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Implications of microalgae enriched *Artemia* nauplii on the growth and survival of *Hippocampus kuda* (Yellow Seahorse)

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Abstract

The need for nutritional sources safer than traditional animal products has renewed interest generally in plants and particularly in microalgae. However, the implication of enrichment culture and type of microalgae to main staple food *Artemia* for seahorses, under a small-scale laboratory set-up remain ambiguous hence, this study. Herewith, the growth and survival of yellow seahorse, *Hippocampus kuda* exposed into *Artemia* nauplii enriched with different green microalgae species, *Chlorella* sp. and *Scenedesmus obliquus* and unenriched *Artemia* nauplii as a live food control for about 4 weeks with the basic water quality parameters maintained in its optimum range; temperature (28°C), pH at 7.8, and salinity (33ppt) were investigated. Among the three (3) treatment of enrichment, the individuals showed maximum growth in terms of length and weight when fed with *Artemia* enriched with *Chlorella*. The result further shows that the male species of *H. kuda* exhibited a greater rate of growth in terms of weight and length compared to the female species as confirmed in the mean difference of 2.150cm and 1.561cm, respectively. Thus, the greater the average weight and length the better effect of microalgae-enriched *Artemia*. Individuals under the *Chlorella*-enriched treatment also showed a 100% survival rate compared to other set-ups. These findings revealed that *Chlorella*-enriched *Artemia* nauplii are suitable food and enhances survivorship and growth of *H. kuda,* thus potentially providing a great and effective way incorporating more cost effective and reliable microalgae enhanced live foods into seahorse culture.

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Introduction

Seahorses had gained popularity in Traditional Chinese Medicine (TCM) and there is an increasing demand for trading. According to Lourie *et al.* (1999), *Hippocampus kuda* is among the highly valued seahorse species in the Philippines, because of its smooth appearance and usually exhibiting pale yellow color that are much preferred by overseas markets of both traditional medicine and curio trade (Celino *et al.*, 2011). The International Union for the Conservation of Nature included *H. kuda* in its list of vulnerable species. To present, the supply of seahorses cannot meet the increasing market demand, especially with the increasing affluence of Asian consumers, the world's biggest market for seahorses (Vincent, 1996). Wild *H. kuda* and other seahorses from Southeast Asia are the only source of these consumer products. Hence, aquaculture could contribute to satisfy the commercial demand for animals while promoting the recovery of wild stocks (Vincent, 1996; Job *et al.*, 2002; Gardner, 2004; Olivotto *et al.*, 2008; Koldewey and Martin-Smith, 2010; Otero-Ferrer *et al.*, 2010). Apparently, a way to rear them in captivity successfully based on a small-scale laboratory setup, feeding regime and implication of enrichment culture of main staple food remain ambiguous hence, this study. In the past, seahorse-culture attempts have mixed results and advancement of technology is required before captive-bred seahorses can be an economically attainable alternative. To date, seahorse rearing and aquaculture cost-efficient and effective in a small-scale setup remains a challenging and daunting task, especially in Mindanao, Philippines.

