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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 anthopogenic 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

habitats) 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 to be later subjected to biochemical composition analysis.

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 filled 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

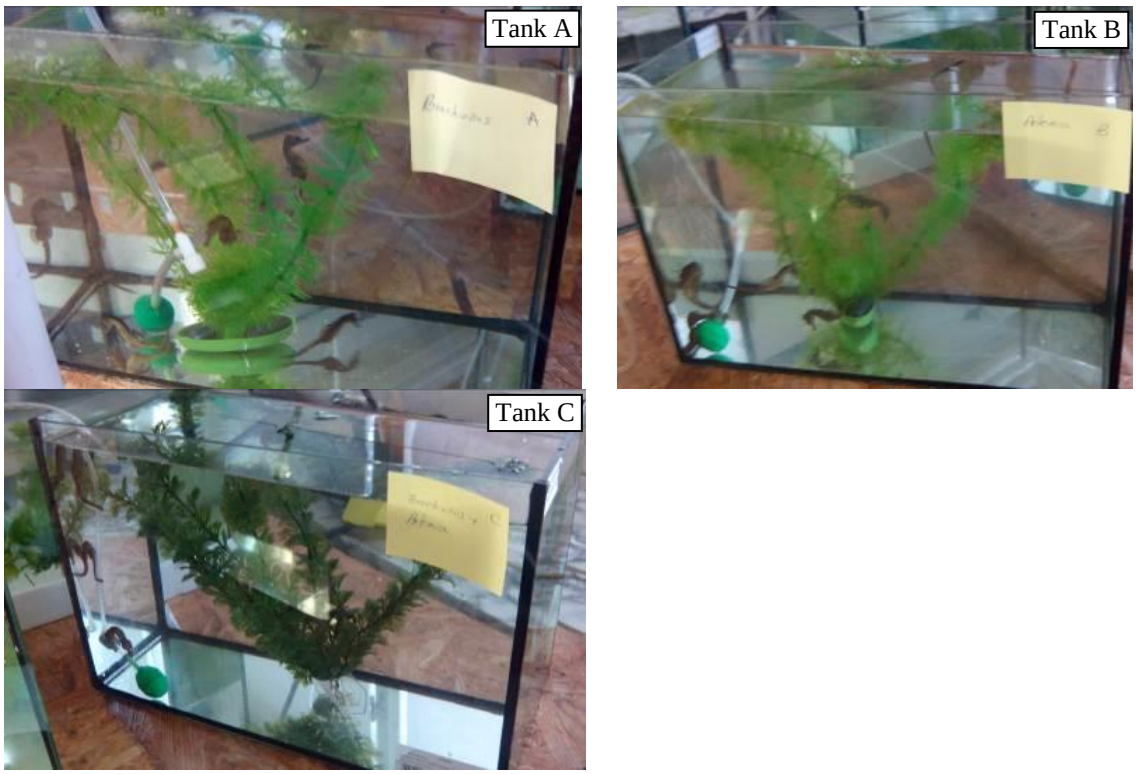

Figure 1. Experimental tanks (A, B, C) set in laboratory conditions (original photo).

Table 1. *H. guttulatus* feeding regime: feeding pattern of the three experimental batches

Date	Tank A	Tank B	Tank C
15.07.2014	200 ml of rotifer culture	200 ml of *Artemia* culture	100 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	100 ml of *Artemia* culture (density 20 ind./ml)
16.07.2014	200 ml of rotifer culture	200 ml of *Artemia* culture	100 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	100 ml of *Artemia* culture (density 20 ind./ml)
17.07.2014	200 ml of rotifer culture	200 ml of *Artemia* culture	100 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	100 ml of *Artemia* culture (density 20 ind./ml)
18.07.2014	200 ml of rotifer culture	200 ml of *Artemia* culture	100 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	100 ml of *Artemia* culture (density 20 ind./ml)
19.07.2014	200 ml of rotifer culture	200 ml of *Artemia* culture	100 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	100 ml of *Artemia* culture (density 20 ind./ml)
	5 individuals removed for biochemical analysis	5 individuals removed for biochemical analysis	5 individuals removed for biochemical analysis
20.07.2014	100 ml of rotifer culture	100 ml of *Artemia* culture	50 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	50 ml of *Artemia* culture (density 20 ind./ml)
21.07.2014	100 ml of rotifer culture	100 ml of *Artemia* culture	50 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	50 ml of *Artemia* culture (density 20 ind./ml)
22.07.2014	100 ml of rotifer culture	100 ml of *Artemia* culture	50 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	50 ml of *Artemia* culture (density 20 ind./ml)
23.07.2014	100 ml of rotifer culture	100 ml of *Artemia* culture	50 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	50 ml of *Artemia* culture (density 20 ind./ml)
24.07.2014	100 ml of rotifer culture	100 ml of *Artemia* culture	50 ml of rotifer culture (density 70 ind./ml) +
(h 12:00)	(density 70 ind./ml)	(density 20 ind./ml)	50 ml of *Artemia* culture (density 20 ind./ml)
	5 individuals removed for biochemical analysis	5 individuals removed for biochemical analysis	5 individuals removed for biochemical analysis

