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Corresponding Author: Dr. Miguel Planas, PhD

Corresponding Author's Institution: Instituto de Investigacions Marinas (CSIC)

First Author: Miguel Planas, PhD

Order of Authors: Miguel Planas, PhD; Miguel Planas, PhD; Alexandro Chamorro, Biologist; Patricia Quintas, Dr Biology; Antonio Vilar, Biologist

Abstract: Knowledge on seahorses is generally scarce but has been increasing in recent years due to their conservation status. Seahorse culture is a quite recent activity in the field of marine aquaculture in most countries attempting it, and captive breeding techniques are available only for some species. With the aim of contributing to the development of breeding in captivity for conservative purposes, broodstocks of the European long-snouted seahorse (Hippocampus guttulatus) were established with 32 wild seahorses captured in Galicia (NW Spain). This study describes the methodologies applied to the maintenance of the broodstocks, with special reference to aquaria design, feeding, growth and breeding. Procedures of seahorse identification (morphologically and genetically) as a tool for broodstock management are also considered. The results achieved during the first year demonstrate a rapid adaptation of wild seahorses to captive conditions. Seahorses were fed exclusively on enriched adult Artemia and displayed high growth rates. However, fatty acid analyses performed on unfertilised eggs of captive broodstock showed a progressive decrease with time in the content of essential fatty acids (DHA, EPA), suggesting the need for improvement in the nutritional quality of broodstock feed.

Establishment and maintenance of threatened long-snouted seahorse, *Hippocampus guttulatus*, broodstock in captivity

Miquel Planas[a,*], Alexandro Chamorro[a], Patricia Quintas[a] and Antonio Vilar[b]

[a]Instituto de Investigaciones Marinas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain
[b]Aquarium Finisterrae, Maseo Marítimo, 15002 A Coruña, Spain

[*]Corresponding author: Tel.: +34 986 214457; fax: +34 986 292762.
E-mail address: mplanas@iim.csic.es (M. Planas).

Abstract

Knowledge on seahorses is generally scarce but has been increasing in recent years due to their conservation status. Seahorse culture is a quite recent activity in most countries attempting it, and captive breeding techniques are available only for some species. With the aim of contributing to the development of breeding in captivity for conservative purposes, captive broodstock of the European long-snouted seahorse (*Hippocampus guttulatus*) were established with 32 wild seahorses captured in Galicia (NW Spain). This study describes the methodologies applied to the maintenance of the broodstocks, with special reference to aquaria design, feeding, growth and breeding. Procedures of seahorse identification (morphologically and genetically) as a tool for broodstock management are also considered. The results achieved during the first year demonstrate a rapid adaptation of wild seahorses to captive conditions. Seahorses were fed exclusively on enriched adult *Artemia* and displayed high growth rates. However, fatty acid analyses performed on unfertilised eggs of captive broodstock showed a progressive decrease in the content of essential fatty acids (DHA, EPA) with time in captivity, suggesting the need for improvement in the nutritional quality of broodstock feed.

Keywords: long-snouted seahorse; *Hippocampus guttulatus*; husbandry; broodstocks; feeding, growth; breeding; aquaria design

1. Introduction

Declines in wild populations of seahorses have occurred, particularly in western Atlantic and Indo-Pacific waters (Alverson et al., 1994; Vincent, 1996, 1997; Baum et al., 2003; Martin-Smith et al., 2004). Some factors responsible for population decrease are considered to be: fishing pressure for commercial trade, bycatch in fisheries, and degradation and loss of habitat (CITES, 2002). There are currently 33 recognised seahorse species (Foster and Vincent, 2004), all of which are currently listed on Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and are included on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2006). Knowledge of the biological characteristics of affected species would be of major importance in conservation actions, development of breeding programmes and recovery of wild populations. Unfortunately, information available on the distribution, biology and rearing of seahorses is generally scarce and limited to a few seahorse species (Foster and Vincent, 2004).

The long-snouted seahorse *Hippocampus guttulatus* Cuvier 1829 is one of two seahorse species found in the north-eastern Altantic Ocean and Mediterranean Sea, and is a relatively large species (Boisseau, 1967; Reina-Hervás, 1989; Forster and Vincent, 2004). According to IUCN (2006), *H. guttulatus* is a 'Data-Deficient' species, which means that there is currently insufficient information available on this species to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status. Direct and by-catch fisheries for *H. guttulatus* occur in Portugal and in southern England and Channel Isles (OSPAR, 2005), with live seahorses contributing to an international aquarium trade. In Spain, it is thought that *H. guttulatus* is not subject to high fishing pressure for international trade, but wild populations have disappeared/reduced in many sites of the coast as reported by fishers, divers and marine naturalists, although there is a lack of investigations to quantify this statement. Whilst information on direct fisheries or by-catch pressure is lacking, these factors must not be discarded as causative of the wild population regression. In addition, the contribution of other factors, such as habitat destruction and pollution, must be also considered (Vincent, 1996).

Project *Hippocampus* started in 2006 as the first coordinated Spanish project focussed on seahorses. The main objectives of the Project are the study of wild populations in some areas of the Spanish coast (Galicia and Canary Islands), to develop a breeding programme in captivity and to assay a genetically controlled repopulation programme in selected natural areas. A

captive breeding approach can be of significant value in conservative programmes, particularly when wild population numbers are very low. The first requirement in the successful captive breeding of seahorses is the availability of broodstock and development of techniques suitable for broodstock maintenance and reproduction. In recent years, research efforts towards the study of wild populations and development of husbandry techniques has progressed for a limited number of seahorse species (Lockyear et al., 1997; Wilson and Vincent, 1998; Payne and Rippingale, 2000; Masonjones and Lewis, 2000; Woods, 2000, 2003 a,b,c; Chang and Southgate, 2001; Job et al., 2002; Perante et al., 2002; Wong and Benzie, 2003; Foster and Vincent, 2004; González et al., 2004). However, the rearing of seahorses in captivity is still in its infancy for many seahorse species, including *H. guttulatus*. This paper provides general information on the establishment of captive broodstock of *H. guttulatus* and the results achieved during the first year of husbandry are discussed in the frame of Project *Hippocampus*.

2. Material and methods

2.1. Collection of seahorses

Adult *H. guttulatus* were hand-caught from 7 April to 7 June in 2006 at three different sites in the coast of Galicia (NW Spain): Ribeira Harbour, Ría of Aldán and Arousa Island (Figure 1). Collection surveys were always initiated in the morning, of duration from 10:00 – 13:00 h. Further details on habitat and collection sites are provided in Table 1. Most seahorses were caught at Ribeira Harbour, which appears to be an important population site. At this site, there is a mud bed with accumulations of *Ulva* sp and anthropogenic debris. In Ribeira, most seahorses were found stationary on the mud, although some were also located in surrounding open sand areas. In Aldán, the site is characterised by a rocky bottom covered with dense, large big masses of the macroalga *Sargassum* (about 1m length and 2 m width), surrounded by small sandy areas. The site in Arousa Island is a sandy bed with small patches of macroalgae, mainly *Sargassum* spp and *Cystoseira* spp. At this site, seahorses were holding onto macroalgae. Canido Beach is characterised by a bottom consisting of sand and small patches of seagrass (*Zostera*). This site was surveyed once (30 May 2006) as it was known that seahorses were frequently sighted here some years ago. However, no seahorses were seen at this site during sampling for this study.

FIGURE 1

Collected seahorses were placed into 20 L plastic bags containing 1/3 seawater and 2/3 pure oxygen. Transportation of the bags to the facilities at the Instituto de Investigaciones Marinas (CSIC) in Vigo (NW Spain) was made in isothermal polystyrene boxes. On arrival, seahorses were gradually acclimatised to the aquaria water for ca. 3-5 hours. Temperature adjustment was always less than 2ºC. Subsequently, the seahorses were weighed and tagged for individual identification. Non invasive samples of tissue were also taken for future genetic studies. Afterwards, groups of 3-4 seahorses were randomly distributed into 53 L sub-aquaria, maintained in quarantine and monitored for some weeks. No therapeutic treatments were applied. During the first days, enriched *Artemia* was offered in excess as describer later.

