was to study the early developmental stages (and concomitant shape changes) of *Hippocampus reidi*, bred in captivity on different diets. Furthermore, the suitability of geometric morphometrics to evaluate shape changes in seahorse fry was assessed. First feeding regimes (day 0 to day 20) consisted of *Artemia* sp. nauplii in experimental group I (group A) and a mix of rotifers (*Brachionus* sp.) and copepods (*Tisbe* sp.) in experimental group II (group M). From day 6 *Artemia* sp. nauplii were also introduced to the mixed plankton diet of group M. In addition, a starvation/hunger treatment (group S) was employed to evaluate growth patterns related to a lack of nutrients. Body development during the first 20 days after fry release was assessed through the measurement of key parameters (wet weight, body height) and geometric morphometrics (based on Procrustes shape coordinates of anatomical landmarks).

The experiments showed higher growth rates (wet weight, body height) in group M (fed with mixed plankton) and a significantly increased survival rate compared to group A (fed with *Artemia*). Principal component (PC) analysis of the seahorses' Procrustes shape coordinates displayed divergent allometric growth patterns in the head region and trunk region in the three groups. Detailed analysis of the trunk region revealed that PC 1 reflected the main developmental axis, whereas PC 2 reflected the nutritional status of the specimens. Analysis of the head region exhibited converged growth trajectories, but seahorses fed only with *Artemia* showed significant growth retardation compared to the group fed with mixed plankton. Moreover, a novel index (average trunk depth to trunk length) to assess condition in seahorse fry was established.

In summary, this study reveals that mixed plankton nutrition (even without artificial enrichment procedures) enhances growth rates and the healthy development of juvenile seahorses and should consequently be considered as the more appropriate food. Furthermore, geometric morphometrics proved to be very suitable to visualize subtle shape changes and thus give insight into the developmental and health status of *Hippocampus* sp. juveniles.



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## Table of Abbreviations

ANCOVA	analysis of covariance
CF	condition factor (based on standard length)
CF <sub>(HT)</sub>	condition factor (based on height)
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CS	centroid size
DHA	docosahexaenoic acid
DL	dorsal fin length
EPA	eicosapentaenoic acid
GBD	gas bubble disease
GM	geometric morphometrics
HL	head length
HT	height
HUFAs	highly unsaturated fatty acids
IUCN	International Union for Conservation of Nature
LM	landmark
NH <sub>3</sub>	ammonia
NO <sub>2</sub> <sup>-</sup>	nitrite ion
NO <sub>3</sub> <sup>-</sup>	nitrate ion
PC	principal component
PCA	principal component analysis
ppm	parts per million
PU	polyurethane
PVC	polyvinyl chloride
SD	snout depth
SnL	snout length
TCM	traditional Chinese medicine
TD	trunk depth average
TD-LT	trunk depth lower trunk
TD-SB	trunk depth at level of swim bladder
TrL	trunk length
TW	trunk width
v/v	volume fraction



## Introduction

Seahorses are very special marine fishes. These fish live in tropical and temperate seas and are well known for their horse-like shape. They use their tails as holdfasts and hide perfectly camouflaged in their shelter. Due to the absence of a caudal fin, seahorses are slow swimmers. Yet, they have one of the fastest feeding mechanisms known. In addition, seahorses display male parental care and have a distinct social behavior as they communicate via sound production and display complex mating rituals. In short, they are a rewarding study object.

Seahorses belong to the genus *Hippocampus* (order: Gasterosteiformes; family: Syngnathidae). The name of the family to which also the pipefishes, pipehorses and sea dragons belong, originates from the Greek “sýn” (together) and “gnáthos” (jaw), indicating that the group displays an elongated pipe-shaped mouth as characteristic (Lourie et al., 1999). The body of a seahorse consists of three main compartments: head, trunk and tail (Lourie, 2003). The head is bent and can be moved in a wide angle, the trunk is curved and the prehensile tail has no caudal fin (Lourie et al., 2004). Seahorses have no scales but an ossified skin. Bony plates appear as a series of ridges and rings (Ginsburg, 1937; cited in Lourie, 2003). Despite their extraordinary body shape seahorses are also known for their distinctive reproduction system. Adult males have a brood pouch posterior to the anal fin, a special compartment into which the eggs of the female are transferred and concealed until the offspring is released. At the point of release seahorse juveniles are well developed and look like a small adult. After exhibiting a planktonic life stage that lasts from a few weeks to several months (Foster and Vincent, 2004; Mai and Velasco, 2012) they become benthic and use their prehensile tail as a holdfast (usually in seagrass, coral and mangrove habitats). Seahorses and other syngnathids are known to employ pivot feeding (Muller, 1987; de Lussanet and Muller, 2007; Van Wassenbergh et al., 2009, 2014), waiting in ambush or slowly approaching until their prey is within striking distance. Then, in a rapid movement the head and snout are rotated cranially (Muller, 1987; Van Wassenbergh and Aerts, 2008). At the end of the dorsal head rotation the snout (and buccal cavity) is widened to suck in their prey (de Lussanet and Muller, 2007; Van Wassenbergh et al., 2013), which consists of crustaceans, other invertebrates and fish fry (Lourie et al., 1999; Foster and Vincent, 2004). This feeding strike is astonishingly fast (5ms in adult *H. reidi*; Van Wassenbergh et al., 2009) and enables seahorses to reach capture success rates of up to 94% (Gemmell et al., 2013). Average lifespans for seahorses are documented between 1.5 and 5 years, depending on the species (Lourie et al., 1999).

In general, the life history of seahorses (sparse distribution, low mobility, small home ranges, low fecundity, extensive paternal care and mate fidelity; Foster and Vincent, 2004) makes them vulnerable to overfishing, habitat destruction and pollution (Vincent, 1996; Foster and Vincent, 2004; Rosa, 2005). It is estimated that more than 20 million specimens (about 56 metric tons dry weight) are caught per year (Vincent, 1996). CITES (Convention on

International Trade in Endangered Species of Wild Fauna and Flora) data alone range from 6 to 10 million annually exported seahorses (wild caught) between 2004 and 2007 (Koldewey and Martin-Smith, 2010). Still demand exceeds supply (Vincent, 1996; FAO, 2010). Between 1990 and 1995 catch declines between 15 and 50% have been reported (Vincent, 1996). Consequently, all seahorse species have been listed in CITES appendix II (CITES, 2004, 2017). Currently the IUCN (International Union for Conservation of Nature) lists 33 seahorse species, of which 11 are labeled “vulnerable”, one “endangered”, one “least concern” and the rest “data deficient” (IUCN, Red List of Threatened Species, 2017). It is estimated that 95% of the global seahorse catches end up as remedies in traditional Chinese medicine (TCM), where dried specimens are thought to benefit respiratory disorders, sexual dysfunction, pain, human skin ailments, high cholesterol levels, excess throat phlegm, goitres, heart disease, lymph node disorders and incontinence (Vincent, 1996; FAO, 2010; Koldewey and Martin-Smith, 2010). The remaining quantity is destined for the aquarium and souvenir trade. While 57% of life-traded seahorses were already captive bred between 2004 and 2008, only about 0.02% of dried specimens happened to be aqua-cultured (Koldewey and Martin-Smith, 2010). Since aquaculture has not been able to produce enough quantities to meet TCM demand (Koldewey and Martin-Smith, 2010), other measures for conservation have to be introduced. Hence, no catch zones, management of fish trawling (Foster and Vincent, 2004) and minimum size limits have been discussed and in part implemented (Foster and Vincent, 2005). Still, the enhancement of seahorse aquaculture techniques to produce sufficient volumes at low cost remains challenging.

Over many years seahorses were considered to be among the most difficult marine fishes to breed (Vincent, 1996). This is annotated to their offspring's need for adequate live food, excellent water quality and their susceptibility to disease. Most *Hippocampus* species exhibit an extended planktonic life phase after they are released from the male's brood pouch (Foster and Vincent, 2004; Mai and Velasco, 2012). Within this life stage they feed on different planktonic species, mostly crustaceans. In captivity they keep this food preference and cannot be weaned to frozen food. Fry of other marine fish is often fed *Artemia* nauplii with good success. Still, many seahorse species have high mortality rates due to the postulated inadequate nutritional profile of their *Artemia* feed (Payne and Rippingale, 2000; Olivotto et al., 2011; Willadino et al., 2012). Consequently, many aquaculturists apply enrichment procedures to enhance the nutrient content of newly hatched brine shrimps. Mostly, commercially available artificial enrichment supplements based on lipid concentrates are used, while natural enrichment with microalgae is scarcely applied. Within *H. erectus* and *H. hippocampus* breeding, survival rates could be improved after wild caught plankton or enriched *Artemia* nauplii together with copepods were used as a starter diet (Gardner, 2004; Bentivegna, 2006). *Hippocampus abdominalis* fry has been successfully bred with enriched *Artemia* as first food

yielding up to 100% survival (Woods, 2003b). However, these findings may only be applicable for this species, due to the large size of its fry (16mm at brood pouch release; Foster and Vincent, 2004) and its capability to hunt and digest bigger food organisms like brine shrimp nauplii. Despite increasing success rates within the aquaculture of *Hippocampus*, a bottleneck for the alleviation of fishing pressure on wild stocks remains: cost effectiveness. Dried wild-caught seahorses are sold between 0.5 and 1.5 USD per piece (Koldewey and Martin-Smith, 2010). Unless seahorses can be produced within that price range, TCM will remain closed as a market place. Promising, however, are reports from Vietnam where *H. comes* is bred for restocking purposes and for the aquarium trade. It is emphasized that production costs to rear an 8 – 10 cm sized specimen for the live trade were about 1 USD in 2009 (FAO, 2010). For aquaculturists in the western world, however, producing at similar unit costs appears to be unfeasible. An additional challenge for the successful captive breeding of seahorses are diseases. Koldewey and Martin-Smith (2010) list the bacterial pathogens *Vibrio* spp. (Alcaide et al., 2001; Balcazar et al., 2010) and especially *Mycobacterium* spp. (Koldewey, 2005; LePage et al., 2015) of most concern. While vibriosis can be controlled with antibiotics, there is no effective treatment for mycobacteriosis (Koldewey and Martin-Smith, 2010). Another syndrome that can cause high mortality rates among juvenile seahorses is a form of gas bubble disease (GBD). Shortly after brood pouch release, excess gas is accumulated in the swim bladder and cannot be expelled or resorbed sufficiently (Woods, 2000; Lin et al., 2010; Manzini, 2010). Affected individuals become positively buoyant, drift towards the water surface and starve. Although hyperbaric treatment is suggested in some cases of gas bubble disease, usually aquaculturists only take preventive measures by keeping optimal culture conditions and hindering seahorses from swallowing excess air (Woods, 2000; Lin et al., 2010; Pawar et al., 2011).