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 so 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

Figure 2. Measuring and weighting *H. guttulatus* individuals from the experimental batches (original photo).

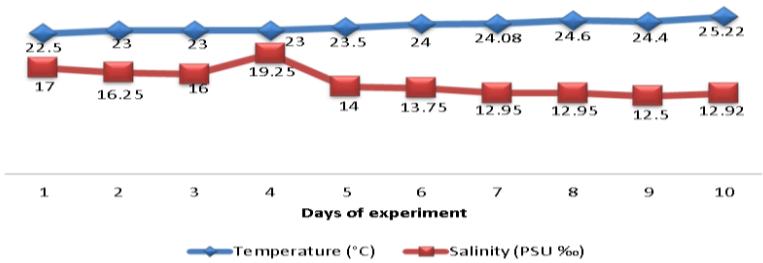

Figure 3. Evolution of temperature and salinity of seawater in experimental tanks during the experiment.

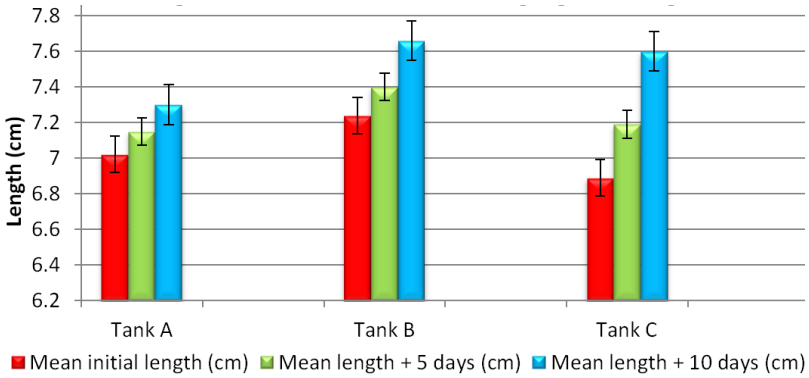

Figure 4. Length variation in relation with feeding regime of *H. guttulatus.*

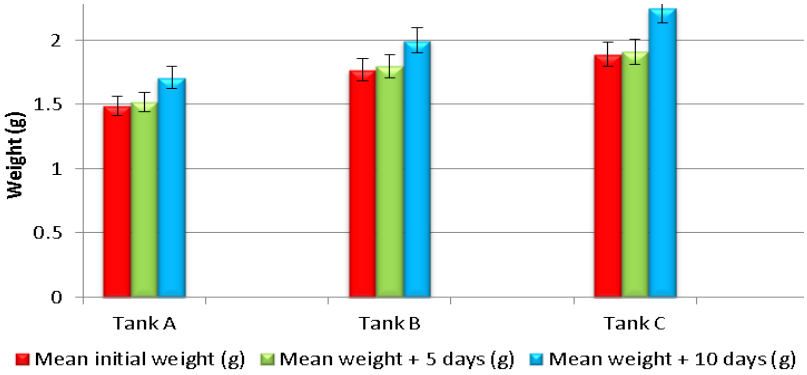

Figure 5. Weight variation in relation with feeding regime of *H. guttulatus*.