2.2. Tagging of seahorses and tissue sampling for genetic analysis

For tagging, a nylon collar (fishing line) with a unique tag code was used for each animal (Figure 2). Fluorescent tags (VI Alpha Tag, Northwest Marine Technology Inc., USA) were used for the identification of each seahorse (Figure 2). Preparation of tags was performed under a binocular microscope, according to a modification of the methodology proposed by Morgan and Martin-Smith (2004). A small piece of plastic electrical wire casing with two holes was cut off. A thin layer of Loctite instant glue was applied to the whole surface of the plastic wire and then the VI Alpha tag was glued on it under a binocular microscope and left to dry. Finally, Loctite glue was applied again to the whole surface of the electrical wire. The tag collar was gently adjusted to the neck of the seahorse by means of a small piece of electric wire carrying a fluorescent VI Alpha tag with an alphanumeric identification code (N00 to N99).

FIGURE 2

Two types of non-invasive tissue samples were taken from each seahorse for further genetic analysis (López, 2006; Pardo et al., 2007; Planas et al., 2007): fin-clipping (dorsal fin) and fleshy skin filaments (fronds)-clipping. Samples of the dorsal fin were taken by clipping a triangular piece of the fin off the posterior corner of the dorsal fin, as described by Lourie (2003a). For comparative purposes, samples of the skin filaments were also taken by clipping a small piece (2-3 mm) of fleshy filaments from the head of each seahorse. Special care was

taken to avoid cutting off of the lower and bony portion of the filaments. Tissue samples were transferred to screw-capped tubes filled with 95% ethanol and maintained in the freezer. An infection preventative treatment with Blastoestimulina (Almirall Prodefarma, Barcelone, Spain) was applied to the sampled area for aiding tissue recovery.

2.3. Aquaria design

Broodstock culture facilities consisted of four experimental units (A, B, C and D) of a design that functioned as autonomous closed systems (Figures 3, 4). Each unit had a total capacity of 630 L and was mounted on a stainless steel structure. Three metacrilate aquaria (160 L capacity; 85 Height x 75 Length x 25 Wide cm) (labelled 1, 2 and 3), were located on the upper part of the structure. A seawater treatment system was located at the bottom of each unit. The treatment system consisted of a filtering system, a cooling unit with electronic thermostat control and pumps.

The three aquaria (1-3) were positioned forming a T-shape. Two of them (1 and 2) were physically separated by a metacrilate plate which could be removed to convert both aquaria in one aquarium (312 L). The third aquarium (3) was independent. For further experimental purposes, each aquarium could be subdivided into three 53 L sub-aquaria (e.g. 1a, 1b and 1c) by using a set of opaque blue removable metacrilate plates. Transparent metacrilate was used in the front sides and opaque blue metacrilate in the lateral sides and bottom. In each aquarium, a 20 W fluorescent tube (4000ºK) located 25 cm above the water surface providing an illumination intensity of approximately 850 -1050 lux. Daily light regime was controlled by a timer.

FIGURES 3 and 4

In each sub-aquarium, an inlet PVC pipe at the surface of the water column was connected to a mobile PVC elbow for adjustment of the direction and depth of the water jet in the aquaria without the entry of air bubbles in the water. A small security overflow outlet window, located 2 cm above the water surface prevented accidental overflow. Outlet waters were drained by overflow through a PVC pipe connected to a PVC tubular grid (1 cm x 0.2 mm openings) located 20 cm above the bottom of the aquaria. The water was collected into an outlet box and drained down towards the seawater treatment system. No aeration was provided.

Seawater temperature was maintained between 15ºC in winter and 17.5ºC in summer and controlled by means of a cooling unit and a thermostat (± 0.5ºC). Temperature was measured in outflow waters, just at the entry into the water treatment unit. This unit provided mechanical, chemical and biological treatment of the seawater in each unit and consisted of a Venturi pump, a skimmer, a mechanical filter (100 µm mesh and perlon) and finally a biofilter. The biofilter consisted of perforated plastic balls and a bed of coral sand and charcoal. Two pumps (PASQ B-30 and PASQ B-A26) distributed the water into aquariums 1 and 2, and 3 respectively. Water flow-through in each aquarium was 3 x 5-6 L/min.

Photoperiods approximating those in the seahorses' natural environment were applied. The daily light regime ranged from 15:30 L: 8:30 D (June-July) to 10 L: 14 D (December-January). The aquaria were covered with black plastic bags during the dark phase. Wastes and uneaten food were removed daily by siphoning the bottom of the aquaria early in the morning. During this cleaning, about 10-15% of the total water was discarded and subsequently replaced with filtered (5 µm) and UV-Light treated seawater. Water quality was checked periodically for $N0_2$, $N0_3$ and NH_4/NH_3 content (0 mg/L) by using Sera Test Kits. Soft plastic plants and plastic ropes (12 mm diameter) were anchored to small stones and placed on the bottom of the aquaria as holdfasts for the seahorses. Salinity and pH levels were 37 ± 2 ppt and 8.0 ± 0.2, respectively.

2.4. Husbandry and feeding

Following the adaptive period to captivity, the seahorses were randomly distributed into the aquaria as follows:

- Aquaria A: One group (A) of 4 males and 4 females in 320 L aquaria (2 pooled 160 L aquaria).
- Aquaria B: Three groups (B1, B2, B3) of 3 males and 1 female in 160 L aquaria each.
- Aquaria C: Three groups (C1, C2, C3) of 1 male and 3 females in 160 L aquaria each.

Seahorses were fed *ad libitum* on live enriched adult *Artemia* only. *Artemia* were offered to seahorses twice daily: in the morning (09:30 -10:30 h) and in the afternoon (16:00-18:00 h), depending on the photoperiod regime. Food levels were adjusted daily (60-150 *Artemia*/seahorse) according to the season/temperature and to visual observations of *Artemia* remaining in the aquaria from the previous day. Adult *Artemia* (length > 5.5 mm; 15-25 days old)

were harvested from the ongrowing of EG *Artemia* nauplii (Inve, Spain). Ongrowing was carried out using mixtures of the microalgae *Isochrysis galbana*, lyophilised *Spirulina* and Prolon (Inve, Spain) (Quintas et al., 2007). Adult *Artemia* was enriched in two steps (0.5 *Artemia*/L): (a) enrichment for 18 h at 26ºC on a mixture of Prolon (50 mg/L), *I. galbana (300 ml/L)* and 5% v/v ACE (*Artemia* Condition Enhancer, Inve), and (b) enrichment on *I. galbana* (1 L/L) for 2-8 hours at 20ºC.

Standard length (SL; cm) of seahorses was measured from digital photographs using image processing software (NIS, Nikon). Standard length was measured as head + trunk+ tail length (curved measurement), as reported by Lourie (2003b). Wet weights (W; g) were recorded at the time of capture (7 April – 7 June 2007), at the end of the reproductive season (31 October 2006) and the onset of the following reproductive season (31 March 2007). Specific growth rates (SGR) were calculated as $SGR = (\ln W_f - \ln W_i)/t*100$, where W_f and W_i are the final and initial weights (g) and t is time (days). Significant correlations between weight and length were provided by Curtis and Vincent (2006), but the function obtained by these authors can not be applied to our seahorses because we measured curved line SL. Consequently, we used our data of weight and curved line SL (pooled sexes) of seahorses in captivity, and the following correlation was obtained: $W= 0.0154*SL^{2.4107}$ (r=0.756). Fulton's condition index (K) was calculated as $K = (W/SL^{2.4107})*100$ (Ricker, 1975).