The longsnout seahorse *Hippocampus reidi* is one of the most heavily exported seahorse species in terms of live trade (Koldewey and Martin-Smith, 2010). Furthermore, it is considered being a flagship species (Rosa et al., 2002) as it is one of the largest species, forming different impressive color morphs and thus is very popular in the aquarium trade (Mai and Velasco, 2012). The longsnout seahorse is labeled “data deficient” by the IUCN (IUCN, 2003). However, some authors reported catch declines: In Brazil, a country known to be a leading exporter of *H. reidi*, fishermen reported a decline in catch quota and a limited capacity to choose seahorses by color (Rosa, 2005; Rosa et al., 2005). Declines are thought to be connected to mangrove destruction, siltation, aquaculture ponds, the use of ichthyocides and overexploitation (Rosa et al., 2005).

It is estimated that about 25% of all CITES documented live trade is made up of *H. reidi* (Evanston et al., 2011). Fortunately, a considerable amount of live traded *H. reidi* are already produced in aquaculture facilities (Koldewey and Martin-Smith, 2010). Still, the breeding of the

longsnout seahorse remains challenging. Although captive held brood pairs frequently produce 200 to 1600 juveniles per clutch (Lourie et al., 1999; Vincent, 1990), a high juvenile mortality is common. Survival rates between 0 and 90% within the first 3 weeks after male brood pouch release are documented (Olivotto et al., 2008; Hora and Joyeux, 2009; Willadino et al., 2012; Pham and Lin, 2013). Besides keeping physico-chemical water parameters close to natural seawater levels, stocking densities (Woods, 2003a; Zhang et al., 2010) and nutrition (Payne and Rippingale, 2000; Olivotto et al., 2008; Otero-Ferrer et al., 2010) have the greatest influence on survival. Juvenile longsnout seahorses seem to require live food organisms like copepods and other zooplankton delivering essential nutrients for a healthy development. Rotifers, for instance, have been considered as a potential supplement in *H. reidi* first feed diets.

Although some studies evaluated the influence of nutrition on growth rates and survival in *H. reidi*, the effects of different feeds on external morphology during ontogeny are not well documented. Still, the quality of administered food is expected to influence morphological development, as nutrient derived skeletal- and organ malformations are known to occur in hatchery reared fish (Cahu et al., 2003 a,b; Planas et al., 2009; Roo et al., 2009). Planas et al. (2009) showed that increased levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in *Artemia* fed to *H. guttulatus* broodstock led to normal skeletal development during embryogenesis. Conversely, head deformations were frequent in juveniles originating from parents fed on *Artemia* without elevated DHA and EPA content. Otero-Ferrer et al. (2010) report significant alterations in snout and trunk length under different feeding regimes in *H. hippocampus* juveniles. Additionally, it is known that seahorses fed with brine shrimp as a single food source lack behind in the formation of the swim bladder complex (Schoell, 2013) and might be more prone to develop a hyper-inflated swim bladder (Abed-Navandi, personal communication, 2012; Manzini, 2010). Histological studies showed that the gas gland and *rete mirabile* were underdeveloped in *H. reidi* affected with this disease (Manzini, 2010). The author hypothesized that *Artemia* food might not supply juvenile seahorses with adequate nutrients to develop a functional swim bladder complex. If, for instance, excessive gas is accumulated in the swim bladder, it will have an effect on trunk shape (in particular trunk depth). Likewise, malnutrition is expected to have specific influence on morphological traits. Consequently, it can be assumed that a close monitoring of shape development allows to identify nutritional and health status. If this assumption proves true, early detection of shape changes that resemble malnutrition or disease could offer the possibility to counteract and thus help to increase overall survival rates in *H. reidi* culture.

Geometric morphometrics (GM) is frequently used to analyze shape variation of biological samples and appeared to be the most suitable approach for the assessment of subtle shape changes. In general, GM is based on the collection of two or three dimensional



coordinates of anatomical landmarks, mathematical elimination of non-shape variation (i.e. variation of position, orientation and scale) and subsequent statistical analysis and graphical visualization (Bookstein 1991, 1997; Adams et al., 2004; Mitteröcker and Gunz, 2009). The advantage of GM compared to traditional morphometrics (that is usually based on point-to-point measurements, angles and ratios) is that shape and especially outlines can be described in more detail (Adams et al., 2004; Verhaegen et al., 2007; Mitteröcker and Gunz, 2009). In addition, quantification and visualization of shape changes can be facilitated. Verhaegen et al. (2007) used a GM approach to evaluate opercular deformities in larval and juvenile gilthead sea bream. The authors concluded that to limit economic losses in farmed *Sparus aurata* GM might be a useful tool in the development of an early detection protocol. Leysen et al. (2011a) implemented the methodology to assess head shape development in *H. reidi*. Still, a detailed analysis of juvenile head and trunk development in the first weeks after brood pouch release has not been undertaken. Due to the occurrence of high mortality rates within the first three weeks of *H. reidi* culture this period appears to be very important for the assessment of juvenile status and health.

To evaluate the influence of nutrition on survival, growth and morphological development in juvenile *H. reidi* two nutritional regimes and one starvation control group were employed immediately after male brood pouch release up to the age of 20 days. The first group received *Artemia* nauplii and the second group received a mixed plankton diet starting with rotifers and copepods, including *Artemia* from day 6 onwards. Samples were taken at defined intervals (0.5 days, 1 day, 2 days, 3 days, 6 days, 10 days and 18 – 20 days). To minimize possible body deformations during fixation the specimens were anesthetized and fixed isoosmotically. To assess developmental shape changes and to bring insight into the seahorse's health status traditional and geometric morphometrics were employed. This approach was chosen to answer following questions:

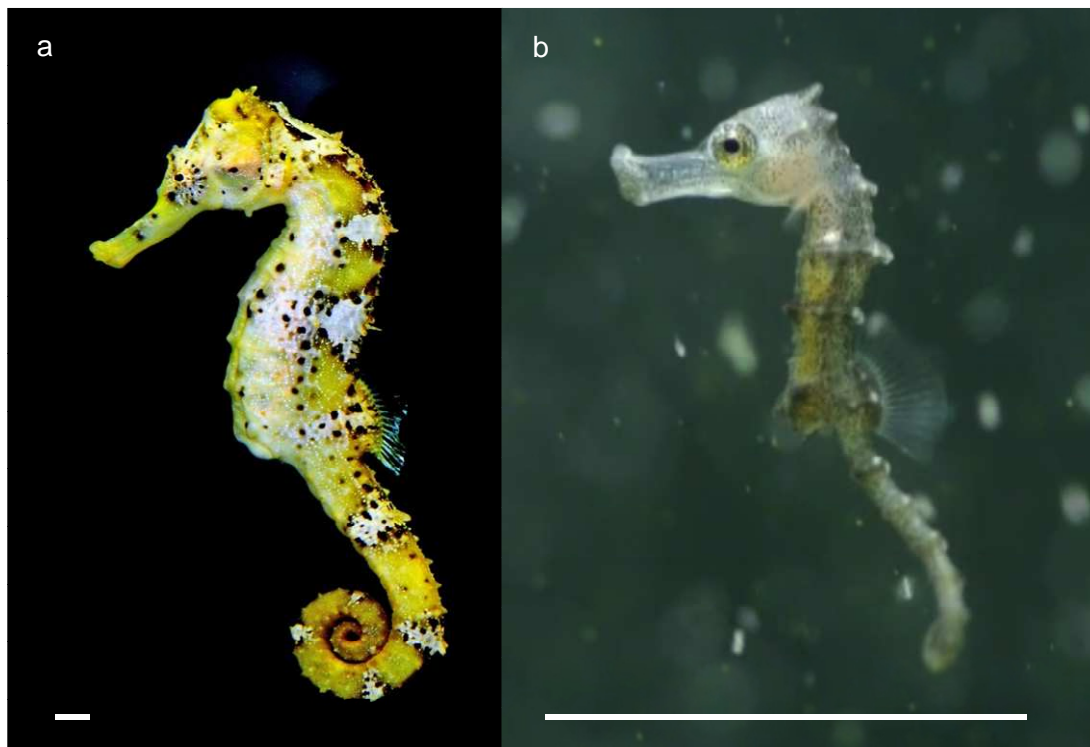
1. How do different first feed regimes affect the growth, shape and survival in *H. reidi*?
2. Does *Artemia* sp. used as single feed lead to a higher percentage of individuals affected with GBD/swim bladder hyperinflation?
3. Is it possible to detect subtle shape changes and thus evaluate nutritional status and health condition in juvenile *H. reidi* by means of geometric morphometrics?



## Materials and Methods

### Study species

*Hippocampus reidi* (Ginsburg, 1933) commonly named longsnout or slender seahorse (Fig. 1) occurs in the western Atlantic Ocean from Cape Hatteras (North Carolina, USA; Lourie et al., 1999) to the state of Rio Grande do Sul in Brasil (Rosa et al., 2005). This species was chosen as a study object, because it is considered being a flagship species (Rosa et al., 2002), sought after as an ornamental species (Mai and Velasco, 2012) and because of the high amount and frequency of offspring it produces in captivity.



**Fig. 1.** *Hippocampus reidi*. Adult (a; photo credit Hans Novak) and juvenile (b; photo credit Helmuth Goldammer). Scale bar indicates 5mm.

## Seahorse breeding

The juvenile seahorses were obtained from a public aquarium in Austria, the “Haus des Meeres” in Vienna. Three pairs of adult *Hippocampus reidi* seahorses (12 – 16 cm, 3 years, captive bred) were kept in a round 1.2 m deep polyethylene container. The offspring of one of those pairs was used in subsequent developmental studies. The fish were held in artificial sea water (salinity 33‰; Reef Crystals, Aquarium Systems Inc., Sarrebourg, France). The other physico-chemical properties were maintained at following levels: pH 8.2;  $\text{NO}_2^- < 0.05\text{ppm}$ ;  $\text{NO}_3^- < 10\text{ppm}$ ;  $\text{NH}_3 < 0.01\text{ppm}$ ; carbonate hardness  $6.5^\circ - 7^\circ \text{dH}$ . Biological filtration was accomplished with a porous glass medium (Siporax, Schott Inc., Mainz, Germany) in a canister filter and opencelled polyurethane (PU) foam. Artificial holdfasts in the containers were provided using polyvinyl chloride (PVC) tubing. Photoperiod was set at 11L:13D using fluorescent lights with a light intensity of 2000 Lux. Water temperature was maintained at  $24^\circ\text{C} \pm 1^\circ\text{C}$ . The animals were fed *ad libitum* twice a day with frozen mysid shrimps (Ruto Inc., Zevenhuizen, the Netherlands) and uneaten food and feces were removed twice a day. To identify the seahorses, fish were marked with necklaces of different color. A specific *Hippocampus reidi* breeding pair was chosen to provide offspring for the study. This pair reproduced every three to four weeks, resulting in 300 to 900 juveniles at each cast. Juveniles were collected, counted and transferred to 3 circular current breeding tanks (Kreisel tanks: Fig. 2) with a volume of 8 liter and a density of about 175 individuals per tank. One control group was held without food (group S), while two different feeding regimes were applied in two experimental groups. Group M was fed live zooplankton (*Brachionus plicatilis* and *Tisbe* sp.) *ad libitum* at an average concentration of 10, respectively 1 animal\* $\text{ml}^{-1}$ . Newly hatched brine shrimp nauplii (*Artemia* sp., Sanders Inc, Utah, USA) were included in their diet from day 6 to the end of the study. The second nutritional group (group A) was solely fed with *Artemia* sp. nauplii (twice a day *ad libitum*) from birth on.