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 ($F=0.545$; $P=0.586 > \alpha = 0.05$) (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 ($F=0.823$; $P=0.462 > \alpha = 0.05$) (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 ($F=1.149$; $P=0.332 > \alpha=0.05$) (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 ($F=0.927$; $P=0.442 > \alpha = 0.05$) (Figure 6d).

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

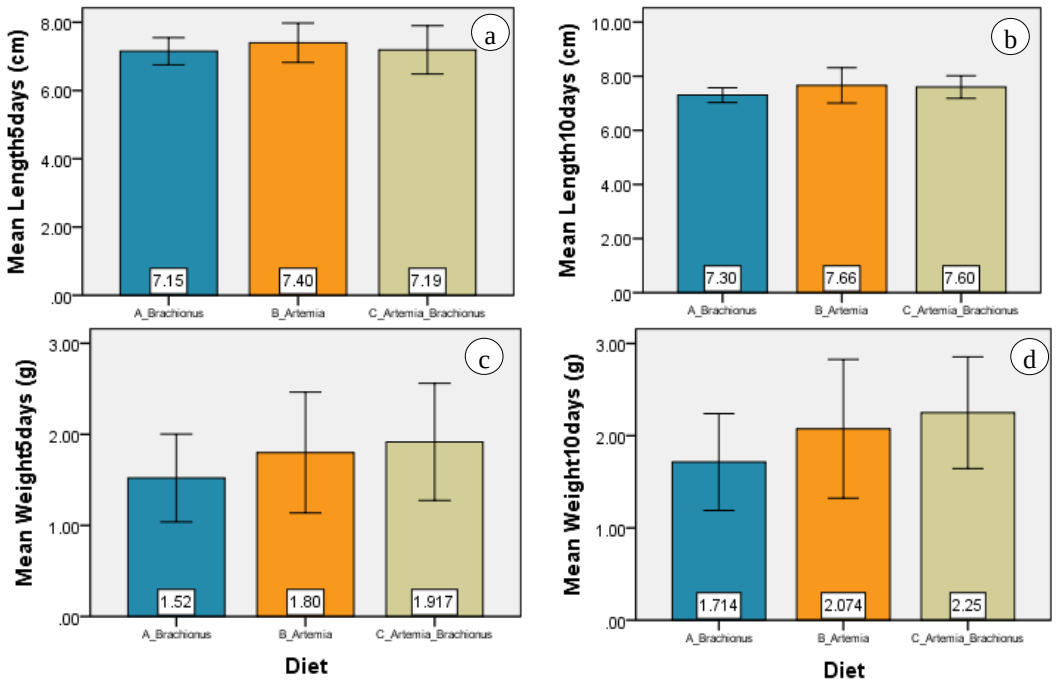

Figure 6. Differences between the three experimental batches: a) mean length after 5 days; b) mean length after 10 days; c) mean weight after 5 days; d) mean weight after 10 days.

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 > α=0.05 for initial length, and F=1.118; P=0.342 > α=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.*

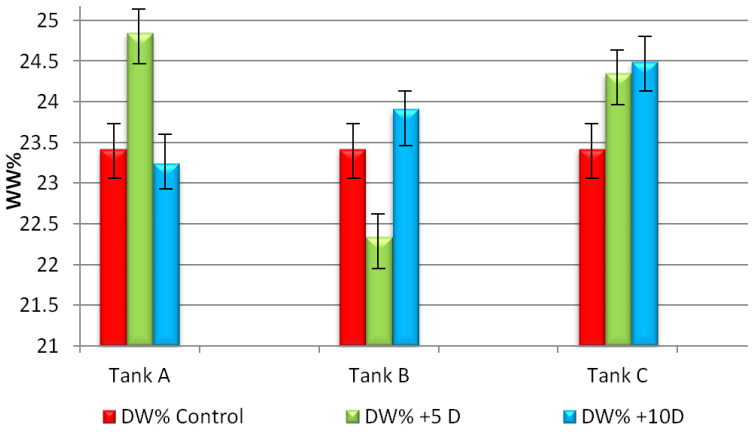

Figure 7. Dry weight (WW%) recorded in *H. guttulatus* individuals in the three experimental tanks.