Most egg batches dropped by females and collected from the bottom of the aquaria were used for genetic analyses but some of them were preserved for fatty acid (FA) content. Lipids were extracted according to Bligh and Dyer (*1959*) and quantified gravimetrically (Herbes and Allen, 1983). Fatty acid composition of lipids was determined by gas-chromatography according to Christie (1992). Lipids were transmetlylated by the method of Lepage and Roy (1986) and then analysed by GC (Perkin Elmer, Clarus 500 gas chromatograph) employing a fused silica capillary column SP-2330 (0.25 mm i.d. x 30 m. Supelco Inc, Bellefonte, PA, USA), programmed from 145 ºC to 190ºC at 1.0 ºC/min, from 190ºC to 210ºC at 5.0ºC/min and then followed by a hold for 13.5 minutes at 210ºC. Nitrogen at 10 psig. was the carrier gas and the flame ionization detector set at 250ºC was used. A programmed temperature injector was used in the split mode (150:1) and heated from 45ºC to 275ºC at 15ºC /min. Peaks were identified by comparison of their retention times with standard FAME mixtures (Supelco: FAME mix). For

quantification purposes, peak areas were automatically integrated and 19:0 fatty acid (Sigma) was used as an internal standard.

Means are given with SD. Statistical analyses were performed with Statistica 6.0 (StatSoft).

3. Results

3.1. Capture sites and collected seahorses

Thirty-two seahorses (16 males and 16 females) were collected from 5 May to 6 June 2006, in the early-middle natural reproductive period (Table 1). All seahorses possessed skin filaments, as is usual in most adult *H. guttulatus*, and did not show external lesions or signs of unhealthy condition. All were adults with the exception of one juvenile female (N31; 3.31 g) found in Arousa Island. None of the males showed signs of pregnancy. Mean weight (mean± SD) of caught seahorses was 10.78 ± 3.64 g (range: 3.31 – 17.05 g) in females and 12.92 ± 2.98 g (range: 6.44 – 18.67 g) in males (Table 2). Measurements of SL were only performed in some of the seahorses captured. The maximum and minimum SL in the measured seahorses was 20.1 cm and 9.6 cm, respectively.

Seahorses were regularly monitored after tagging with VI Alpha tag collars. Most seahorses behaved normally, without symptoms of stress or lesions. As the months progressed, the surface of some tags were colonised by diatoms, impeding easy reading of the code. The collars were cleaned in a solution of pure acetic acid and brief ultrasonication.

Sampling of seahorses for non-invasive tissue collection was made without any apparent detrimental effect on the fishes. The samples provided sufficient amount of biomass and DNA for further genetic analysis. Fin-clipping proved more problematical than fleshy skin filament clipping because the seahorses usually contracted the fin over the body during handling. Full recovery of dorsal fin tissue was observed in 4-6 weeks after clipping. Recovery of fleshy filaments was not monitored.

3.2. Feeding and growth

Newly established seahorses were interested in the *Artemia* offered from the beginning. Most of them accepted this prey and started to feed in the following 24 - 48 h post-capture. In

some cases, the onset of feeding was delayed for longer, although all seahorses were actively feeding after one week. Due to the changes applied to the temperature and light regimes, the amount of *Artemia* daily offered ranged from 65 to 125 per seahorse. In general, most of the food offered each day was ingested but a small portion (<10-20%) of the total amount was trapped by the outlet filter tubing and consequently not accessible to seahorses. Seahorses generally did not accept *Artemia* older than 21 days (9.2± 0.9 mm). Due to unknown reasons, most of these *Artemia* were rejected after capture or simply not captured.

Growth rates of seahorses were high during the whole period of study, as supported by the data on weight and length, and condition index (Table 2). Condition index was higher in males than in females, although the differences were statistically not significant. In both sexes, the values of K at the end (31 Oct 2006) and at the beginning (1 Mar 2007) of the breeding season were similar. Figure 5 shows the growth performance (SGR) of seahorses according to the treatment applied. Significant differences were found only between reproductive and non-reproductive seasons (ANOVA, $p<0.001$) and between aquaria (reproductive season only) (ANOVA, $p<0.05$). Average specific growth rates (% SGR) were higher during the breeding season, particularly in group A (4 males / 4 females; SGR>0.30). Growth rates decreased (SGR<0.12) after the breeding period, with small differences between groups.

FIGURE 5

3.3. Courtship behaviour and reproduction

During the breeding season, mature and receptive seahorses showed a pale and silver skin colour. Changes in skin colour were pronounced until the end of November, particularly during courtship. Courtship mostly occurred early in the morning, during the first 1-2 hours of the light period of the day, but also extended occasionally all day. In some cases, greetings engaged 3 or 4 seahorses of either sexes or, occasionally several males. Frequently, males initiated courtship with females but it was not infrequent to observe mature females looking for males to court. However, egg transfer was never observed.

Twenty unfertilized egg batches were collected from the bottom of the aquaria from 29 June to 20 Nov 2006. The number of eggs per batch ranged from 5 to 538 (160 ± 152). Eggs were released by the females during the first 90 min of the light phase. Only two batches were collected in the afternoon. Eggs were characterized by a thin and fragile external membrane

and a big yolk surrounded by numerous oil droplets with a characteristic orange colour. Average long and short axes size of these freshly released eggs were 2.44 ± 0.28 and 1.30 ± 0.16 mm, respectively, whereas long and short axes of yolk measured 1.73 ± 0.21 and 1.23 ± 0.35 mm, respectively.

Fatty acid analyses performed on 10 egg batches showed two clearly different profiles (Figure 6). Profile I corresponded to two egg batches collected in late June and early July, seven weeks after the capture of the fish in the wild. These eggs had a relatively high level (35%) of ω3 HUFA (t-Test; $p<0.001$), mainly due to their content in DHA (22:6ω3, 18%) ($p<0.05$) and EPA (20:5ω3; 11%) ($p<0.001$), and a low content in ω6 FA (10%) ($p<0.001$), mainly linoleic acid (LA; 18:2ω6; 4.5%) ($p<0.001$) and arachidonic acid (ARA; 20:4ω6; 4%) ($p<0.05$). Eggs collected from seahorses maintained in captivity for more than eleven weeks were included in profile II, characterized by a lower level (25%) in ω3 FA and a higher level (18%) in ω6 FA, due to a decrease and an increase in the level of ARA (8%) and LA (13%), respectively. Both profiles also showed clear differences in DHA/EPA ($p<0.001$) and ω3/ω6 ratios ($p<0.001$).

FIGURE 6

Two broods were collected in September and November post-parturition, with a total of 326 and 453 young (including embryos and premature dead young). Only 11 and 118 of these were live young respectively. Further genetic analysis identified the progenitors as male N01 and females N13 and N16 for the first and second batch, respectively. Female N13, the original mate of male N01, died on the 23 August 2006, being immediately replaced by female N16.

