Effects of Different Live Feed Diets Applied to the Long-Snouted Seahorse (*Hippocampus guttulatus* Cuvier, 1829)

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Abstract

This paper aims to point-out the effects of applying different live feed diets to the long-snouted seahorse (*Hippocampus guttulatus* Cuvier, 1829) of the Romanian Black Sea coast. The response of seahorses to *Brachionus plicatilis*-based, *Artemia salina*-based and combined diet is analysed, from the development, ethologic and biochemical point of view. Three experimental tanks were set in laboratory conditions, for a 10 day period. The different batches of seahorses collected from the natural environment were placed in the three tanks, after previous acclimation. Subsequently, one batch (Tank A) was fed exclusively with *B. plicatilis*, one with *A. salina* (Tank B), and one with a 50%/50% combined mixture of the two invertebrates (Tank C). The results obtained indicated a linear length and weight increase of *H. guttulatus* in all three feeding regimes, the final length (+10 days) being higher than the +5 days length and initial length. The biochemical composition of the three batches showed some variations. From the ethologic point of view, differences in the behavior of *H. guttulatus* in the three tanks were observed. Future research is required, focusing mainly on extending the experimental period (more than 30 days), separating males and females and diversifying the prey fed to seahorses.

Keywords: long-snouted seahorse, *Brachionus plicatilis*, *Artemia salina*, biochemical composition, behavior

Introduction

The long-snouted seahorse (*Hippocampus guttulatus* Cuvier, 1829) is a representative species of the Romanian coast, due to its charismatic appearance and extraordinary biology. Although it is not a commercial fish in Romania, it is subjected to harvesting to be sold as curio or for the aquarium business (Nenciu et al., 2013). Research has also confirmed that seahorses, like most of marine species, absorb the xenobiotics reaching the aquatic environment, which makes them vulnerable to anthropogenic pressures (Nenciu et al., 2014a, 2014b).

The conservation state of *H. guttulatus* is not very well known, as the International Union for Conservation of Nature (IUCN) (Woodall, 2012) rates it as “Data Deficient”. In the Black Sea region, it is listed as “Endangered” by Turkey, “Vulnerable” in Georgia and the Ukraine, and “Least Concern” in Romania (Yankova, 2012). This species has a low dispersion and migration movements are limited (Caldwell and Vincent, 2013), reducing their ability to colonize new habitats, emphasizing the importance of preserving the habitats they occupy today. There is no global estimate of the size of *H. guttulatus* populations, nor any assessment of trends in these populations. There are data only for two locations in Ria Formosa, Portugal (Gamito, 2008), and France, the Arcachon Basin (Grima, 2011). Similarly, there are no such estimates for the Black Sea, which confirms the need to conduct studies in this regard. *H. guttulatus* inhabits relatively small areas in shallow coastal waters, lagoons and estuaries (0.5 to 15 m) (Curtis and Vincent, 2005), but may make seasonal migrations to deeper waters (30 m+) (Garrick-Maidment et al., 2013). Adults inhabit different habitat types across multiple types of sediment, preferring areas covered by macroalgae and seagrass, which they cling to, but they were also observed fixed on artificial holdfasts (Curtis and Vincent, 2005; Woodall, 2009).

*H. guttulatus*, like many other seahorse species, is a monogamous species, at least within a reproductive cycle (Woodall, 2009), which reduces their chances of mating when the partner disappears (it dies, it is fished etc.). However, *H. guttulatus* matures quickly, it has a fast growth rate and short time between generations, suggesting that it can recover quickly when pressure factors (direct - fishing, indirectly - accidental catch, destruction of
herring) are removed, but they can be vulnerable to long periods of poor stock recovery (Curtis and Vincent, 2005). The breeding period extends from March to October, but depends on temperature, and gestation lasts about 21 days (Curtis, 2007).