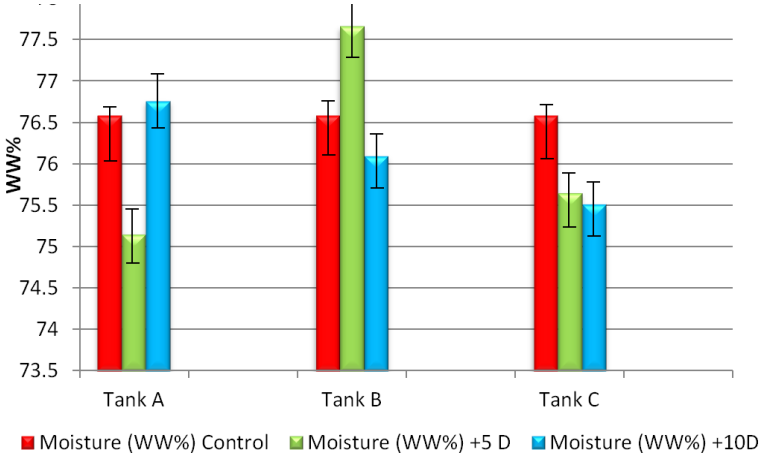

Figure 8. Moisture (WW%) recorded in *H. guttulatus* individuals in the three experimental tanks.

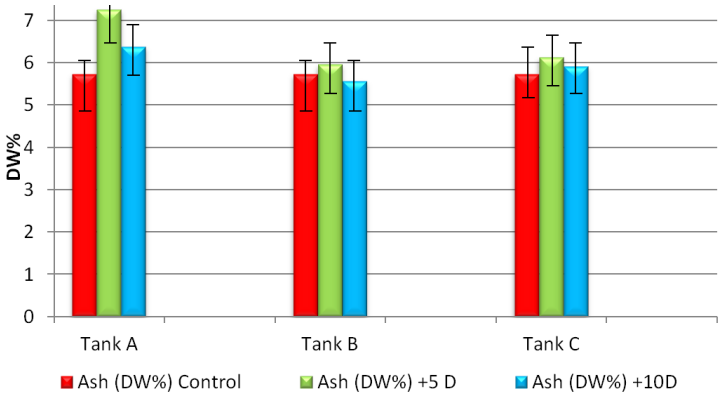

Figure 9. Ash (DW%) recorded in *H. guttulatus* individuals in the three experimental tanks.

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

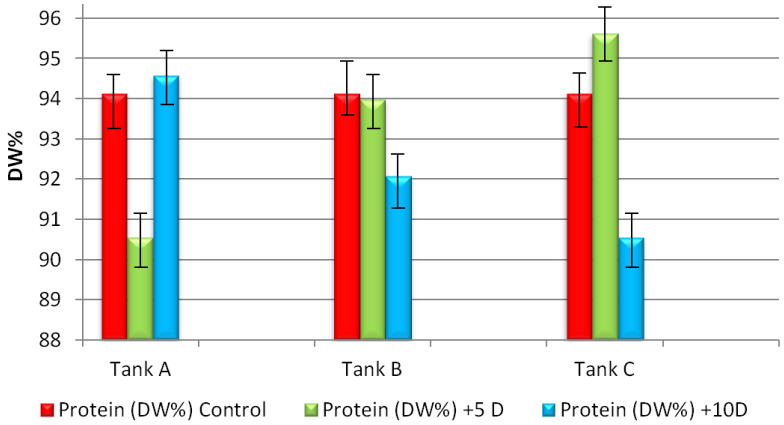

Figure 10. Protein content of *H. guttulatus* individuals in the three experimental tanks.

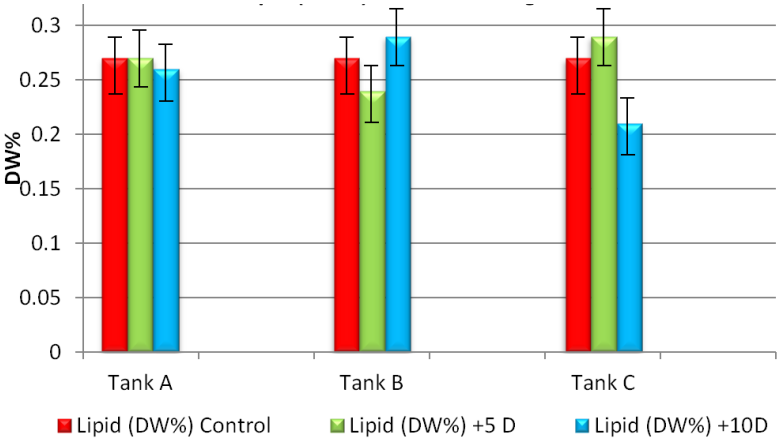

Figure 11. Lipid content of *H. guttulatus* individuals in the three experimental tanks.