3.4. Broodstock diseases

In summer, some fish exhibited symptoms of infection by ciliates, becoming anxious, shaking the body or rubbing the head with the tail. The symptoms ceased after a 3-days bath treatment (1 h) containing a mixture of 36% formaldehyde (0.1 mL/L) and malachite green (0.4 mg/L). In addition, five seahorses died during the period of study. One female died due to myxosporidioses that completely invaded all the internal organs and four seahorses died due to a tail rot-like disease. The first symptom was a loss of prehensility in the tail, followed by whitening and tissue erosion starting at about 1-2 cm above the tip of the tail. In most cases that

316 portion of skin began to flake or lift up, accompanied by the presence of a ring of ulcers. As the
317 disease progressed, the tip of the tail became white and the loss of coloration advanced further
318 up the tail, with death occurring several days later. In female N02, the disease progressed for 6
319 weeks with a gradual loss of the last few segments of her tail, followed by death. Unsuccessful
320 attempts were made to control the disease by topical treatments (formalin + malachite green,
321 antibiotics, iodine or fresh water baths). Microbiological studies performed on affected
322 seahorses did not reveal a consistent presence of pathogenic bacteria (Pintado, unpub. data).
323

324 **4. Discussion**

325 Where repopulation initiatives using captive-bred seahorses are planned, the production of
326 genetically-controlled seahorses is advantageous to potentially maintain the original genetic
327 identity and diversity of the area being repopulated. Thus, as we are determining in Project
328 *Hippocampus*, the use of appropriate microsatellite loci is valuable for genetic diversity analysis,
329 parentage studies, the development of conservation plans, and management of seahorse
330 broodstock in captivity. *Hippocampus guttulatus* and the short-snouted seahorse *H.*
331 *hippocampus* occur sympatrically. The presence of skin filaments in *H. guttulatus* has been
332 used as a criterion for identification but it has been reported that it is an unreliable character
333 because *H. hippocampus* also has potential to grow skin filaments (Garrick-Maidment, 1998;
334 Curtis, 2006). All the seahorses captured in the present study corresponded to the *H. guttulatus*
335 morphological type, with a mane of skin filaments, and this identification was genetically
336 confirmed (Pardo et al., 2007). López (2006) and Pardo et al. (2007) characterized the first 12
337 specific polymorphic microsatellite loci in *H. guttulatus*. This study used non-invasive samples of
338 skin filaments and dorsal fin. Both types of tissue sampling provided sufficient amount of DNA
339 for analysis. However, fleshy filament sampling was more convenient because it is less time
340 consuming and less stressful for the fishes. Population and family genetic analyses of
341 broodstock was also carried out by means of microsatellite markers, to avoid inbreeding and
342 losses of genetic diversity (López et al., 2007), yielding information in the future about the
343 mating pattern in captivity. The simultaneous use of genetic and physical identification with VI
344 Alpha tag collars would appear to fulfil the necessities for an adequate management of captive
345 *H. guttulatus* broodstock.

346 The performance of the seahorses in terms of growth and condition factor demonstrates
347 that a captive feeding regime based exclusively on enriched adult *Artemia* is adequate to
348 support growth in *H. guttulatus*. Unfortunately, there is no information available on natural
349 growth for comparative purposes, but the maximum weight (22.43 g) and size (23.52 cm SL)
350 achieved in our laboratory exceed any record previously reported, both in the wild and in
351 captivity. During the breeding season, the increase in growth rates and condition factors were
352 probably the result of temperature increase, commensurate with the increase in feeding rates
353 and body weight gain due to gonad maturation in females and pouch development in males.
354 However, a negative consequence of temperature increase was the appearance of some
355 diseases, mainly tail rot. Tail rot is a complex illness of unknown origin but typically associated
356 with captive seahorses (Garrick-Maidment and James, 2002). Attempts made to ascertain the
357 cause of this disease have been unsuccessful to date and no further symptoms of tail-rot have
358 been observed since then in our facilities. The only death caused by myxosporidan infection
359 may have been contracted before the time of capture in the wild. The affected seahorse never
360 showed any surface skin lesions, but post-mortem examination revealed a generalised invasion
361 of the internal organs by white microsporidan cysts (> 2mm in diameter). Similar cysts of *Guglea*
362 *heraldi* or spores of the myxosporidan *Sphaeromyxa sabrazesi* have been earlier observed in *H.*
363 *erectus* and *H. guttulatus* (Bellomy, 1969; Vincent and Clifton-Hadley, 1989). In this study, the
364 non-appearance of the gas bubble disease, another common illness in seahorses (Garrick-
365 Maidment, 1997), which appears to be related to the presence of air bubbles in the water and
366 shallow tank depths, appears to have been avoided by the design (water inlet pipe and high
367 water column) applied to our aquaria.
368 The reported breeding season for wild *H. guttulatus* extends from March to September-
369 November depending on latitude (Boisseau, 1967; Reina-Hervás, 1989; Curtis, 2007), although
370 Curtis and Vincent (2006) reported that the proportion of reproducing males varies by season,
371 with a peak in June - August. Surprisingly none of the males collected in the present study were
372 pregnant. The first egg batch was collected in the laboratory seven weeks after capture. These
373 eggs probably matured in captivity since recent results in our laboratory suggest that the female
374 interclutch interval is 28-31 days at 16-17ºC. This period is similar to previous interbrood
375 intervals reported in wild males (Boisseau, 1967; Lozano-Cabo, 1979; Kuiter, 2001) but higher
376 than the female interclutch interval (21 days) in Ria Formosa lagoon, where a weak temperature

effect was determined by Curtis (2007). Information on batch fecundity for *H. guttulatus* in captivity is not available, although Boisseau (1967) reported a maximum brood size *in situ* of 581, which is consistent with the maximum egg batch size (538) and maximum brood (419) achieved in our laboratory. Regarding female maturation, there are some interesting findings in the present study that need to be highlighted. The origin (female) of egg batches could be only determined in aquaria B (1 female/3 males). In the other aquaria, the identification of originator females was attempted through genetic analyses but the amount of DNA utilised was insufficient (C. Bouza, pers. comm.). However, the data that was obtained showed that not all females matured and that some of them did not release any eggs during the whole breeding season. On the other hand, maturation of females did not extend over the entire breeding season. The causes of these different maturation patterns among females are unknown. Possible relationships between maturation/egg production and seahorse size, courtships partners, mating patterns and other factors (nutritional, environmental) should be considered in the future. Breeding of *H. guttulatus* in captivity is currently under study in our laboratory, and recent results show a large increase (up to 9 fold) in the egg batch production, probably due to the enhancement in the biochemical composition of the food, particularly in the content in DHA.

Most marine organisms are rich in ω3 HUFA and poor in LA, reflecting the fatty acid profile of their natural food. This pattern is also evident in fish eggs (Tocher and Sargent, 1984; Tocher, 2003). To our knowledge, the present study provides the first published information on the FA content of eggs or gonads in seahorses. Egg batches analysed had an unexpected high level of LA and a relatively low content of ω3 HUFA, particularly in eggs of Profile II. Prior to enrichment, adult *Artemia* were grown for at least two weeks on a culture mixture with a high content of *Spirulina*, a fresh-water cyanobacterium rich in palmitic acid (PA; 16:0), gamma-linolenic acid (GLA; 18:3ω6) and gamma-linoleic acid (LA; 18:2ω6) (Colla et al., 2004). The levels of thoses FAs in the *Spirulina* source used in our study were 50.5, 22.8 and 17.5, respectively. It is feasible that Spirulina may be responsible for the high content of LA in seahorse eggs and also in the *Artemia* (12-13%, data not shown). This finding is not uncommon for some marine fishes. For example, high levels in LA have been reported in ovaries (wild and cultivated fish) and eggs (cultivated fish) of the white sea bream *Diplodus sargus* (Cejas et al., 2003), probably due to the feeding regime, although the source could not be confirmed. The fatty acid content of eggs could be explained by assuming that those eggs partially reflected the

previous feeding regime of the females in the wild (low LA and high ω3 FA levels). On the contrary, eggs of Profile II showed FA levels in agreement with the feeding regime in the laboratory. Although, the diet used for the ongrowing of *Artemia* seems to be adequate for the growth of adult seahorses, the previous findings must be considered in the future to improve the biochemical composition of eggs. The analysis of FA in freshly released eggs of wild seahorses would greatly contribute to the progress in the diet improvement, particularly during the reproductive season.

Courtship behaviour in *H. guttulatus* was very similar to the description provided by Woods (2000) in *H. abdominalis.* Competition between males for a female seems to be usual in seahorses. Forster and Vincent (2004) suggested that this conventional sex role is due to the relative time it takes seahorses to prepare for mating. However, we have also occasionally observed the opposite (aquaria C), with several females competing for the only available male. To what extent availability of several mates might affect mating patterns or reproductive success in long-snouted seahorse has not been reported but it is known that mating and egg transfer is prevented in *H. abdominalis* when multiple seahorses of the same sex participate in the courtship (Woods, 2000). The results indicate that in such situation other biological parameters would be also affected. For instance, seahorses maintained in larger groups, including several males and females (aquaria A), would have growth enhanced with respect to seahorses that must compete with others for a unique mate (aquaria B and C). Although these results are still preliminary and need to be confirmed, the knowledge of this type of information would be interesting in the management of captive seahorse broodstock.