In Romania, the first experiments conducted on the breeding and rearing in captivity of seahorses were carried out by the National Institute for Marine Research and Development (NIMRD) “Grigore Antipa” Constanta, in 2008, under the project “2 Mai - Durankulak Area - Conservation of Marine Biodiversity and Public Awareness”, financed by the European Union under PHARE 2005 Bulgaria-Romania Cross-border Cooperation (coordinated by NGO “Mare Nostrum”), when the first results of this kind for the Black Sea were obtained (Zaharia et al., 2010). The concrete results of the experiments conducted, expressed as a percentage of animal survival and animals released into the sea, have shown that the breeding and subsequent rearing of these fish in captivity is feasible. However, the major drawback in rearing H. guttulatus was supplying the most appropriate diet for the fry, as many individuals died of starvation before reaching maturity due to the lack of a small-sized food alternative. Under these circumstances, the current research was an attempt to determine which live diet is better accepted by seahorses in a controlled environment, for future rearing in captivity.

Materials and Methods

The different batches of seahorses collected from the natural environment were placed in the three tanks, after previous acclimation. Subsequently, one batch was fed exclusively with Brachionus plicatilis, one with Artemia salina, and one with a 50%/50% combined mixture of the two invertebrates. The control batch was represented by ten adult H. guttulatus individuals collected by divers from the Olimp area (southern Romanian coast, rocky area, covered by seagrass and perennial algae). They were transported alive to the NIMRD premises and euthanized using MS 222 (Tricaine methanesulfonate, 250 mg/L concentration) (AVMA Guidelines for the Euthanasia of Animals, 2013). Subsequently, they were sexed, measured and weighed, then frozen for euthanizing the organisms in order to count them.

Thirty H. guttulatus individuals were collected from the same sampling location (Olimp) and acclimated for 24 hours in a large 80 L tank with aeration. No food was administered during the acclimation period. The duration of the experiment was 10 days (15-24 July 2014). Photoperiod period 16 h L : 8 h D, similar to normal summer conditions at the Romanian Black Sea. Three experimental tanks were set in the laboratory, with water parameters similar to the natural environment in terms of temperature, salinity and light, and filled with 10 L of seawater (1 L/seahorse). All three tanks were properly aerated and provided with artificial plants as holdfasts for the seahorses. Water was changed daily, so as to provide a constant density of prey in the experimental tanks, thus temperature and salinity were consistent with values in the natural environment.

Tank A contained 10 H. guttulatus individuals (6 males, 4 females) fed exclusively on the rotifer B. plicatilis. Tank B contained 10 H. guttulatus seahorse individuals (3 males, 7 females) fed exclusively on the brine shrimp A. salina. Tank C contained 10 H. guttulatus individuals (5 males, 5 females) fed on a 50-50% B. plicatilis and A. salina diet (Figure 1). Food was given daily, at a density of 70 ind./ml for rotifers and 15 ind./ml for brine shrimp (Table 1).

In the feeding cycle of seahorses, phytoplankton serves as food for the rotifers and the brine shrimp, enriches the nutritive value of the rotifers and A. salina and detoxifies the water in which seahorses are kept, by assimilating or neutralizing the toxic substances such as ammonia and pesticides (Nita et al., 2011). For this experiment, 5 L Nannochloropsis sp. and Tetraselmis sp. cultures were used, obtained from pure strains purchased previously within NIMRD from a specialized laboratory in the U.S.A., in sealed plastic containers.

The rotifer B. plicatilis was obtained from a strain provided by the courtesy of the Central Fisheries Institute in Trabzon (CFRI). It was subsequently reared in the NIMRD laboratories (Zaharia, 2002; Alexandrov, 2008; Nita et al., 2011; Maximov, 2012). The “exploitation” culture system was used, rotifers were only partially harvested and fed to the seahorses, while the volume difference in the tank was fed with Nannochloropsis sp. culture, maintaining the rotifer density at 70 rotifers/ml. Rotifers were counted by analyzing samples at the stereomicroscope (40x) to determine the density: 1 ml of the culture was put on a Petrie dish and drop of Lugol solution was used for euthanizing the organisms in order to count them.