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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References

Alexandrov L. 2008. Obtaining live feed in aquaculture (Obtinerea hranei vii in acvacultura), Ed. Punct Ochit Constanta, 150 p. (*in Romanian*);

AVMA Guidelines for the Euthanasia of Animals: 2013 Edition, ISBN 978-1-882691-21-0: 32. Accessed online 30.08.2014 https://www.avma.org;

Caldwell, IR and Vincent ACJ. 2013. A sedentary fish on the move: effects of displacement on long-snouted seahorse (*Hippocampus guttulatus* Cuvier) movement and habitat use. Environmental Biology of Fishes. 96 (1): 67-75, DOI: 10.1007/s10641-012-0023-4;

Curtis, J.M.R. and Vincent, A.C.J. 2005. Distribution of sympatric seahorse species along a gradient of habitat complexity in a seagrass dominated community. Marine Ecology Progress Series 291: 81-91, DOI: 10.3354/meps291081;

Curtis, J.M.R. 2007. Validation of a method for estimating realized annual fecundity in a multiple spawner, the long-snouted seahorse (*Hippocampus guttulatus*), using underwater visual census. Fisheries Bulletin 105: 327-336;

Gamito, S. 2008. Three main stressors acting on the Ria Formosa lagoonal system (Southern Portugal): Physical stress, organic matter pollution and the land–ocean gradient. Estuarine, Coastal and Shelf Science 7 (4): 710-720, DOI: 10.1016/j.ecss.2007.11.013;

Garrick-Maidment, N., Durant E. and Newman J., 2013., Year 5 report on the Seahorse Tagging Project at South Beach, Studland Bay in Dorset run by The Seahorse Trust, accessed online 15.10.2014 http://www.theseahorsetrust.org;

Grima, D. 2011. Etat des connaissances et ressources d'informations sur les hippocampes du Bassin d'Arcachon. Extrait du rapport remis à la mission pour le parc marin du bassin d'Arcachon et son ouvert. Arcachon, France, Accessed online 10.12.2014: http://www.peaubleue.org/data_folio/data_kcfinder/files/Grima2011HippoArcachonbd.pdf;

Gurkan S., Taskavak E., Sever T.M. and Akalin S. 2011. Gut contents of two European seahorses *Hippocampus hippocampus* and *Hippocampus guttulatus* in the Aegean Sea, Coasts of Turkey. Pakistan J. Zool., vol. 43 (6): 1197-1201;

Kitsos, M.S., Tzomos, T.H., Anagnostopoulou, L. and Koukouras, A., 2008. Diet composition of the seahorses, *Hippocampus guttulatus* Cuvier 1829 and *Hippocampus hippocampus* (L., 1758) (Teleostei, Syngnathidae) in the Aegean Sea. J. Fish Biol., 72: 1259-1267, DOI 10.1111/j.1095-8649.2007.01789.x;

Léger P., Bengtson D.A., Sorgeloos P., Simpson K.L. and Beck A.D. 1987. The nutritional value of *Artemia*: a review. *Artemia* Research and its Applications. 1987. Vol. 3. Ecology, Culturing, Use in aquaculture. Sorgeloos P., Bengtson D. A., Decleir W., and Jaspers E. (Eds). Universa Press, Wetteren, Belgium. 556 p.;

Lowry O.H., Rosebrough N. J., Fare A. Z. and Randall R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275;

Manescu S. 1978. Sanitary and environmental chemistry (Chimia sanitara si a mediului) Ed. Medicala, Bucharest, 54 p. *(in Romanian);*

Maximov V. 2012. Sustainable management of turbot (Managementul durabil al calcanului la litoralul romanesc), Editura Boldas Constanta, ISBN 978-606-8066-40-0, 79-90 (*in Romanian*);