Most seahorse species are considered monogamous as mating occurs exclusively between the same two partners in a single breeding season. The natural mating pattern for *H. guttulatus* is unknown. Visual observations showed that males in captivity do not remain faithful to a single female for courtships (unpub. data). However, faithful mating can not be confirmed in this study because only one male (N01) became pregnant, but more recent results support genetic monogamy (Planas, unpub. data). Genetic analyses were performed by López et al. (2007) on the two batches of young released by male N01 allowed the assignment of maternity/paternity of the progenitors. These revealed that male N01 mated first with female N13 and later with female N16 (after the death of N13), giving birth to young on 3-10 Sept and 2-9 Nov, respectively.

In conclusion, the husbandry of adult *H. guttulatus* does not seem to be problematic when strict control is applied to water quality, cleaning and feeding. The aquaria design used appears to fulfil the captive requirements for this species. Whilst enriched *Artemia* are appropriate for growth, improvements must be made in the nutritional quality of *Artemia* or other feeds used to enhance the captive breeding performance of *H. guttulatus*.

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578

Table 1 – Information on the sites and seahorses (*Hippocampus guttulatus*) caught in 2006, and growth (W - wet weight; SL- standard length).

CAPTURE INFORMATION							W (g)			SL (cm)		
ID TAG	DATE (d/m/y)	SITE[a]	DEPTH (m)	HABITAT[b]	Behaviour	SEX	CAPTURE	31Oct06	1Mar07	31Oct06	1Mar07	DEATH
N00	07/04/06	R	9	1	Holdfasted to *Ulva*	♂	-	14.33	16.40	18.60	19.51	
N01	07/04/06	R	9	1	Stationary inside a tin with *Ulva*	♂	-	19.80	18.18	17.31	18.22	
N02	07/04/06	R	9	1	Laying on the bottom	♀	-	-	-	-	-	04/09/06
N03	26/04/06	A	6	2	Swimming above *Sargassum*	♂	-	22.42	24.74	19.84	20.38	
N04	26/04/06	A	6	2	Swimming in sandy bed	♀	-	15.44	16.10	18.40	18.96	
N05	05/05/06	R	7	1	Stationary on mud bed	♂	13.84	19.85	22.93	17.99	18.87	
N06	05/05/06	R	7	1	"	♀	10.55	16.25	18.37	17.73	18.76	
N07	05/05/06	R	7	1	"	♀	10.04	14.44	18.42	17.66	19.51	
N08	05/05/06	R	7	1	"	♂	14.99	17.36	16.65	18.64	19.37	
N09	16/05/06	R	6-8	1	"	♀	11.58	15.95	16.00	18.09	19.17	
N10	16/05/06	R	6-8	1	"	♂	11.02	17.50	22.13	17.47	19.04	
N11	16/05/06	R	6-8	1	"	♀	9.30	-	-	-	-	23/08/06
N12	16/05/06	R	6-8	1	Swimming on mud bed	♂	15.77	21.90	23.82	18.79	19.91	
N13	16/05/06	R	6-8	1	Stationary on sand bed	♀	17.05	-	-	-	-	13/10/06
N14	16/05/06	R	6-8	1	Swimming on sand bed	♂	10.21	17.56	20.23	19.46	19.84	
N15	16/05/06	R	6-8	1	Swimming on mud bed	♂	14.95	19.61	22.39	17.75	18.76	
N16	16/05/06	R	6-8	1	Stationary on mud bed	♀	14.38	17.39	19.83	18.75	19.11	
N17	16/05/06	R	6-8	1	Swimming on mud bed	♂	10.15	17.34	19.83	17.91	18.99	
N18	16/05/06	R	6-8	1	Stationary on mud bed	♀	13.54	15.59	17.12	18.74	19.17	
N19	16/05/06	R	6-8	1	"	♂	6.44	16.17	20.03	16.16	17.32	
N20	16/05/06	R	6-8	1	"	♂	15.31	-	-	-	-	07/08/06
N21	16/05/06	R	6-8	1	"	♂	13.35	21.50	22.90	19.57	19.62	
N22	16/05/06	R	6-8	1	Swimming on mud bed	♀	12.64	12.75	14.09	17.84	19.03	
N23	16/05/06	R	6-8	1	Stationary on sand bed	♀	11.22	16.67	17.46	19.23	20.89	
N24	16/05/06	R	6-8	1	Swimming on mud bed	♂	12.01	17.62	19.05	17.65	18.28	
N25	16/05/06	R	6-8	1	"	♀	11.38	12.63	14.52	16.78	18.05	
N26	07/06/06	I	4-6	3	Holdfasted to algae	♂	18.67	22.43	24.71	21.59	23.52	
N27	07/06/06	I	4-6	3	Holdfasted to algae	♀	6.59	11.01	14.06	15.43	17.39	
N28	07/06/06	I	4-6	3	Holdfasted to algae	♀	12.06	13.93	14.66	17.20	17.80	
N29	07/06/06	I	4-6	3	Swimming between rock and sand	♂	9.34	-	-	-	-	20/09/06
N30	07/06/06	I	4-6	3	Holdfasted to algae	♀	6.40	10.65	13.86	15.51	16.42	
N31	07/06/06	I	4-6	3	Holdfasted to algae	♀	3.31	6.71	10.05	14.55	16.12	

[a]R- Ribeira Harbour; A – Ria of Aldán; I – Arousa Island

[b]1- Mud with *Ulva* sp surrounded by sand; 2- Rocky bed with dense patches of *Sargassum* surrounded by small areas of sand; 3- Sand with patches of macroalgae (*Sargassum, Cystoseira*)

Table 2 – Wet weight, standard length and Fulton's K condition index in male, female and pooled sexes of seahorses (*Hippocampus guttulatus*) maintained in captivity. Dead seahorses were not included in the analyses. Data are mean ± SD.

Sex	Date	Wet Weight (g)	Standard Length (cm)	Condition Index (K)
Females	31/10/2006	13.80 ± 3.01	17.38 ± 1.44	1.391 ± 0.137
	01/03/2007	15.73 ± 2.58	18.49 ± 1.31	1.388 ± 0.161
	Max - Min	19.83 - 6.71	20.89 - 14.55	1.699 – 1.055
Males	31/10/2006	18.96 ± 2.50	18.48 ± 1.34	1.673 ± 0.243
	01/03/2007	21.00 ± 2.79	19.40 ± 1.43	1.648 ± 0.259
	Max - Min	24.74 - 14.33	23.52 – 16.16	2.071 – 1.221
Pooled	31/10/2006	16.47 ± 3.77	17.95 ± 1.47	1.537 ± 0.243
	01/03/2007	18.46 ± 3.76	18.97 ± 1.42	1.513 ± 0.248

Table 3 – Batches of unfertilized eggs and young seahorses (*Hippocampus guttulatus*) obtained in 2006 in aquaria A, B and C. The origin (male/female) of the batch, and the date and time are provided.