**Fig. 2. Kreisel tanks.** Juvenile *H. reidi* cultured in a special rearing tank. A circular water flow is created with rising air bubbles on the left side of each tank (photo credit: Daniel Abed-Navandi).

## Sample collection and preparation

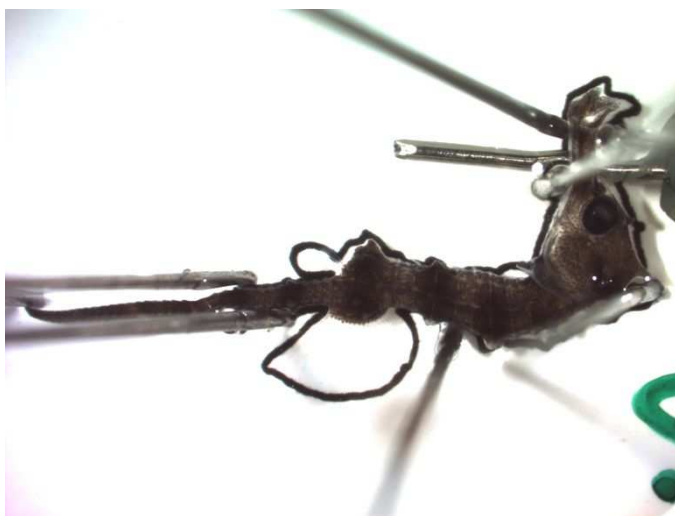
Juvenile seahorses were collected from each treatment group at defined intervals: 0.5 days, 1 day, 2 days, 3 days, 6 days, 10 days and 18 – 20 days post male brood pouch release, respectively. The first individuals were sampled right after their first swim bladder filling, but prior to their first food uptake (at 0.5 days). Sample size was 5 to 15 individuals per group and interval. To anesthetize the seahorses, clove oil at a final solution of 50 ppm was used. Due to the limited solubility of clove oil in water, a 10% stock solution in ethanol (v/v) was prepared. The amount of ethanol (500ppm in the final anesthetic solution) was used to improve solubility of clove oil in water and is considered harmless for fish within a limited period of time. Munday and Wilson (1997) showed that even a tenfold higher concentration of ethanol had no visible effect on damselfish (*Pomacentrus amboinensis*) within a 15 minute observation period.

After administering the anesthetic in 33‰ seawater, seahorse fry was anesthetized within half a minute, but the fish's active osmotic regulation collapsed and resulted in shrunken and deformed seahorse bodies within less than 2 minutes. Subsequently, all further clove oil dilutions in seawater were prepared to a final salinity of 12‰ at which no osmotic shock and subsequent deformations of the seahorse bodies were observed.

Different clove oil concentrations were tested and a 50ppm clove oil solution (in 12‰ artificial sea water medium) was found to be most efficient and additionally provided a moderate way of anesthetizing seahorse fry. Similar observations on the efficacy and usability of clove oil on fish species have been reported by other authors (Munday and Wilson, 1997; Otero-Ferrer, 2010).

Wet weight measurements were conducted with a fine scale immediately after anesthesia. Before weighing, excess water was removed by dabbing the specimens on each lateral side gently on a piece of paper. Fixation and preparation of the seahorses was done using insect pins, dental wax plates and shape templates. To keep the bodies in a standardized position 8 to 10 needles were gently pinned along the outline of the specimens that were placed on shape templates reinforced with underlying wax plates (Fig. 3). These templates were established in 5 different sizes. Finally, a solution of clove oil and 4% formalin was added on to the anesthetized seahorses. Fixation time was 3 hours in 4% formalin solution, followed by 2 hours fixation in 10% formalin solution (12‰ salinity). The hardened specimens were stored in 1.5ml airtight vials in 10% formalin solution. After the fixation process length measurements (Tab. 1 – 3) were performed with a digital caliper and by tpsDIG2 software (Rohlf, 2010a). This approach was chosen to minimize measuring artefacts originating from the high flexibility of a live seahorse body. Comparison of height measurements in live vs. fixed specimens (of a later brood of the same seahorse couple) showed a reduction of body height between 4.9% in the oldest and 10.5% in the youngest age group. Similarly, a reduction of body weight between

5.3% in 20 day old and 11.5% in the up to 1 day old seahorses could be observed due to fixation.



**Fig. 3. Fixation and preparation of a seahorse.** An anesthetized *H. reidi* specimen mounted on a shape template using insect pins to keep the body in a reproducible position. After positioning the specimen, a formalin solution was added for fixation.

**Tab. 1. Measuring definitions** (adapted from “Measuring seahorses”, Lourie, 2003; LM definitions: see Tab. 2 and 3).

Head length (HL)	distance between head-LM15 (snout tip, upper jaw) and mid-point of the cleithral ring
Snout length (SnL)	distance between head-LM15 and pre-orbital spine
Snout depth (SD)	the narrowest distance between the dorsal and ventral surfaces of the snout
Trunk length (TrL)	distance between mid-point of the cleithral ring and trunk-LM28 (straight-line measurement)
Trunk depth at level of swim bladder (TD-SB)	$(\text{distance LM3-LM24} + \text{distance LM5-LM22}) \cdot 0.5$
Trunk depth lower trunk (TD-LT)	distance between LM8 and LM19
Trunk depth average (TD)	$(\text{TD-SB} + \text{TD-LT}) \cdot 0.5$
Dorsal fin length (DL)	distance between LM10 and LM12
Height (HT)	distance between head-LM6 and tip of outstretched tail

## **Imaging and geometric morphometric analysis**

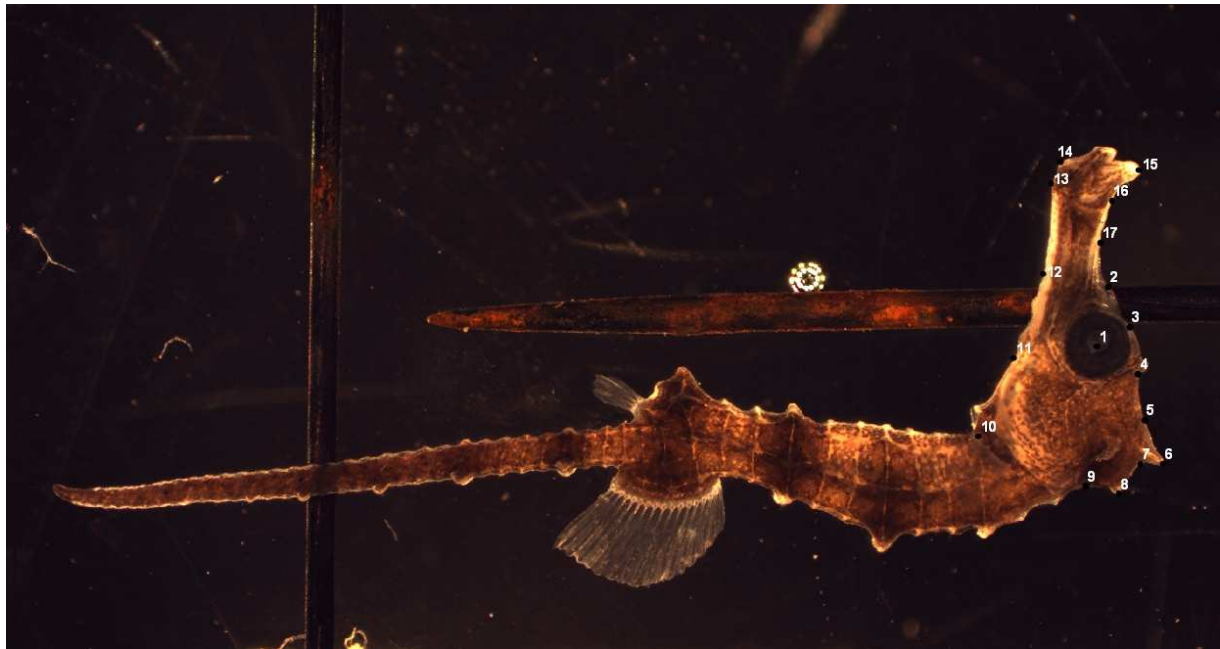
Production of images was performed using a Nikon SMZ 1500 Binocular microscope with an OPTOCAM-I microscope camera and OptoCapture 2.1 software. The pictures were taken from the left lateral side of the seahorses covered by a layer of water. To bring the samples in planar position insect pins were laterally placed under the snout and tail of the fishes (Fig. 4; Fig. 5).

The geometric morphometric analysis performed is based on the transformation of biological shape information into a Cartesian landmark coordinate model and subsequent statistical analysis. Landmarks (LMs) are defined “as homologous points bearing information on the geometry of biological forms” (Bookstein, 1991). In contrast semi-landmarks are landmarks on smooth surfaces or body outlines (that cannot be identified as homologous points) and are thus estimated (Mitteröcker and Gunz, 2009).

In total 212 images were digitized applying 17 LMs for the head analysis (Fig. 4; Tab. 2) and 29 LMs for the trunk analysis (Fig. 5; Tab. 3) using tpsUtil (Rohlf, 2010c) and tpsDIG2 (Rohlf, 2010a). To remove all variation that is not shape related (i.e., variation due to position, size and orientation of landmark configurations) a generalized Procrustes superimposition was performed. This involves a three-step calculation on the landmark configurations:

1. Translation: landmark configurations were aligned by their centroid (geometric center: coordinates are calculated from the arithmetic mean of all landmark coordinates).
2. Scaling: landmark configurations were scaled to an equal centroid size (CS):  $CS = \sqrt{\text{summed squared Euclidean distances from each point to the centroid}}$  (calculated by dividing the landmark coordinates by CS).
3. Rotation: landmark configurations were rotated until the squared differences between corresponding landmarks are minimized (iterative least squares fit procedure).

The generated Procrustes shape coordinates were statistically analyzed through a principal component analysis (PCA) in tpsRelw (Rohlf, 2010b) and corresponding scatter plots were produced in PAST, version 2.17 (Hammer et al., 2001).