The brine shrimp A. salina was obtained from dormant eggs (cysts) purchased from the market (JBL Artemio Pur) and then incubated in seawater with aeration. Cylindrical tanks were filled with seawater and aerated. The eggs were placed in these tanks, in a concentration of 1 g/L. They were put to hatch for 22-24 hours, with strong aeration. After hatching, the nauplii were counted to establish the density, using the same technique as for rotifers, using the stereomicroscope (40x): 1 ml of the culture was put on a Petrie dish and a drop of Lugol solution was used for euthanizing the organisms in order to count them. The A. salina nauplii density was kept at 20 individuals/ml. This density dropped at 15 ind./ml in the adult stage, as 350 ml of Tetraselmis sp. were added every other day to feed the growing brine shrimps.

Every day, the tanks were siphoned to remove feces and unconsumed food and fresh water from the pumping system was added. A density of 70
rotifers/ml in a 10,000 ml water volume resulted in a rotifer density of 1.4 rotifers/ml of water in the experimental tank, while a density of 20 ind./ml in *A. salina* resulted in a brine shrimp density of 0.4 brine shrimps/ml of tank water. Thus, food was available at will for all seahorses involved in the experiment. No mortalities were recorded during the experimental period.

Sexual differences in seahorses are observed at 95 days of age at a length range between 6 and 7 cm (Ortega-Salas *et al.*, 2006), which demonstrates that all *H. guttulatus* individuals used in this experiment...
were mature adults, as male and females were easily identified. The three batches of seahorses were sexed, weighed and measured alive at the beginning of the experiment, then, after five days of separate feeding five individuals were removed from each tank, euthanized using MS 222, then measured and weighed. *H. guttulatus* individuals were subsequently frozen for biochemical composition analysis. The remaining five seahorses in each tank were measured and weighed alive and placed again in the tanks. At the conclusion of the other five days, they were removed, euthanized using MS 222, measured and weighed and frozen for biochemical composition analysis (Figure 2).

After completion of the experimental interval, the biochemical composition of the three *H. guttulatus* batches and of the control batch was analyzed using standard methods (Rosioru et al., 2014): moisture and dry weight - by drying in an oven at 105°C for 24 h; ash - by burning in an oven at 550°C for 6 h (Manescu, 1978); protein content (% dry weight) - by using the Lowry method (Lowry et al., 1951); lipid content was assessed in lyophilized tissue according to “Les notes techniques de l’URAPC” - NT/URAPC/ 96-01-02-03, IFREMER (Razet et al., 1996). The live feed’s biochemical composition was referenced from literature (Leger et al., 1987; Onciu, 1998).

The results were statistically interpreted using IBM SPSS Statistics v. 20, applying the One-Way Anova (Analysis of Variance) parametric test.

**Results**

The temperature and salinity of seawater in experimental tanks were monitored, but no corrections were made, so as to recreate the conditions of the natural environment. During the 10 days of experiment, temperature and salinity had an inversely proportional relationship: while temperature increased, salinity dropped (Figure 3).

Concerning the biometrics, it was noted that length increase in *H. guttulatus* individuals was linear in all three feeding regimes, the final length (+10 days) being higher than the +5 days length and initial length, respectively (Figure 4). The maximum increase in length was reported for the combined diet (Tank C), followed closely by the *A. salina* diet (Tank B). The minimum increase was registered by seahorses fed with *B. plicatilis*.

With reference to weight variation, the maximum increase was reported for the combined diet (Tank C), followed by the brine shrimp diet (Tank B) and the rotifer diet (Tank A) (Figure 5). This can be explained by the higher lipid ratio contained by *A. salina* (18.9% dry weight) (Léger et al., 1987) as
compared to *B. plicatilis* (12% dry weight) (Onciu, 1998).