Aquaria	Origin	Date	Time	Eggs
A	nd	1-Jul	8:10	111
A	nd	4-Jul	8:00	32
A	♀ N30	13-Sep	8:00	80
A	nd	17-Sep	12:00	34
A	nd	10-Nov	17:35	343
B2	♀ N04	6-Jul	8:05	225
B2	♀ N04	8-Sep	10:15	331
B2	♀ N04	20-Nov	10:15	459
B3	♀ N25	29-Jun	8:15	44
B3	♀ N25	15-Aug	12:00	5
B3	♀ N25	7-Oct	8:30	15
C1	nd	25-Jul	8:00	176
C1	nd	27-Sep	9:00	125
C1	nd	28-Sep	17:40	21
C3	nd	29-Jun	8:15	249
C3	nd	25-Jul	8:00	64
C3	nd	22-Aug	9:00	103
C3	nd	12-Oct	8:30	100
C3	nd	2-Nov	9:00	538
C3	nd	3-Nov	13:00	140
				Embryos[a] /Young
C2	♂ N01 / ♀ N13	3-sep	9:00	315/0
C2	♂ N01 / ♀ N13	10-sep	13:00	0/11
C2	♂ N01 / ♀ N16	2-nov	18:30	10/0
C2	♂ N01 / ♀ N16	3-nov	8:30	171/0
C2	♂ N01 / ♀ N16	6-nov	8:30	30/10
C2	♂ N01 / ♀ N16	8-nov	9:00	90/108
C2	♂ N01 / ♀ N16	9-nov	10:15	4/34

[a] Including dead and premature young; nd – not determined (3 females in the aquarium)

LIST OF FIGURES

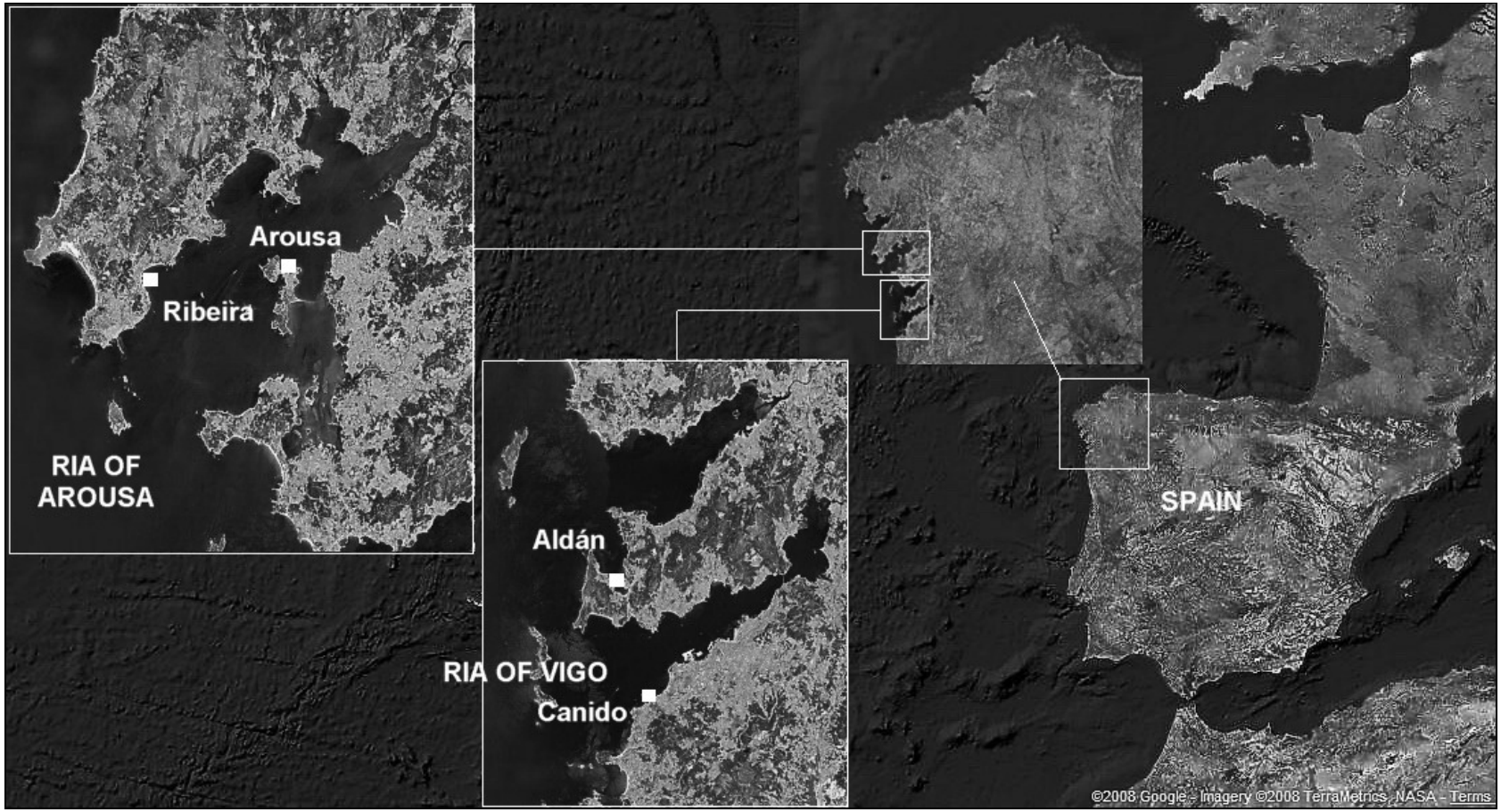
Arousa
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Figure 1