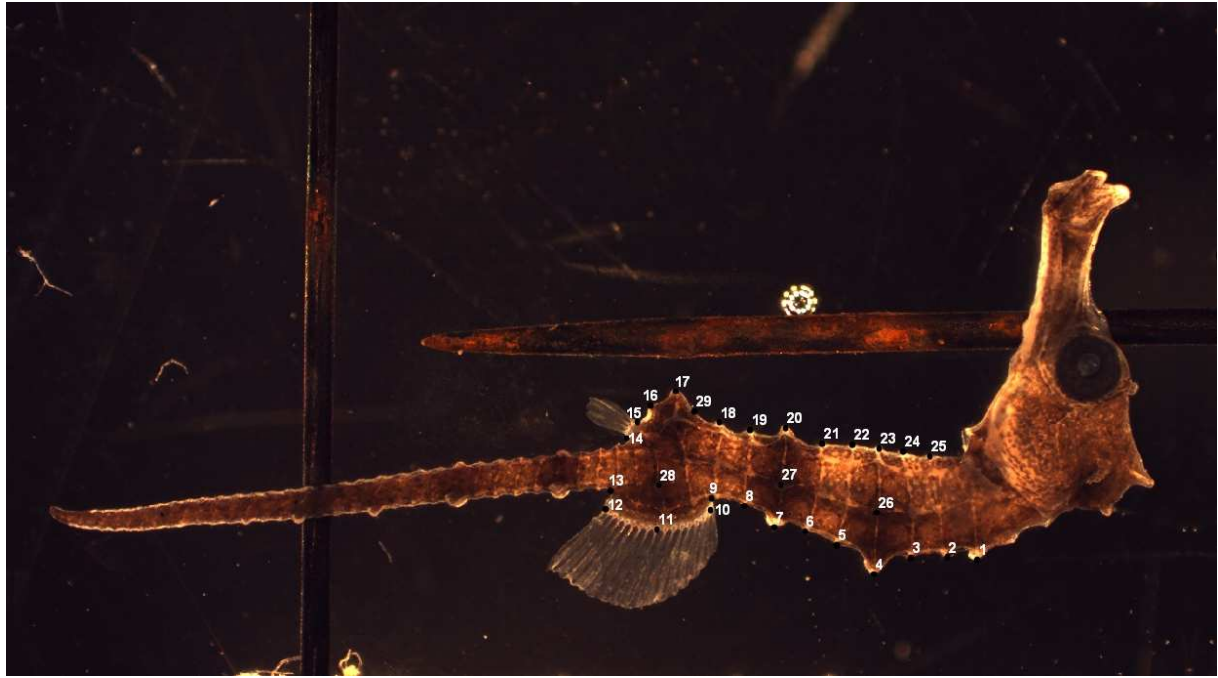
**Fig. 4. Landmark locations (head).** Lateral view of a 20 day old *H. reidi* showing the position of 17 landmarks set in the head region (insect pins were used to bring the samples into a planar position).

**Tab. 2.** Landmarks (LMs) and semi-LMs chosen for the analysis of the head region.

LM number	Definition
1	center of the orbit
2 – 5 and 16 + 17	sliding semi-LMs along dorsal head outline set equidistantly between LM6 and LM15
6	most terminal point of the prominent juvenile head spine at the supraoccipital region*
7	lowest point of the depression between LM6 and LM8
8	most terminal point of the protrusion at the first nuchal plate (coronet)
9	lowest point of the depression between LM8 and the protrusion at the second nuchal plate
10	ventral base of the pectoral fin
11 + 12	sliding semi-LMs along ventral head outline, set equidistantly between LM10 and LM13
13	most ventro-caudal point of the dentary bone (lower jaw)
14	most ventro-rostral point of the dentary bone
15	most dorsal point of the premaxillary bone (snout tip, upper jaw)

\*...supraoccipital region: Head shapes of the juveniles including spine formation undergo distinct changes within the 3 weeks of observation. It appears that during head ontogeny underlying bone, cartilage, tendon and muscle structures are altered significantly (Roos et al., 2009; Van Wassenberg et al., 2009; Leysen et al., 2011a,b). It can be hypothesized that LM6 that at first sight looks to be the developing coronet is actually just a prominent head spine most probably the pre-coronet spine that lies superior to the supraoccipital bone. Subsequently, it is assumed that the actual coronet will develop from the first head protrusion posterior to LM6.





**Fig. 5. Landmark locations (trunk).** Lateral view of a 20 day old *H. reidi* showing the position of 29 landmarks set in the trunk region (insect pins were used to bring the samples into a planar position).

**Tab. 3.** Landmarks (LMs) and semi-LMs chosen for the analysis of the trunk region.

LM number	Definition
1 – 8	most dorsal points at the level of corresponding trunk rings (trunk ring 1 to 8)
9	depression between dorsal tubercle of trunk ring 9 and the base of the first dorsal fin ray
10	base of the first dorsal fin ray
11	base of the ninth dorsal fin ray
12	base of the last dorsal fin ray
13	dorsal base of the tail
14	posterior base of the anal fin
15	anterior base of the anal fin
16 + 29	sliding semi-LMs equidistantly set along the ventral body outline between LMs 15 + 17 and LMs 17 + 18, respectively
17	tip of most ventral tubercle at trunk ring 10
18 – 25	most ventral points at the level of corresponding trunk rings (trunk rings 9 to 2)
26	junction of lateral trunk ridge and trunk ring 4
27	junction of lateral trunk ridge and trunk ring 7
28	median trunk spine of the last trunk ring



## Results

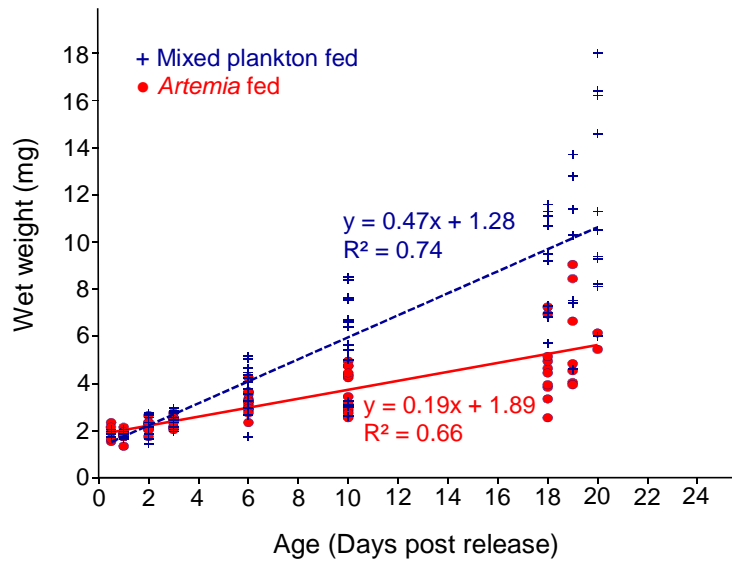
Seahorses of both nutritional groups showed active behavior and food uptake from their brood pouch release until the end of the observation period. No floaters could be observed. However, individuals of both groups showed signs of positive buoyancy after being transferred from the circular current tank to a holding container (where they were kept for later anesthesia). The affected seahorses seemed to have a marginally overfilled swim bladder as they slowly drifted towards the surface, as soon as they stopped swimming downwards.

### Survival rates

*Hippocampus reidi* juveniles exhibited very different survival rates between the two nutritional groups. The overall survival within the 20 day observation period in mixed plankton fed seahorses was 48.8% whereas survival in *Artemia* fed seahorses was only 4.7%. Compiled data of two broods of the same seahorse couple with a total offspring number of 1360 yielded an overall survival of 50.9% in group M while survival in group A was 2.4% after 20 days. About 30% of the seahorses in group M reached the adult state (about 4 months after brood pouch release).

### Weight increments

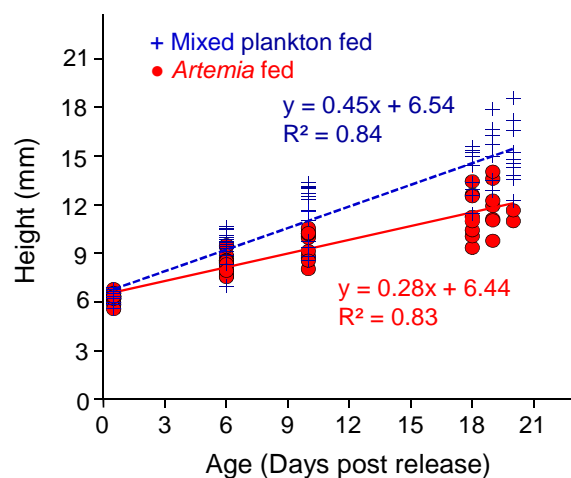
*Hippocampus reidi* fry averaged  $1.77 \pm 0.17$  mg (mean  $\pm$  standard deviation) in wet weight 4 hours after paternal brood pouch release (up to this point the seahorses did not receive any food). Until day 6 of the study weight increments showed no obvious difference between the two nutritional groups (Fig. 6). From day 6 onward weight variances were significant (F and T test;  $p < 0.05$ ). On day 6 *Artemia* (group A) fed seahorses had an average wet weight of  $3.25 \pm 0.44$  mg, whereas mix plankton (group M) fed seahorses weighed  $3.88 \pm 0.94$  mg. On day 10 group A showed an average weight of  $3.86 \pm 0.88$  mg and group M  $5.84 \pm 2.04$  mg. The final 3 weight measurements were taken on days 18, 19 and 20 of the study. *Hippocampus reidi* juveniles fed with *Artemia* nauplii reached  $5.33 \pm 1.73$  mg in weight (day 18 – 20: pooled values), whereas the mixed plankton fed group reached  $10.21 \pm 3.37$  mg (day 18 – 20: pooled values). Within-group variation of weight increased strongly at the end of the experiment. A one-way ANCOVA showed that the slopes and adjusted means differed significantly between the two feeding regimes.



**Fig. 6. Weight increments of *H. reidi* juveniles.** Data points and lines reflect two nutritional regimes: *Artemia* nauplii (red) and mixed plankton (blue) fed seahorses. Mix plankton fed seahorses display a considerably higher mean wet weight from day 6 onwards.

### Body height increments

Height data were collected after *H. reidi* juveniles were fixed in formol. From day 6 onwards height development was different between seahorses of the two nutritional groups (Fig. 7) also shown by significant inter-group variances (F and T test;  $p < 0.05$ ). Group A had a mean height of  $8.34 \pm 0.98$  mm, whereas group M had a mean height of  $9.49 \pm 1.88$  mm on day 6. The means of 10 day old individuals were  $9.57 \pm 1.92$  mm in group A and  $11.57 \pm 3.36$  in group M. The oldest specimen had means of  $11.53 \pm 2.58$  mm and  $14.77 \pm 3.44$  mm (group A and M, respectively). A one-way ANCOVA showed that the slopes and adjusted means differed significantly between the two feeding regimes.