However, the differences in length and weight reported after 5 and 10 days, respectively, between the three experimental tanks were not statistically significant.

After applying the One-Way ANOVA (Analysis of Variance) parametric test, it resulted that for the variable “mean length” after 5 days the means of the three groups (*H. guttulatus*, Tank A = 7.15 cm, *H. guttulatus*, Tank B = 7.40 cm, *H. guttulatus*, Tank C = 7.19 cm) did not differ significantly among each other (F=0.545; P=0.586 > α = 0.05) (Figure 6a). Similarly, after 10 days, the means of the three groups (*H. guttulatus*, Tank A = 7.30 cm, *H. guttulatus*, Tank B = 7.66 cm, *H. guttulatus*, Tank C = 7.60 cm) did not differ significantly among each other (F=0.823; P=0.462 > α = 0.05) (Figure 6b).

For the variable “mean weight”, the values recorded after 5 days did not differ significantly among the three batches: *H. guttulatus*, Tank A = 1.52 g, *H. guttulatus*, Tank B = 1.80 g, *H. guttulatus*, Tank C = 1.91 g (F=1.149; P=0.332 > α=0.05) (Figure 6c). In a similar manner, the mean weights measured after 10 days of controlled feeding did not record statistically significant differences among the three diets administered: *H. guttulatus*, Tank A = 1.71 g, *H. guttulatus*, Tank B = 2.07 g, *H. guttulatus*, Tank C = 2.25 g (F=0.927; P=0.442 > α = 0.05) (Figure 6d).

Concerning the biochemical composition, the mean values obtained after 5 and, respectively, 10 days of differentiated feeding were referred to the

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**Figure 3.** Evolution of temperature and salinity of seawater in experimental tanks during the experiment.

**Figure 4.** Length variation in relation with feeding regime of *H. guttulatus*.

**Figure 5.** Weight variation in relation with feeding regime of *H. guttulatus*. 
mean value recorded by the control batch, as *H. guttulatus* individuals were similar in length and weight (statistically confirmed, $F=0.896; P=0.420 > \alpha=0.05$ for initial length, and $F=1.118; P=0.342 > \alpha=0.05$, for initial weight).

Dry weight (DW) and moisture (WW%) recorded some differences between tanks and moments of the experiment (DW = dry weight, WW = wet weight). The highest dry weight value was recorded in *H. guttulatus* individuals from Tank A after 5 days (24.85%), while the lowest in Tank B after 5 days (22.34%) (Figure 7). In a complementary manner, the lowest moisture value was recorded in *H. guttulatus* individuals from Tank A after 5 days (75.15%), while the highest water content was recorded in samples from Tank B after 5 days (77.66%) (Figure 8).

Ash (DW%) values were the highest in the *B. plicatilis* diet (Tank A), while brine shrimp (Tank B) and combined (Tank C) diets recorded similar values (Figure 9).

Protein content varied differently after 5 days and 10 days. Thus, after 5 days of feeding, the highest protein content was recorded in *H. guttulatus* individuals from Tank C (combined), while the lowest was recorded in seahorses from Tank A (rotifer diet). However, after 10 days, these values shifted: the highest protein content was recorded in *H. guttulatus* individuals from Tank A (rotifer diet), while the lowest in seahorses in Tank C (combined) (Figure 10). Lipid content was the highest in the *A. salina* diet (Tank B) after 10 days, as well as in the combined diet (Tank C) after 5 days (Figure 11).

**Discussion**

From the ethologic point of view, differences in behavior in the three tanks were observed. The batch fed with *B. plicatilis* was the less active, after food was administered they did not detach from the holdfasts and no strikes were visible, probably due to the smaller size of the prey. On the other hand, in Tank B and Tank C, where *A. salina* nauplii and metanauplii were introduced, all *H. guttulatus* individuals became immediately mobile and strikes were visible as they hunted on the larger sized brine shrimps.