Moreover, one of the fundamental bottlenecks in attempting to build up financially practical and organically fruitful commercial seahorse aquaculture is that of giving adequate amounts of nutritionally stable live food. Seahorses are voracious predators, relying entirely on live, moving food. In their natural habitat, seahorses are visual predators that target live prey, for example, amphipods, copepods, mysid shrimp, and caridean shrimp (Reid, 1954; Lovett, 1969; Tipton and Bell, 1988). They will ingest anything that fits into their mouth – mostly zooplankton, small crustaceans and also small fishes – ambushing their prey by inhaling rapidly through their snout. In the captive condition, aquaculturist, researchers, and commercial culturist have depended heavily on the said cultured live foods (Correa *et al.*, 1989). Be that as it may, refined the extensive amounts of live foods required by seahorses in commercial culture proved to be difficult and expensive, and harvesting wild live food is reliant upon an erratic resource. There are recounted reports of aquarists effectively feeding seahorses on artificial foods, such as fish flake and goldfish granules. In addition, there are likewise broad reports of commercial seahorse culturists utilizing artificial foods to some degree, such as shrimp and fish meal based diets (Chen, 1990; Forteath, 2000). But, many researchers have tried enhancing live foods such as *Artemia* with microalgae. The main applications of microalgae for aquaculture are associated with nutrition (as sole component or as food additive to basic nutrients) for coloring the flesh of salmonids and for inducing other biological activities. Microalgae are required for larval nutrition during a brief period, either for direct consumption in the case of molluscs and peneid shrimp or indirectly as food for the live prey fed to small fish larvae (Muller-Feuga, 2000). The most frequently used species are *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema* and *Thalassiosira*. Combination of different algal species provides better balanced nutrition and improves animal growth better than a diet composed of only one algal species (Spolaore *et al.,* 2006). In order to be used in aquaculture, a microalgal strain has to meet various criteria, such as ease of culturing, lack of toxicity, high nutritional value with correct cell size and shape and a digestible cell wall to make nutrients available (Raja *et al.*, 2008; Patil *et al.*, 2007). Protein and vitamin content is a major factor determining the nutritional value of microalgae. There is no concise account yet of what particular species of microalgae is suitable for enrichment or what particular combination would yield maximum growth and survival based on a small-scale laboratory setup hence, this study takes precedence.

This is a pilot study. Basically, this looked into the improvement of rearing of *H. kuda* from Mindanao, based on a small-scale cost-efficient laboratory set-up. Specifically, the utilization of different types of microalgae enriched *Artemia* as a source of food were examined to determine their implication on survival and growth. The present study also identified the most suitable feeding regime of *H. kuda* species in order to improve the efficacy and viability of seahorses. Results of the study is pertinent for aquaculture and tailor-fit conservation efforts for the said species.

Materials and methods

Sampling area, specimens and identification

Seahorse samples were readily available in the laboratory at Premier Research Institute of Science and Technology (PRISM) and donated as live bycatch samples from fishermen. Hence, such opportunity was taken advantage to study the effect of microalgae enriched *Artemia* nauplii on the growth and survival of seahorse, *Hippocampus kuda*. The seahorses were reported to come from Tubod, Lanao del Norte, Philippines. Identification of samples was done through illustrated keys, Guide to the identification of Seahorses (Lourie *et al*., 2004) and consultation of experts. All seahorses were sexed and initial weights and lengths were obtained, and subtracted to succeeding measurements.

Experimental set-up

The aquarium dimension was 50 by 35 by 40 mm in size with three (3) compartments. There were mainly three (3) set-ups, with pair of seahorses for each set-up: Set-up 1: Fed with *Artemia nauplii* enriched with *Scenedesmus obliquus*; Set-up 2: Fed with *Artemia nauplii* enriched with *Chlorella* sp.; and Set-up 3: The control fed with unenriched *Artemia nauplii*. Each set-up was provided with a strong aeration. Experimental parameters such as temperature, salinity, and pH were maintained (Table 1). Based on the experimental design triplicates were conducted.

Hatching and enrichment of Artemia cyst

Artemia nauplii cysts were used for feeding *H. kuda* species of seahorse. The corion of the cysts was removed by decapsulation according to the method of Sorgeloos *et al.,* (1986). Hatching of the decapsulated cysts were done daily in 500 mL improvised conical container (plastic bottled water) in filtered (2µ) and aerated sea water at room temperature. Twenty-four (24) hours after hatching, the nauplii were transferred to the enrichment system.

The enrichment of nauplii was done by feeding with the respective axenic microalgae (*Scenedesmus obliquus* and *Chlorella* sp.) in the logarithmic phase at a concentration of 1.5 $\times 10^6$ cell.ml^{-1} for 24hours. Pure cultures of Microalgae in their respective culture media were also readily available in the laboratory. Subcultures were made to ensure availability throughout the duration of the experimental process. The enriched *Artemia* nauplii were harvested using an improvised siphon made out of drinking straw, washed thoroughly to remove the debris before feeding to the seahorse.