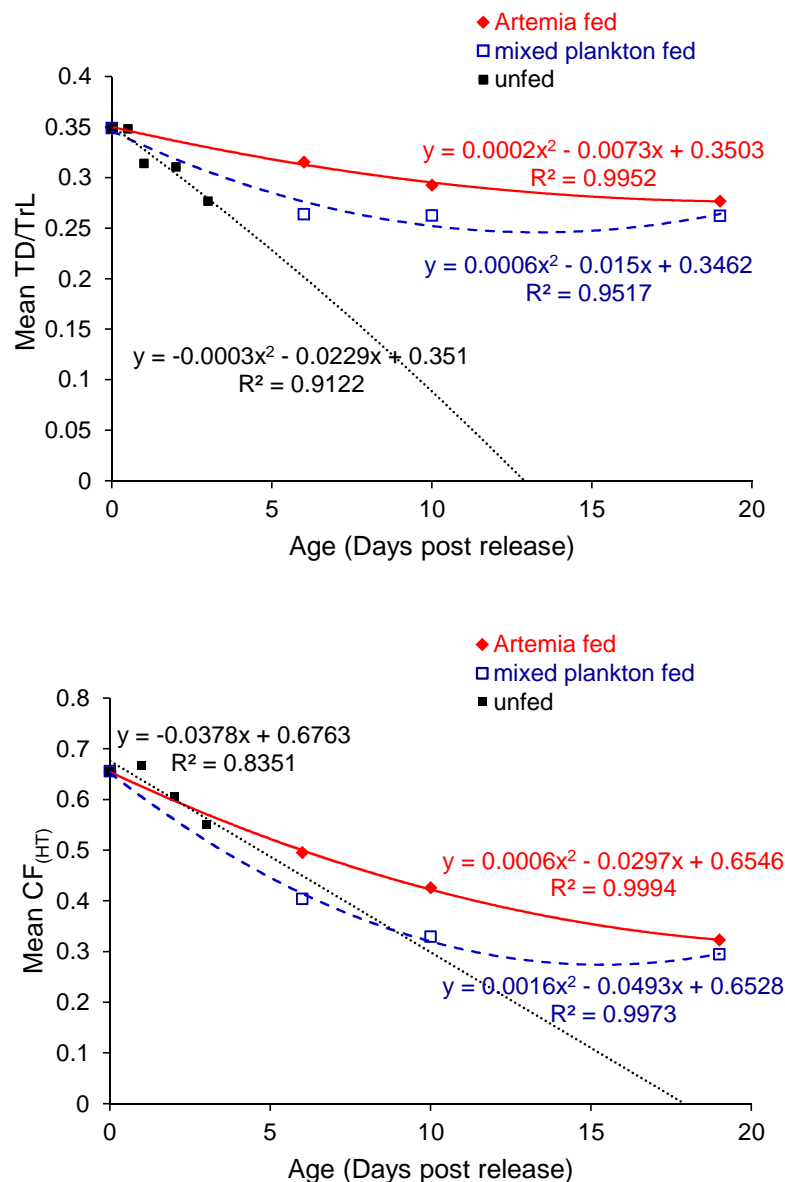


**Fig. 7. Height increments of *H. reidi* juveniles.** *Artemia* nauplii (red) and mixed plankton (blue) fed seahorses.

## Body shape and fitness: condition indices

To calculate the condition factor (CF) the seahorses height (Pham and Lin, 2013) and weight post fixation were used as:  $CF_{(HT)} = 100 \cdot [\text{mass(g)}/\text{height}^3(\text{cm}^3)]$ . In juvenile *H. reidi*,  $CF_{(HT)}$  decreased sharply from 0.66 in freshly released to 0.31 in 20 day old specimens showing a considerable range (Tab. 4). In comparison to the *Artemia*-fed seahorses, the condition factor in mixed plankton fed seahorses was smaller and displayed a greater decline during the observation period (Fig. 8).

Trunk depth was measured at the level of the swim bladder (TD-SB) and the lower trunk (TD-LT) to calculate an average trunk depth  $[TD = (TD-SB + TD-LT) \cdot 0.5]$ .



**Fig. 8. Condition factor (CF) and ratio average trunk depth (TD) to trunk length (TrL).** Data points reflect means of two nutritional regimes and an unfed control: *Artemia* fed (red), mixed plankton fed (blue) and unfed (black).

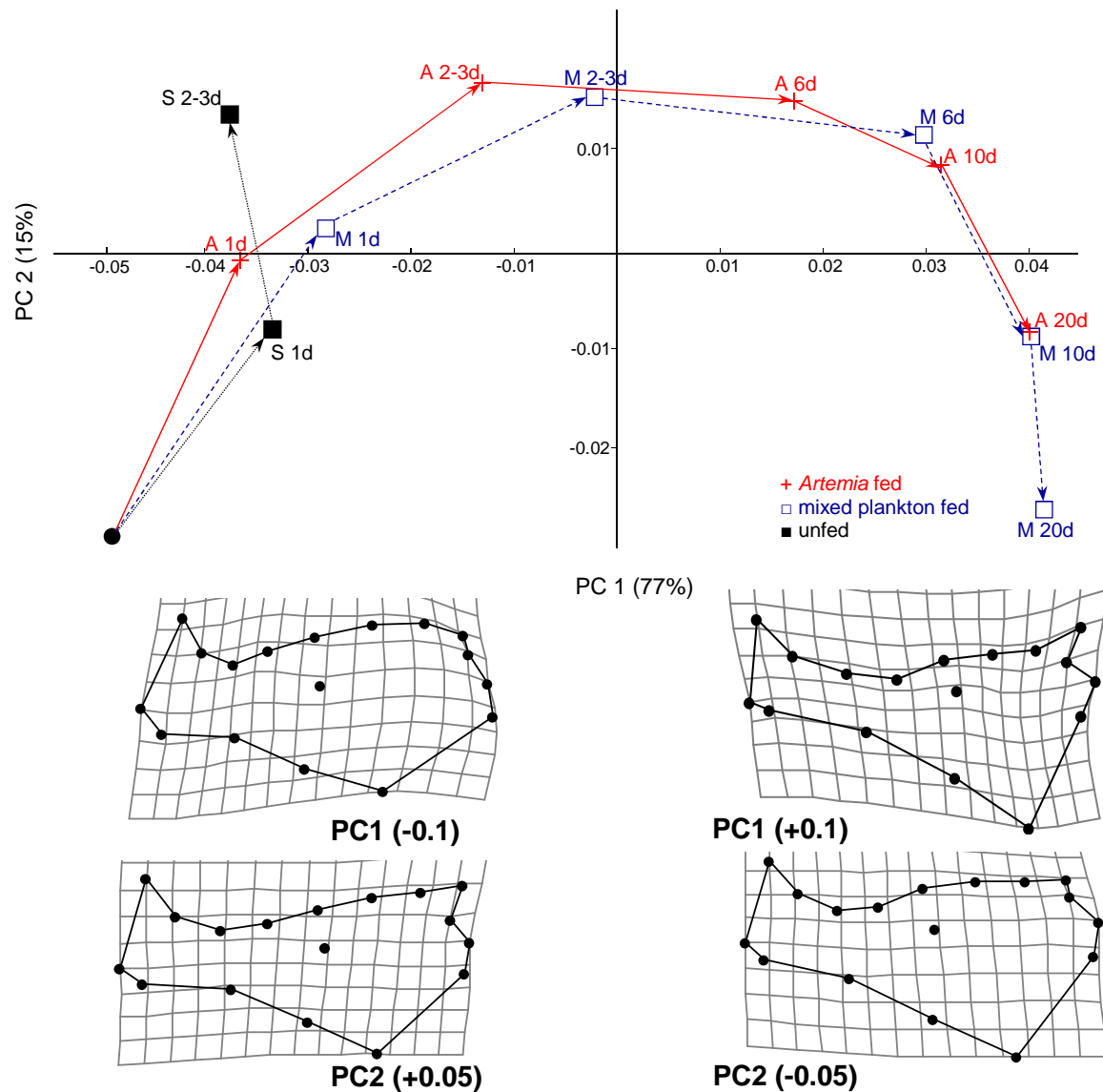
Similarly to  $CF_{(HT)}$ , the ratio of the average trunk depth (TD) to trunk length (TrL) showed a greater decline with age and was smaller in group M compared to group A (Fig. 8). The unfed control group showed the greatest drop in TD/TrL within the 3 days of observation. Within the mixed plankton fed specimens the TD/TrL-decline leveled off at 0.26 and the TD/TrL-means were constant from day 6 to day 20. *Artemia*-fed specimens displayed a mean TD/TrL of 0.32 at day 6, 0.29 at day 10 and 0.28 from day 18 to day 20. A maximum difference between the two dietary treatments was observed at day 6. Compared to  $CF_{(HT)}$ , the values of the TD/TrL-ratio had a narrower range (Tab. 4).

**Tab. 4.** Mean ( $\pm$  standard deviation)  $CF_{(HT)}$  and TD/TrL-ratio of juvenile *H. reidi* fed with *Artemia* sp. (A), mixed plankton (M) and unfed control (S).

Nutritional group   Age (days)	TD/TrL ( $\pm$ SD)	$CF_{(HT)}$ ( $\pm$ SD)
0	0.350 $\pm$ 0.039	0.656 $\pm$ 0.134
A 6	0.316 $\pm$ 0.012	0.496 $\pm$ 0.058
A 10	0.293 $\pm$ 0.019	0.426 $\pm$ 0.170
A 20	0.277 $\pm$ 0.026	0.323 $\pm$ 0.047
M 6	0.264 $\pm$ 0.009	0.404 $\pm$ 0.031
M 10	0.263 $\pm$ 0.013	0.330 $\pm$ 0.031
M 20	0.263 $\pm$ 0.016	0.295 $\pm$ 0.021
S 1	0.327 $\pm$ 0.024	0.667 $\pm$ 0.086
S 2	0.311 $\pm$ 0.030	0.607 $\pm$ 0.062
S 3	0.277 $\pm$ 0.022	0.550 $\pm$ 0.071

## Geometric morphometrics: Head analysis

The main between-group shape differences of the head region were described in both principal component 1 (PC1) and principal component 2 (PC2). PC1 contributed 77% and PC2 15% to total variance, respectively. The PCA scatter plot of the mean landmark configurations and the corresponding deformation grids depicted distinct changes in snout length and depth (Fig. 9). Furthermore an altered supraoccipital and coronet region could be observed. According to PC1 (the main axis of shape change with growth) snout length increased and snout depth decreased. In the medium aged group (6 to 10 days) the “pre-coronet spine” showed significant growth as depicted in the deformation grid of PC2 (Fig. 9).