However, the biochemical composition resulted in a higher protein content of the batch fed with *B. plicatilis*, somehow unexpected after observing the feeding behavior of seahorses, which seemed to prefer larger-sized prey (*A. salina*). These values can be explained by the higher protein content in *B. plicatilis* (63% dry weight) (Léger et al., 1987) as compared to *A. salina* (54.3% dry weight) (Léger et al., 1987). Due to its biochemical composition, the rotifer *B. plicatilis* is a quality source of food (total lipid content 12% DW, protein 63% DW) when reared with *Nannochloropsis* sp. (Onciu, 1998).

On the other hand, a proximate analysis of *A. salina* revealed an equilibrated high-protein diet indicating that macronutrient requirements are probably satisfied for most predators, seahorses included. The brine shrimp’s (fed with *Tetraselmis* sp.) biochemical composition is the following: total lipid content 18.9% DW, protein 52.2% DW in *A. salina* nauplii (Léger et al., 1987). Lipid content in *H.
guttulatus individuals was the highest in the A. salina diet (Tank B) after 10 days, as well as in the combined diet (Tank C) after 5 days, explainable by the higher lipid ratio contained by A. salina as compared to B. plicatilis (12% DW) (Onciu, 1998).

The influence of environmental factors must not be overlooked, as the evolution of temperature and salinity during the 10 days of the experiment may have influenced the feeding behavior of H. guttulatus individuals and consequently their metabolic activity. The evolution of the protein content in the three batches stands out: apart from Tank A (H. guttulatus fed exclusively with the rotifer B. plicatilis), in Tank B (H. guttulatus fed with A. salina) and C (combined diet) the protein content dropped significantly after 10 days of controlled feeding, from 93.98% DW to 92.07% DW and from 95.62% DW to 90.54% DW, respectively. When correlating this drop in protein...
content with temperature and salinity, it can be noted that exactly after 5 days of experiment temperature started to rise (from 23.5°C to 25.22°C), while salinity dropped (from 14 PSU‰ to 12.92 PSU‰), suggesting that the metabolism of *H. guttulatus* may have been slowed down.

**Conclusions**

This research revealed that length increase in *H. guttulatus* individuals was linear in all three feeding regimes, the final length (+10 days) being higher than the +5 days length and initial length, respectively. The maximum increase in length was reported for the combined diet (Tank C), followed closely by the *A. salina* diet (Tank B). The minimum increase was registered by seahorses fed with *B. plicatilis*. With reference to weight variation, the maximum increase was reported for the combined diet (Tank C), followed by the brine shrimp diet (Tank B) and the rotifer diet (Tank A).

The preliminary conclusions of this research are that a combined diet of rotifers (*B. plicatilis*) and brine shrimp (*A. salina*) is the most recommended for rearing seahorses (*H. guttulatus*) in captivity, due to the advantages that the two invertebrates have separately. On the one hand, rotifers develop greater densities and have a higher protein content (reflected in the protein content of the batches analyzed), while brine shrimps have a higher lipid content and are easier to prey on, being larger and more visible in the tanks. Other diets should also be tested in captive conditions, as studies have indicated that Amphipoda, Anomura, Decapoda and Mysidacea seem to be the dominant prey categories in the wild (Kitsos et al., 2008; Gurkan et al., 2011).

On the other hand, seawater temperature and salinity influence the metabolism of *H. guttulatus*. The increase of temperature above 23°C and salinity drop below 14 PSU‰ are likely to have caused the changes in the biochemical composition of *H. guttulatus*.

Further research is required during a longer time frame (at least 30 days), in order to monitor how the length-weight relationship develops in time in correlation with the diet applied, as well as the variation of the biochemical composition of *H. guttulatus* under different feeding regimes, also considering the influence of environmental factors. In addition, sex differences should be considered, as well
as different developmental stages (juveniles vs. adults). The ultimate aim is to find the optimal diet for rearing seahorses in captivity using live prey that can be easily obtained in artificial environments.

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