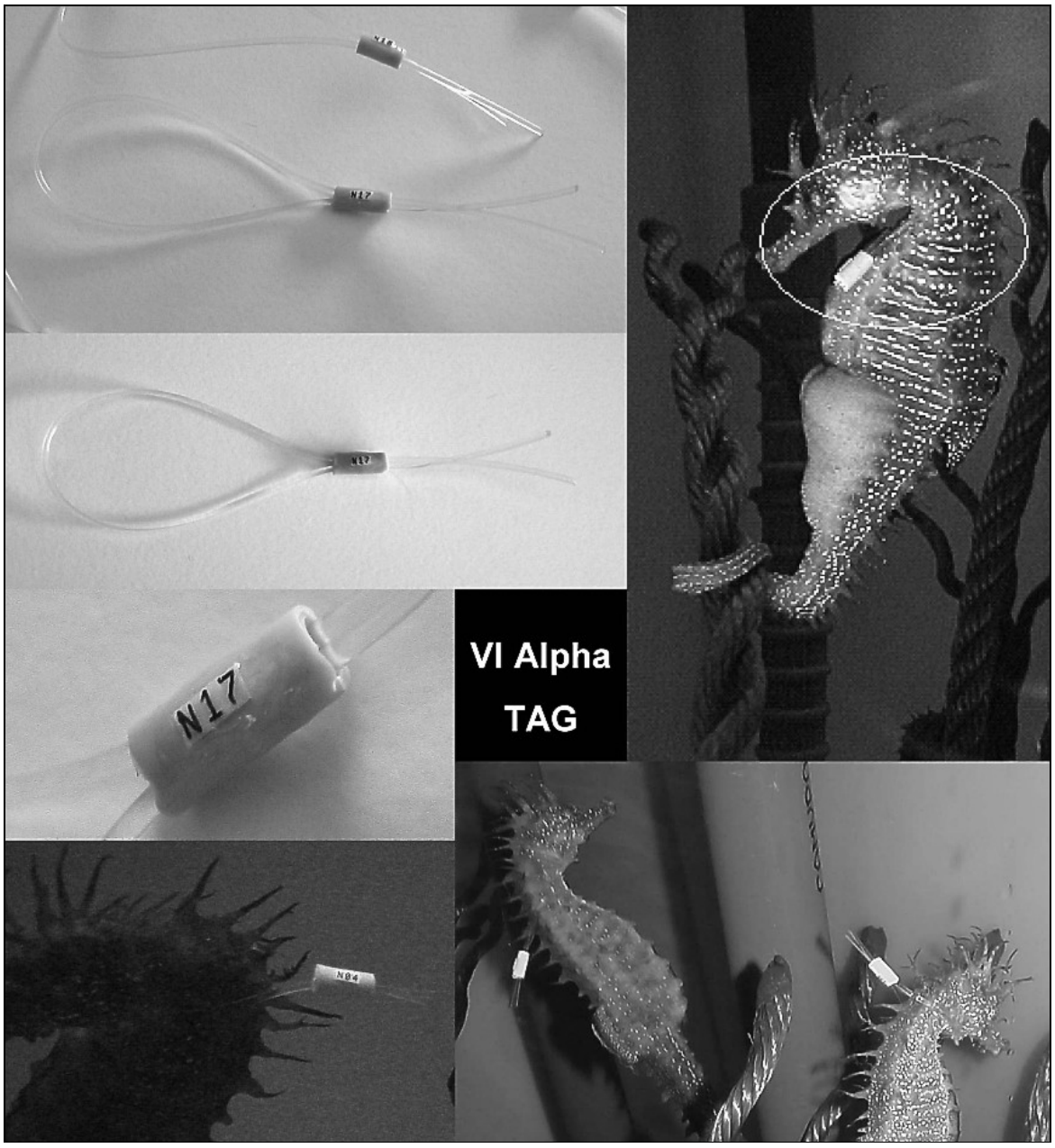


Figure 2

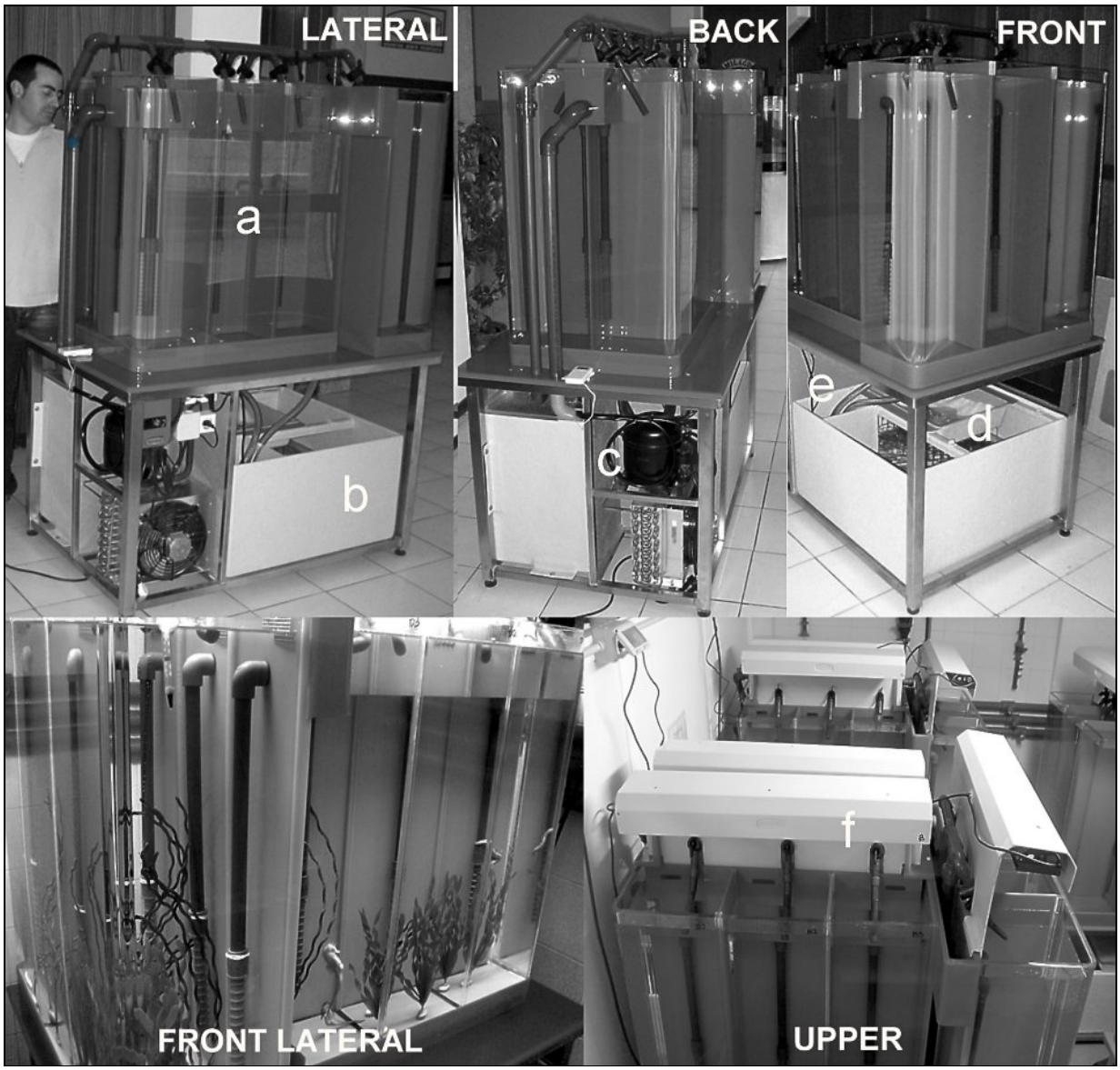
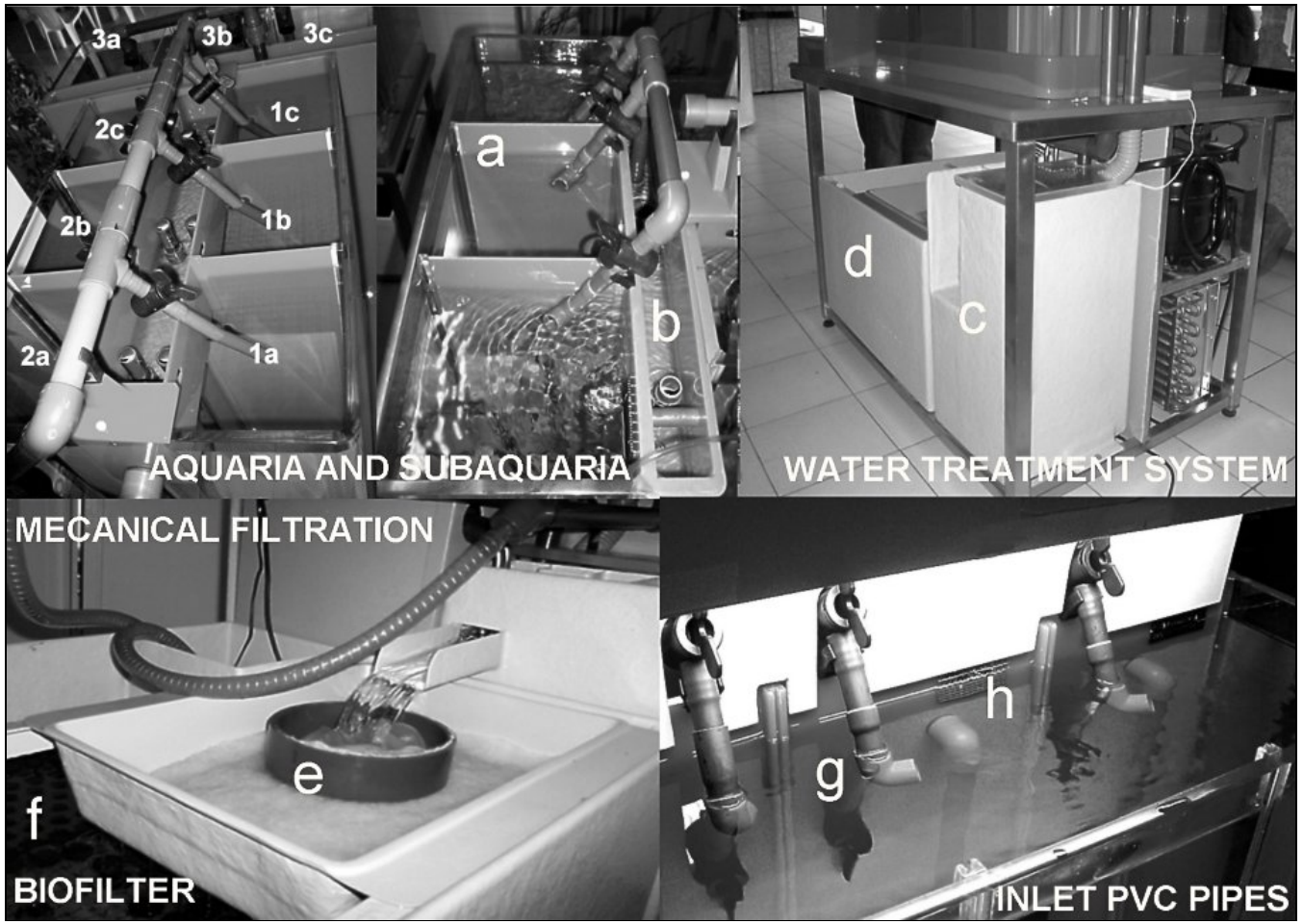