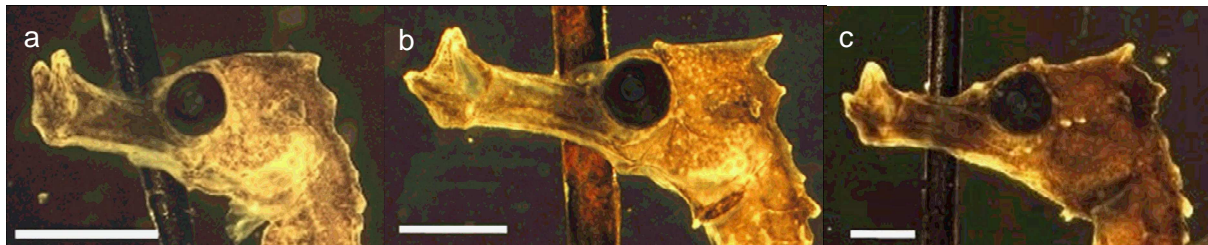


**Fig. 9. Principal component analysis of mean Procrustes shape coordinates (head).** Analysis is based on a set of 17 landmarks representing the head region of juvenile *H.reidi* at different ages (group M, blue arrows,  $n = 89$ ; group A, red arrows,  $n = 81$ ; group S, black arrows,  $n = 28$ ). Scatter plot: PC 1 (x-axis) PC 2 (y-axis). PC1 contributed 77% and PC2 15% to total variance. Shape changes are visualized as deformation grids of the respective PC1 and PC2 scores (values shown below each grid).

This shape change was also depicted via light microscopy (Fig. 10b; Fig. 13e and h). In the oldest age group an elevated coronet region was visualized via PC1 (Fig. 9). The size of the developing coronet increased corresponding with the age and feeding regime of the samples as 20 day old mixed plankton fed seahorses showed the most prominent coronet region.

### Effect of nutrition on head shape development

The mean head shape of group A at the age of 10 days is very similar with the mean head shape of group M at the age of 6 days. Likewise, the head shapes of group A at the age of 20 days do correspond with the head shapes of group M at the age of 10 days. Thus, as shown by the PCA, *Artemia* fed seahorses have the shape development as the mixed plankton fed seahorses, but lack behind in time (Fig. 9).



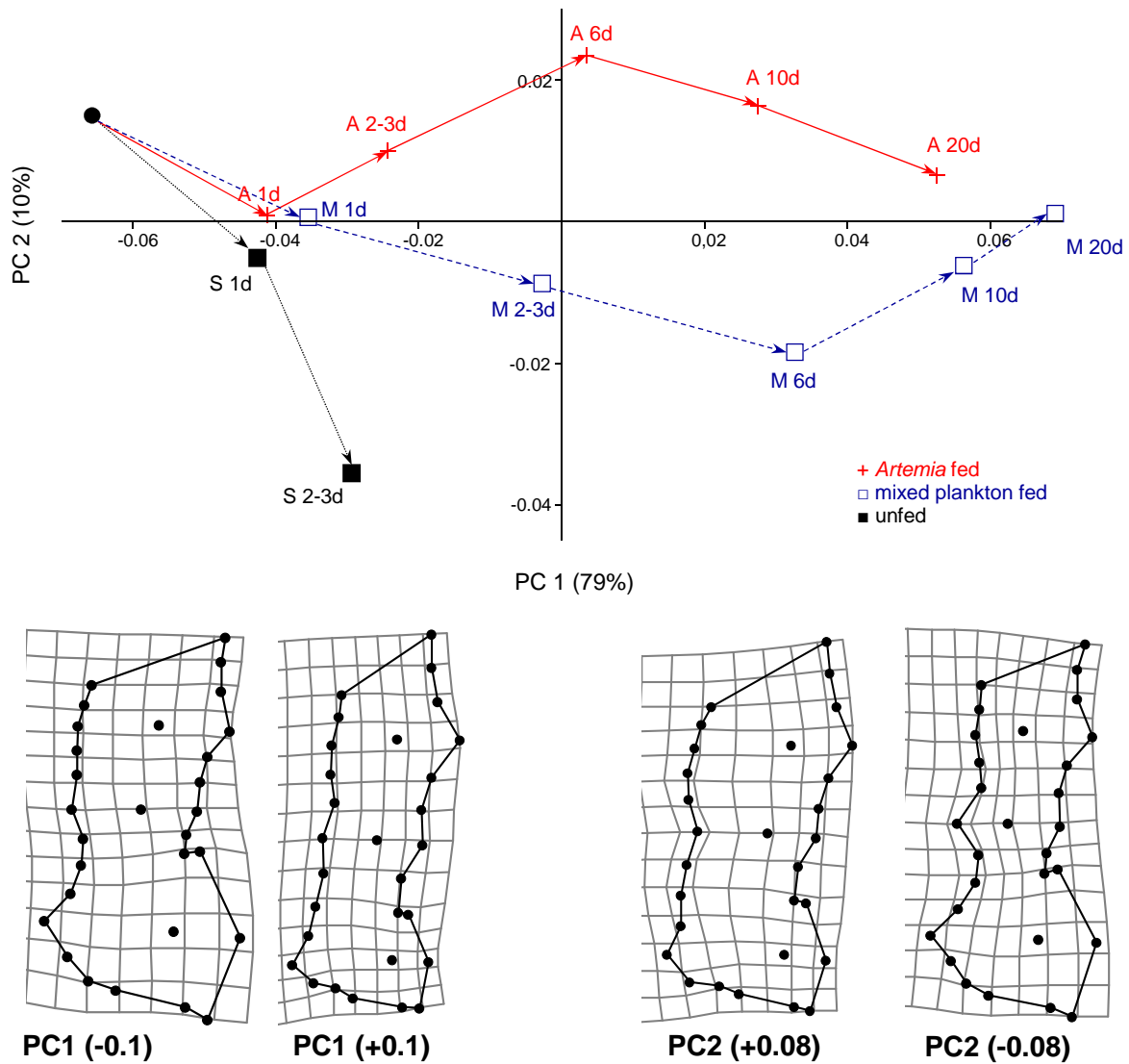
**Fig. 10. *Hippocampus reidi* head shape changes.** Typical growth patterns in juvenile *H. reidi*. Images show individuals from group M at the age of 0 (a), 10 (b) and 20 (c) days. Scale bar indicates 1mm.



## Geometric morphometrics: Trunk analysis

The main shape change in the trunk region was shown by PC1 and PC2 (79% and 10% of total variance respectively). Along PC1 the trunk shape becomes elongated, corresponding to the age of the seahorses, whereas trunk depth increases along PC2, as illustrated in figure 11. In addition, the length of the base of the dorsal fin related to overall trunk length decreased with age.

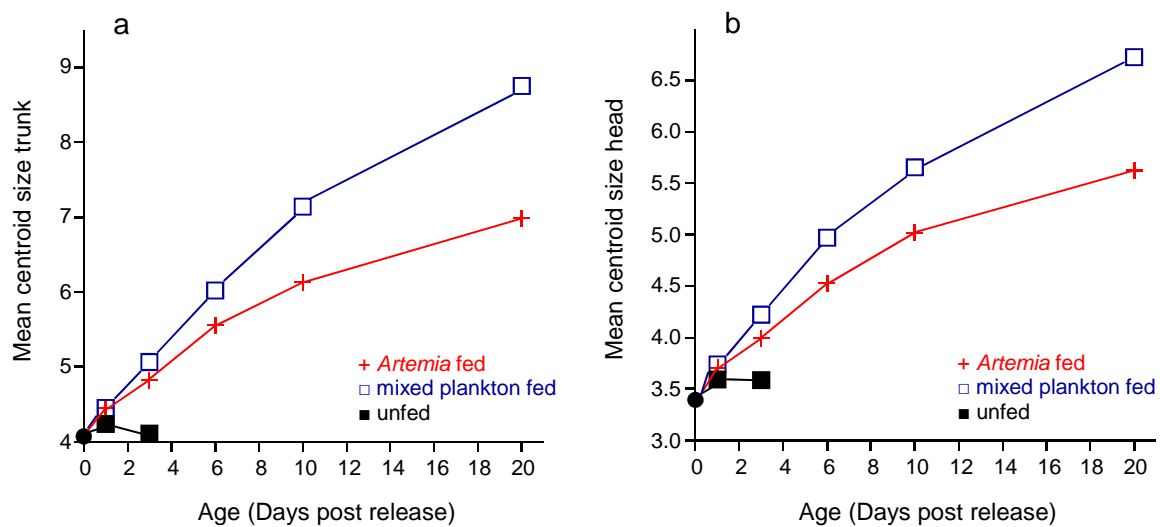
In contrast to the PCA of the head region, the PCA of the trunk region follows a different direction along PC2 in group A and group M, with a maximum difference at day 6. Compared to mixed plankton fed seahorses, *Artemia* fed individuals show a developmental pattern that differs in a greater trunk depth (both in the anterior and in the posterior part of the trunk) and a compressed dorsal fin.



**Fig. 11. Principal component analysis of mean Procrustes shape coordinates (trunk).** Analysis is based on a set of 29 landmarks representing the trunk region of juvenile seahorses. Scatter plot: PC 1 (79.3% of variance) against PC 2 (10.1% of variance). Shape changes are visualized as deformation grids of the respective PC1 and PC2 scores.