Nenciu (Zaharia) M. I., Rosioru D., Coatu V., Oros A. and Rosoiu N. 2013. Characterization of the Environmental Conditions of the Long-snouted Seahorse Habitat at the Romanian Coast. J. Environ. Prot. Ecol, 14 (4): 1695-1702;

Nenciu M.I., Coatu V., Oros A., Rosioru D., Tiganus D. and Rosoiu N. 2014a. Pollutant Bioaccumulation in the Long-Snouted Seahorse at the Romanian Coast. J. Environ. Prot. Ecol, 15 (4): 1650-1659;

Nenciu M.I., Rosioru D., Oros A., Galatchi M. and Rosoiu N. 2014b. Bioaccumulation of heavy metals in seahorse tissue at the Romanian Black Sea coast: 14th International Multidisciplinary Scientific Geoconference SGEM 2014, at Albena, Bulgaria, Volume: volume 2: 547-554, DOI: 10.5593/SGEM2014/B32/S15.072;

Nita V., Zaharia T., Maximov V. and Nicolae C. 2011. Obtaining the necessary living food for rearing the turbot (*Psetta maeotica*) in its early life stages, Lucrari Stiintifice, Seria D, Vol. LIV, U.S.A.M.V. Bucharest;

Onciu T. 1998. The culture of invertebrates used as feed for economically valuable fish and crustaceans - focus of the Romanian mariculture (Cultura unor nevertebrate utilizate in hrana pestilor si crustaceilor cu valoare economica - obiect al mariculturii romanesti, *PhD Thesis*, Bucharest Institute of Biology, 180 p. (*in Romanian*);

Ortega-Salas A.A. and Reyes-Bustamante H. 2006. Fecundity, survival, and growth of the seahorse *Hippocampus ingens* (Pisces: Syngnathidae) under semi-controlled conditions, Rev. Biol. Trop, Vol. 54, DOI: 10.15517/rbt.v54i4.14082;

Razet D., Faury N., Geairon P., Soletchnik P. and Goulletquer P. 1996. Les Notes Techniques de l'URAPC- NT/URAPC/96-01-02-03, IFREMER, 39;

Rosioru D., Abaza V, Dumitrache C., Filimon A. and Timofte F. 2014, Ecological and Biochemical Aspects of *Mytilus galloprovincialis* from the Romanian Black Sea Coast, 14th International Multidisciplinary Scientific Geoconference SGEM 2014, at Albena, Bulgaria, Volume: volume 2: 569-576, DOI: 10.5593/SGEM2014/B32/S15.075;

Woodall, L.C. 2009. The population genetics and mating systems of European seahorses, *PhD thesis*. Royal Holloway, University of London;

Woodall, L. 2012. *Hippocampus guttulatus*. The IUCN Red List of Threatened Species. Version 2014.3.

<www.iucnredlist.org>. Downloaded on 28 December 2014;

Yankova, M. 2012. Black Sea Fish Checklist: A Publication of the Commission on the Protection of the Black Sea Against Pollution. Accessed online 7.12.2014: http://www.blacksea-commission.org/_publ-BSFishList.asp;

Zaharia T. 2002. Research for elaborating the breeding and rearing technology of flounder and turbot, with the aim of rehabilitating natural populations (Cercetari pentru elaborarea tehnologiei de reproducere si crestere a cambulei si calcanului pentru reabilitarea populatiilor naturale). *PhD thesis*. "Lower Danube" University of Galati (*in Romanian*);

Zaharia T., Alexandrov L., Popa M. and Bilal I. 1998. Observations on the behavior of turbot larvae during early life stages (Observatii privind comportamentul larvelor de calcan in primele etape de dezvoltare). Cercetari Marine/Recherches Marine 29-30, ISSN 0250-3069: 287-293 (*in Romanian*);

Zaharia T., Maximov V., Micu D. and Nita V. 2010. Measures for the management for the protection of the Black Sea horse *Hippocampus ramulosus* LEACH, 1814 at the Romanian littoral. Muzeul Olteniei Craiova. Oltenia. Studii si comunicari. Stiintele Naturii. Tom. 26, No. 2/2010 ISSN 1454-6914: 247-252.