Figure 3


3a
3b
3c
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2b
1b
2a
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AQUARIA AND SUBAQUARIA
a
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WATER TREATMENT SYSTEM
MECANICAL FILTRATION
e
f
BIOFILTER
h
g
INLET PVC PIPES

Figure 4

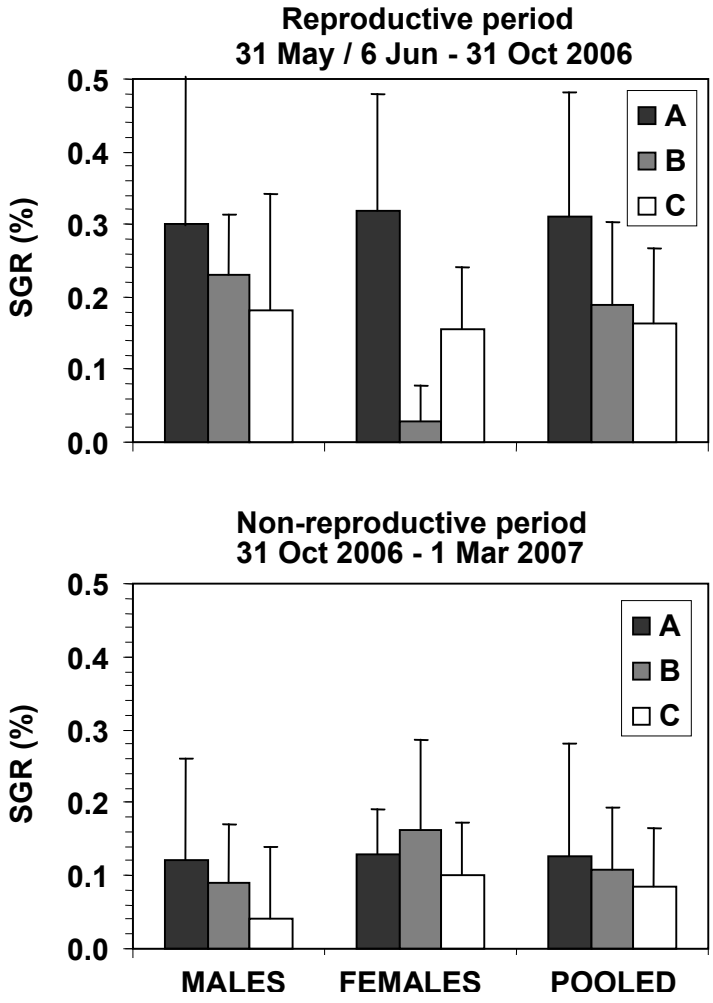
Reproductive period
31 May / 6 Jun - 31 Oct 2006
SGR (%)
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
Non-reproductive period
31 Oct 2006 - 1 Mar 2007
SGR (%)
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
MALES
FEMALES
POOLED

Figure 5

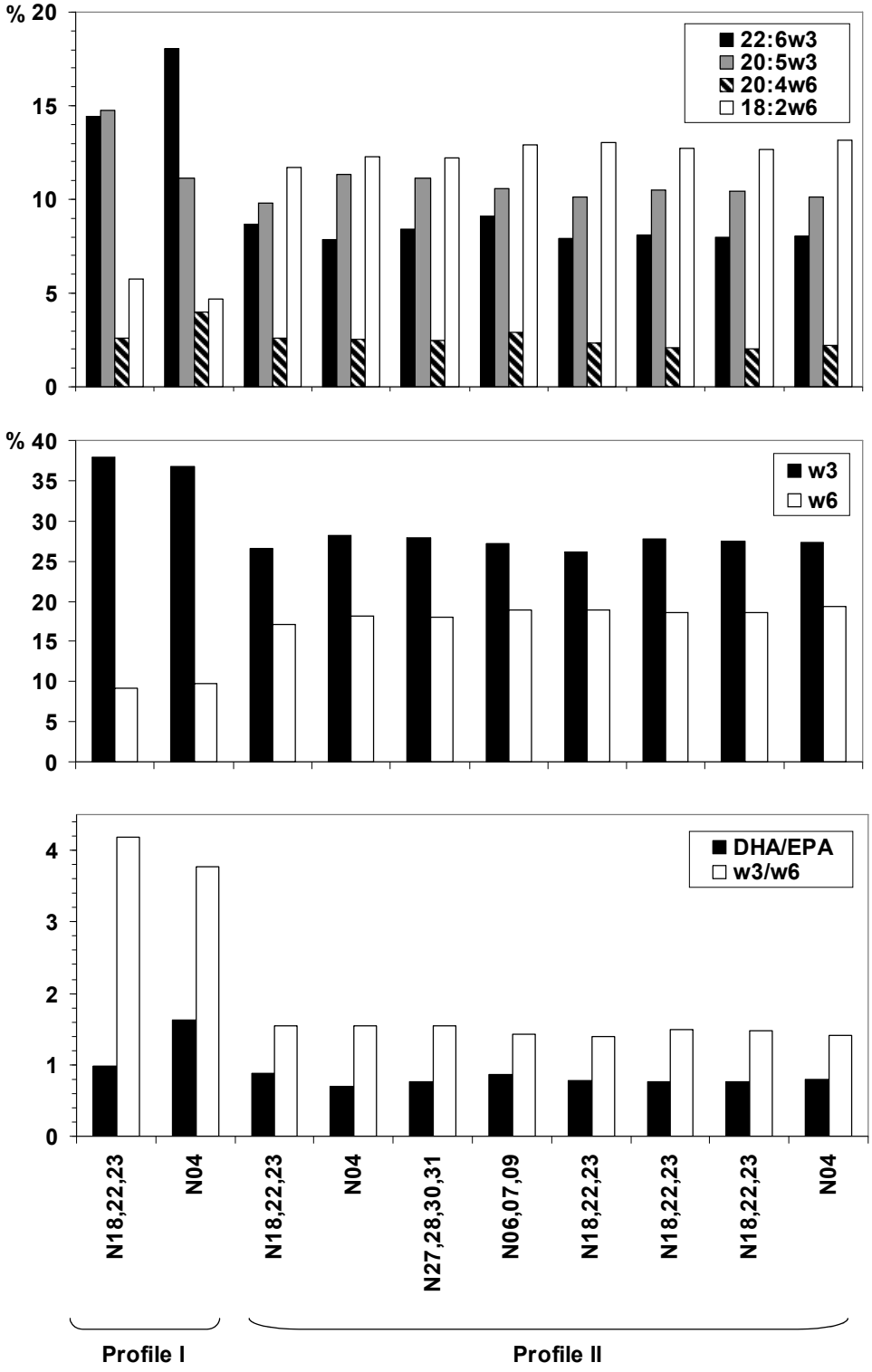
% 20
15
10
5
0
22:6w3
20:5w3
20:4w6
18:2w6
% 40
35
30
25
20
15
10
5
0
w3
w6
4
3
2
1
0
DHA/EPA
w3/w6
N18,22,23
N04
N18,22,23
N04
N27,28,30,31
N06,07,09
N18,22,23
N18,22,23
N18,22,23
N04
Profile I
Profile II

Figure 6