## Head and trunk growth

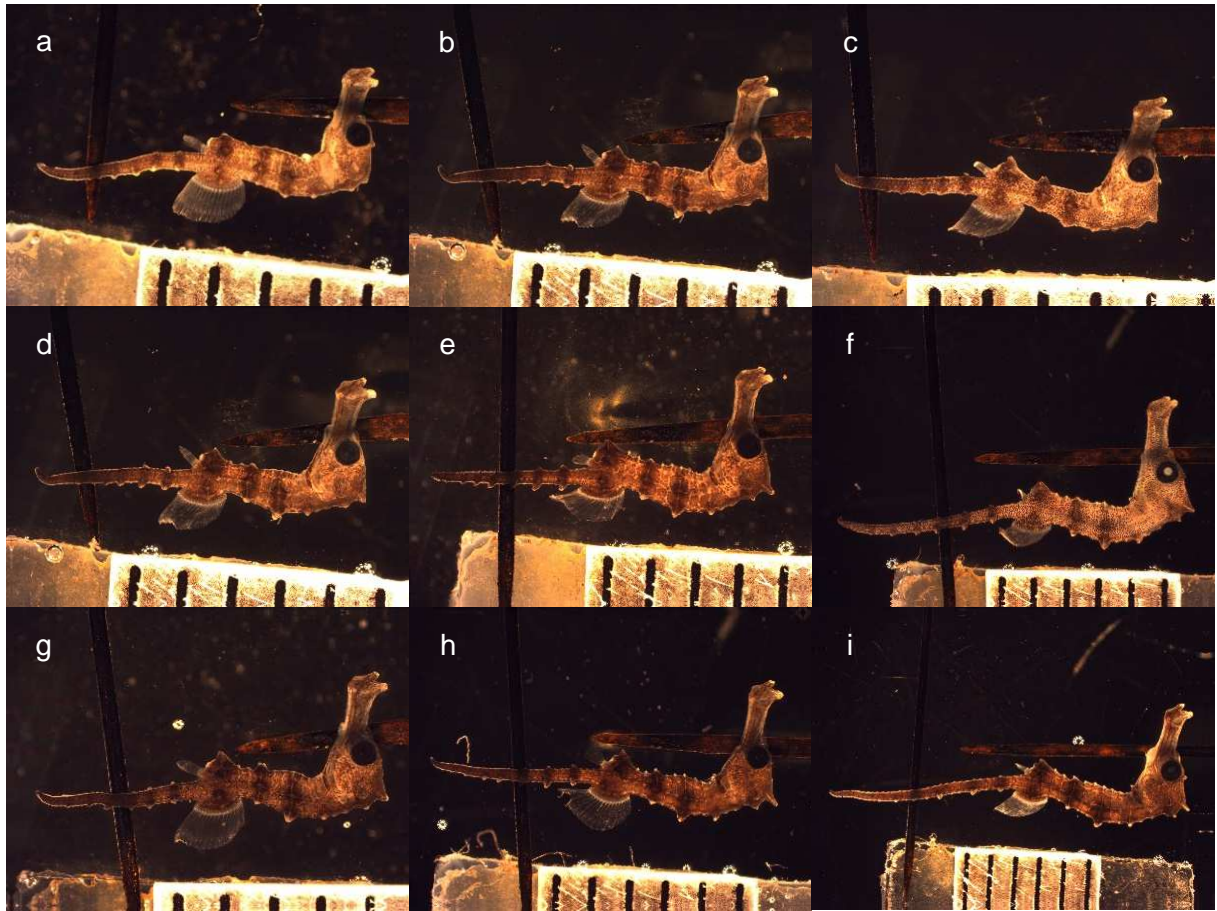
The mean centroid sizes of both head and trunk regions of *H. reidi* juveniles showed a clear food-dependent growth rate (Fig. 12). While centroid sizes of unfed seahorses were stagnant, centroid sizes of head and trunk of the two nutritional groups increased until the end of the measurements at day 20. From day 6 onwards mixed plankton fed specimens show higher centroid sizes compared to the *Artemia* fed group also correlating with the previously described weight data.



**Fig. 12. Development of centroid sizes in juvenile *H. reidi* in three different feeding groups. (a) trunk, and (b) head.**

## Qualitative and quantitative body shape changes

Besides shape data originating from geometric morphometrics analysis, a direct shape comparison via binocular microscope provided comparable results (Fig. 13). After brood pouch release seahorses exhibited a more compact body shape compared to grown individuals. The ratio head length to trunk length averaged 1.02 in the youngest specimens, 0.98 and 0.95 in the 6 to 10 day old individuals and 0.95 and 0.90 in the 18 to 20 day old individuals (of group A and M, respectively). Snout length in relation to head length was higher in the medium aged and oldest aged groups (HL/SnL:  $2.19 \pm 0.09$ ,  $n = 27$  and  $2.25 \pm 0.15$ ,  $n = 27$ , respectively) compared to the youngest group ( $2.66 \pm 0.21$ ,  $n = 14$ ). Alterations in the supraoccipital and coronet region (landmarks 6 to 8) became evident in the largest individuals of both nutritional groups, but being most pronounced in individuals of group M. In addition, a significant rostral shift of the developing cheek spine could be observed in the oldest age group.



**Fig. 13. *Hippocampus reidi* body shape changes.** Sequence of *Hippocampus reidi* pictures taken at different time intervals. Unfed seahorses at day 0, 2 and 3 (a – c); *Artemia* fed individuals at day 2, 6 and 18 (d – f); Mixed plankton fed individuals at day 2, 6 and 20 (g – i).



## Discussion

Within this study, the effects of two different feeding regimes - *Artemia* sp. as a single feed and mixed plankton plus *Artemia* sp. feed - on the growth patterns and survival of juvenile *H. reidi* were evaluated using traditional and geometric morphometrics. The use of mixed plankton resulted in higher survival rates and enhanced growth.

### Survival and growth

At the end of the 20 days of observation, mixed plankton fed individuals showed an average survival rate of 49% and a mean wet weight of 10 mg. In contrast, the *Artemia*-only feed group exhibited a lower survival rate and wet weight (5% and 5 mg, respectively). Within-group weight variations were higher than previously reported (Olivotto et al., 2008) and could be due to food competition at the used stocking densities of 20 individuals per liter. Some individuals of both nutritional groups had buoyancy problems due to a slightly overfilled gas bladder, still no casualties could be related to that condition. Thus, an increased occurrence of gas bubble disease due to *Artemia* as single feed could not be observed as described in *H. guttulatus* (Palma et al., 2013) and postulated in *H. reidi* (Manzini, 2010; Schoell, 2013).

The reported survival rates are similar to earlier studies (Olivotto et al. 2008; Willadino et al. 2012; Pham and Lin, 2013). Still the maximum weight and height attained after 20 days was lower in the present study. The explanation might be that the food was not artificially enriched and a far higher stocking density was used. Olivotto et al. (2008) used Algamac 2000 as enrichment for rotifer- and *Artemia* feed, together with an extended photoperiod to increase the time in which seahorses could feed. The copepods he used were fed with the micro-algae *Isochrysis galbana*. He reported a survival rate in *H. reidi* of 35% (within 21 days of culture). Willadino et al. (2012) used 5 different feeding protocols in a 14 day long *H. reidi* rearing trial, gaining the best results (33% survival) with a copepod- and DC DHA Selco enriched *Artemia* feed. Pham and Lin (2013) used rotifers and *Artemia* nauplii enriched with Dan's Feed (a blend of commercially available enrichment products, natural astaxanthin and plant extracts) in a 28-day feeding study culturing the offspring of two *H. reidi* couples achieving about 27% and 84% survival, depending on broodstock pair (captive-bred and live-caught, respectively). Hora and Joyeux (2009) reported excellent survival in *H. reidi* of 92.7% at 20 days of culture using wild caught plankton and Super Selco enriched *Artemia salina* as food sources. Wild caught plankton, however, is not available in many places and alternative food sources have to be used by most breeders.

The survival rate of 5% achieved in the *Artemia*-only feed group (group A) is also similar to previously published data (Willadino et al., 2012). Within *H. reidi* breeding it seems that specimens lack the ability to fully digest *Artemia* nauplii within the first few days after pouch

release (Abed-Navandi, personal communication, 2013) as undigested *Artemia* can be found in seahorse feces. Therefore the capacity for nutrient uptake appears to be limited. Together with the brine shrimps variability in nutrients and content in essential HUFAs (Léger et al, 1986; Payne and Ripplingale, 2000) even after enrichment procedures (McEvoy et al., 1995) the retardation in growth and lower survival in *Artemia*-only fed seahorses could be explained.

To this end - at least in *H. reidi* breeding - the use of rotifers and copepods within the first week of culture seems to be the best alternative to reach adequate survival rates. Even unenriched (not artificially enriched) plankton as shown in this study gives good survival and growth rates in the longsnout seahorse. If one takes into account, that high stocking densities (up to 20 times higher than in other studies) were employed, the survival rate can be considered excellent and thus be relevant for successful commercial aquaculture.

### **Head shape and growth**

A principal components analysis of landmark coordinates from samples of different age gave detailed information on developmental shape changes. Interestingly the head shape development was similar in both nutritional groups. Group A and M showed allometric growth patterns, that started with a compact head shape characterized through a thick, short snout and an only marginally developed head spination. Head spines started to grow 3 to 6 days after pouch release, which was especially seen in the spine above the supraoccipital bone. It is assumed that the development of the head (and body) spines could be an adaptation to the planktonic life stage and serve as defense mechanism against predators (Abed-Navandi, personal communication, 2013). Additionally, the snout started to elongate and became thinner. From day 6 (group M) and day 10 (group A) to the end of the study the main change happened in the coronet region. The tubercle forming the future coronet started to grow. In contrast it appeared that the spine above the supraoccipital became smaller (relatively to head length) and shifted rostrally. Although developmental patterns were similar, *Artemia*-fed specimens showed growth retardation. The mean head shape of group A at day 20 resembled the mean head shape of group M at day 10. This retardation was also reflected in the head size.

The snout (relatively to head length) elongated in medium aged (6-10 days) and older (20 days) individuals. Roos et al. (2010, 2011) found that suction performance (suction volume together with suction flow velocity) increased with decreasing snout length. Otherwise, a shorter snout takes more time to reach the prey by head rotation. Hence, the authors concluded that in seahorse ontogeny snout dimensions are always a trade-off between the maximum attainable suction volume on one side and the minimal time to reach the prey by head rotation. Therefore in the early ontogenetic phase, when seahorses live pelagic, it is assumed that a short snout increases the hunting success of nearby available and favored

food sources (i.e. evasive prey like copepods). When seahorses start their benthic life stage, they use their prehensile tails to grab holdfasts in sea grass beds or other sheltered surroundings (mangroves, corals etc.). In those habitats water turbulences are usually weaker. Consequently, evasive prey organisms would detect the seahorses much earlier. Therefore an elongated and narrow snout to minimize the distance towards their targeted prey and to reduce the hydrodynamic disturbances while approaching their prey is postulated favorable (Gemmell et al., 2013). As the juveniles grow and the snout elongates and seahorses start to use a kind of ambush tactics, a slightly longer snout seems to be advantageous. Gemmell et al. (2013) showed that in *Hippocampus zosterae* head and snout morphology allows approaching their prey with minimal hydrodynamic disturbance during a feeding strike. Although *H. zosterae* is smaller than *H. reidi*, ontogenetic changes in snout shape and the mechanisms of prey capture are comparable in both species. Thus, it appears that snout elongation in *H. reidi* helps to enhance capture success through reducing water deformations and the prey's subsequent ability to detect the predator and initiate a flight response.

### **Trunk shape and growth**

The divergence of the growth trajectories between the 2 nutritional regimes started at day 2, reached a maximum at day 6 and leveled off constantly until day 20, where the developmental trajectories led to more similar phenotypes again. A maximum relative trunk depth (TD) was found in group A around day 6 and a minimum relative TD in group M around day 6. Furthermore, mixed-plankton fed seahorses already displayed an elongated trunk around day 6 - a shape *Artemia*-fed individuals took 10 days to develop. Similarly the even more elongated shape of 20 day old individuals of group A corresponded to 10 day old individuals of group M. Again the growth retardation of *Artemia*-fed specimens was clearly seen in the PCA. Generally, the most pronounced TD alterations in juvenile *H. reidi* were displayed in the upper part of the trunk (TD-SB) and the lower part of the trunk (TD-LT). The main underlying anatomical structures of the anterior and posterior part are the swim bladder complex and intestine as can be seen in translucent juveniles. Consequently, relative TD in those areas seems to be originating primarily from the filling status of the swim bladder and/or the intestine.

Within the first 6 days of the study the rotifer and copepod diet seemed to give adequate nutrients for seahorse survival, but to some extent the shape of the trunk developed into a direction that resembled hypotrophy. This state was clearly displayed in individuals of the starvation group (as displayed in a very thin lower and upper trunk). Rotifers and copepods seem to be better digested than *Artemia* (Otero-Ferrer, 2010, and references therein; Blanco et al., 2013) and could therefore not expand the gut like undigested brine shrimp can do. When *Artemia* was added to the mixed plankton diet, the trunk depth increased and approached developmental trajectories displayed by the *Artemia*-fed group. Additionally it has been

reported, that brine shrimp as a single food source can increase the incidence of the gas bubble disease via a disturbed formation of the swim bladder complex (Manzini, 2010). Schöll (2013) analyzed the development of the swim bladder in juvenile *H. reidi* originating from the same brood stock as in the present study. She found that seahorses fed with mixed plankton showed substantial obliteration of their *ductus pneumaticus* at day 3 after brood pouch release. By contrast, same-aged *Artemia*-fed individuals showed no signs of obliteration. Even at day 4 the ductus was still present, although it already started to degenerate. Schöll's (2013) results correspond well with the growth retardation of the *Artemia*-fed group in the present study. Moreover, it is known that juvenile seahorses have the tendency to accidentally swallow air while hunting for food organisms (Woods, 2000; Pawar et al., 2011). It appears that the *ductus pneumaticus* is functional for a longer period in *Artemia*-fed specimens. This circumstance could lead to a higher filling level of the swim bladder and consequently a higher TD-SB.

It appears that trunk morphology gives insight into the developmental status and health of the juvenile seahorses. Zhang et al. (2011) published a possible indicator in the assessment of quality in *H. erectus* juveniles: The ratio trunk width (TW, measured at the half of trunk length) to trunk length (TrL), from which the authors deducted a 0.190 threshold for juvenile survival (5 day old individuals below that threshold showed zero survival on day 45 of the study). TW was not measured in this study, because the focus was laid on the areas of the trunk that undergo greater shape change during early development (initial inflation of the swim bladder, first food uptake etc.). Consequently, trunk depth was measured at the level of the swim bladder (TD-SB) and at the lower trunk (TD-LT) to calculate an average trunk depth [ $TD = (TD-SB + TD-LT) \cdot 0.5$ ]. This ratio corresponds with Zhang's (2011) TW to TrL ratio and with the condition factor, but shows less variation than the latter. Hence, the ratio TD to TrL could be proposed as an alternative indicator for the health of juveniles. Within *H. reidi* breeding, the establishment of a lower survival threshold (that indicates hypotrophy and possible starvation) and an upper threshold (indicating a possible overfilling of the swim bladder or gas bubble disease of the gut) appears promising.

In the current study mix-plankton fed specimens showed the highest survival and moderate TD/TrL-values compared to the *Artemia*-fed and the unfed seahorses. Mean TD/TrL-values of 0.26 reflected a healthy development in 6 to 20 day old *H. reidi*. Both, higher and lower TD/TrL-ratios (*Artemia*-fed and hypotrophic seahorses, respectively) appeared to negatively influence survival. Given the higher range of the condition factor  $CF_{(HT)}$ , observed in the present study, it has been concluded that the ratio TD to TrL is a more consistent indicator of juvenile health when size and weight variations are high.



### **Innovative fixation technique for comparability and reproducibility**

Body deformations due to fixation artefacts were one of the main methodical challenges in the current study. Fixation artefacts, like contraction of the trunk, bending of the head or variances in the size of the snout gape, were most pronounced in newly released individuals. Therefore a method was developed, that reduced fixation artefacts to a minimum. This was accomplished by using an isosmotic clove oil solution for anesthesia and shape templates together with insect pins during the fixation process. As a result, the seahorses were preserved in a standardized posture (head at right angles towards trunk and outstretched tail, as recommended by Lourie, 2003). Still the height of the seahorses decreased by 9.6% in the up to 10 day old and by 4.9% in the 20 day old individuals during the fixation process.

Apparently other studies on juvenile seahorses did not mention the possible occurrence of fixation artefacts and subsequent measuring inaccuracy. Choo and Liew (2006) for example describe allometric growth patterns in *H. kuda* and are cited within other developmental studies (Leysen et al., 2011a; Roos et al., 2010). However, Choo and Liew (2006) fixed their juvenile seahorses in 70% ethanol prior to subsequent measurements whereas Leysen et al. (2011a) and Roos et al. (2010) used neutralized buffered formalin. It has to be expected that samples suffered from “fixation-specific” shape changes (Nadeau et al., 2009; Fox, 1996, cited in Martinez et al., 2013) that are likely to be more pronounced in the youngest specimens (calcification of the bony plates is not well advanced at early life stages). Therefore, the comparability of these studies appears to be limited unless preservation related shrinkage (Nadeau et al., 2009) and possible shape changes are also quantified.

Consequently, future studies should consider the use of equal fixation and preparation methods to increase accuracy and comparability. A suitable fixation method is suggested above.



## Conclusion

This study revealed that the use of unenriched rotifers and copepods in the first 6 days after brood pouch release followed by *Artemia* sp. nauplii as an additional food source proved to be an adequate nutrition for *Hippocampus reidi*. First feed regimes on *Artemia* sp. as a single food source delayed seahorse development and reduced survival severely. Thus, the use of mixed plankton as a starter diet seems to act as a “kick-starter” for juvenile seahorse development. The mediated developmental boost within the first 6 days of culture, compared to the *Artemia* single feed diet, shows an ongoing effect in terms of weight and size gain until the end of the study.

Despite previous recommendations in seahorse aquaculture to keep stocking densities at 1 individual per liter (Zhang et al., 2010; Pham and Lin, 2013), survival rates of up to 50% have been achieved at stocking densities of 20 individuals per liter within the present study. Still size and weight variations are higher compared to other studies (this could be a result of food competition at these high stocking densities).

Besides traditional morphometric analysis, geometric morphometrics (GM) was applied to evaluate developmental trajectories and health status in *H. reidi*. It was discovered that standard morphometric methods, like calculation of the condition factor, might not be sufficient in the assessment of juvenile health if weight and height variations are high. Alternatively, the use of another ratio: average trunk depth (TD) to trunk length (TrL), was found to better reflect the developmental status (due to lower variance).

GM-analysis gives high-resolution information on shape variation. Even the nutritional differences and health status of juveniles can be assessed through interpretation of PCA scatter plots and thin plate spline deformation grids. Still, interpretation of the PCA plots and deformation grids have to be made carefully keeping in mind that intraspecific variation (i.e. offspring of different breeding pairs) has to be considered. However, GM-analysis following a meticulous preparation, fixation and landmark data acquisition proved to be a powerful tool in the assessment of developmental trajectories in *H. reidi* and could be further applied not only in developmental studies, but possibly also in the aquaculture of the genus *Hippocampus*.

To minimize fixation artefacts, an isosmotic formol solution was used in the present study. During fixation, templates and insect pins were employed to keep the samples in the intended position. For future studies, it is recommended to use isosmotic fixation and further develop methods to limit fixation artefacts and increase comparability.



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### Photo credits

Fig.1a: Novak H., (2012). Adult *Hippocampus reidi*. Haus des Meeres, Vienna.

Fig.1b: Goldammer H., (2013). Juvenile *Hippocampus reidi*. Haus des Meeres, Vienna.

Fig. 2: Abed-Navandi D., (2012). Kreisel tanks. Haus des Meeres, Vienna.

## Zusammenfassung

Die Zerstörung von Lebensräumen und zunehmender Fischereidruck sind zur Hauptbedrohung für Seepferdchen (Gattung *Hippocampus*) geworden. Daher wurden diese besonderen marinen Fische im Jahr 2004 in den CITES Anhang II aufgenommen und verstärkte Bemühungen zu deren Aquakultur unternommen.

Ziel dieser Arbeit war es, die frühen Entwicklungsstadien (und begleitende Gestaltveränderungen) von *Hippocampus reidi* Nachzuchten unter verschiedenen Ernährungsbedingungen zu untersuchen. Des Weiteren wurde die Eignung von geometrischer Morphometrie zur Beurteilung von Formveränderungen der Seepferdchenbrut überprüft.

Das Startfutter (Tag 0 bis Tag 20) bestand aus *Artemia* sp.-Nauplien in der ersten Testgruppe (Gruppe A) und aus einer Mischung aus Rotiferen (*Brachionus* sp.) und Copepoden (*Tisbe* sp.) in der zweiten Testgruppe (Gruppe M). Ab Tag 6 wurden der Gruppe M in die Mischplanktondiät ebenfalls *Artemia* sp.-Nauplien beigemischt. Zusätzlich wurde eine Hungerkontrollgruppe (Gruppe S) durchgeführt, um nahrungsmangelinduzierte Wachstumsmuster evaluieren zu können. Die Entwicklung während der ersten 20 Tage nach Freisetzung der Fischbrut wurde durch die Messung von Schlüsselparametern (Lebendgewicht, Körperhöhe) und geometrischer Morphometrie (basierend auf Prokrustes-Gestaltkoordinaten anatomischer Landmarken) beurteilt.

Die Versuche zeigten höhere Wachstumsraten (Lebendgewicht, Körperhöhe) in Gruppe M (gefüttert mit Mischplankton) und eine signifikant erhöhte Überlebensrate verglichen mit Gruppe A (gefüttert mit *Artemia*). Eine Hauptkomponentenanalyse der Landmarkenkoordinaten der Seepferdchen ergab divergente allometrische Wachstumsmuster im Rumpf- und Kopfbereich der Testgruppen. Eine Detailanalyse der Rumpfregion zeigte, dass die erste Hauptkomponente die hauptsächliche Entwicklungsachse und die zweite Hauptkomponente den Ernährungszustand der Testexemplare beschreibt. Die Auswertung der Kopfregion wies konvergente Wachstumstrajektorien auf; allerdings zeigten die mit *Artemia* als Einzelfutter ernährten Seepferdchen eine deutliche Wachstumsverzögerung gegenüber der mit Mischplankton gefütterten Gruppe. Um den Entwicklungsverlauf der Seepferdchenbrut zu beurteilen, wurde zudem ein neuer Index (Rumpfdicke zu Rumpflänge) etabliert.

Zusammenfassend erschließt sich aus dieser Studie, dass eine Ernährung aus Mischplankton (auch ohne künstliche Anreicherungsprozeduren) die Wachstumsraten und eine gesunde Entwicklung juveniler Seepferdchen fördert und daher als das geeignetere Futter anzusehen ist. Zudem erweist sich die geometrische Morphometrie als gute Methode, kleinste Gestaltveränderungen darzustellen und folglich Einblick in den Entwicklungs- und Gesundheitszustand juveniler *Hippocampus* sp. zu geben.