Collection of data

Filtered seawater was changed daily and debris that settled at the bottom were siphoned out without disturbing the animals. For the growth measurement, the researcher used the water displacement method in measuring the weight of the seahorse and standard length method as well as a photosoftware in measuring the length of the juveniles. Standard length (SL) would measure the length of head+trunk+tail (Lourie, 2004) see Fig. 1. from initial day to the termination of the experiment. Survival rate (%) was calculated using a formula:

$$Survival\ Rate\ (\%) = \frac{\text{Final Number of Surviving Seahorse}}{\text{Initial Number of Seahorse}} X\,100$$

Statistical analysis

To analyze and interpret the data, descriptive statistics were utilized such as arithmetic mean as a measure of average and standard deviation as a measure of variability of numerical observations. To test the hypothesis for significant difference in the growth of *H. kuda* using different microalgae-enriched *Artemia*, One-Way Analysis of Variance (ANOVA) was used with p – value <0.05 considered as significant. Moreover, post hoc test for multiple comparisons (Scheffe's Test) was used to determine which among the different microalgae-enriched *Artemia* differ significantly in terms of mean weight and length of *H. kuda*. On the other hand, to test the hypothesis for significant difference in the growth of *H. kuda* between the male and female species across different microalgae-enriched *Artemia*, Independent Samples t test was utilized, Statistical Package for the Social Science (IBM SPSS Statistics 20) was used.

Results and discussion

Effect of Microalgae-enriched Artemia on the growth of H. kuda in terms of length

The growth and survival of seahorse, *Hippocampus kuda* was studied across different microalgae-enriched *Artemia*. Different types of microalgae and *Artemia* nauplii were naturally propagated in culture tanks. The cyst *Artemia*, was enriched with microalgae until it hatched.

Table 1. Controlled parameter measurements of the three (3) set-ups.

Set-up	pH	Water Temperature(°C)	Salinity (ppt)
Set-up 1: *Scenedesmus obliquus* enriched *Artemia*	7.8	28	33
Set-up 2: *Chlorella* sp. enriched *Artemia*	7.8	28	33
Set-up 3: Unenriched *Artemia*	7.8	28	33

Table 2. Summary of the mean measurements for *H. kuda*, seahorse (male and female) reared under different microalgae enriched *Artemia*: Head Length (HL); Trunk Length (TrL); Tail Length (TL); Standard Length (SL).

	Mean HL (cm)	Mean TrL (cm)	Mean TL (cm)	Mean SL (cm)
Control (*Artemia*)				
Male	2.3136	3.7128	5.5608	11.5872
Female	1.809	2.3590	4.7880	11.1950
Chlorella-Enriched *Artemia*				
Male	2.9388	4.3768	8.1208	15.4364
Female	2.5686	4.2446	6.6906	13.7040
Scenedesmus-Enriched *Artemia*				
Male	2.6094	3.9856	7.3012	13.8602
Female	2.8154	3.1330	5.1790	11.1274

Table 2 shows the summary of the mean Head Length (HL), Trunk Length (TrL), Tail Length (TL), and Standard Length (SL) of the *H. kuda* male and female individuals that were exposed to three different microalgae enriched *Artemia* feeding treatment by ranging the values from Week 0 to Week 4 of experimentation.

It was found out that male and female individual in the control set-up has a mean head length (HL) of 2.2316 cm and 1.8090 cm; mean trunk length (TrL) of 3.7128 cm and 2.3590 cm; mean tail length (TL) of 5.5608 cm and 4.7880 cm; and a mean standard length (SL) of 11.5872cm and 7.95600 cm, respectively. On the *Chlorella* enriched *Artemia* set-up, male and female individual has a mean head length (HL) of 2.9388 cm and 2.5686 cm; mean trunk length (TrL) of 4.3768 cm and 4.2446 cm; mean tail length (TL) of 8.1208 cm and 6.6906 cm; and has a mean standard length (SL) of 15.4364 cm and 13.5038 cm, respectively. Male and female individual exposed in *Scenedesmus obliquus* enriched *Artemia* set-up has a mean head length (HL) of 2.6094 cm and 2.8154 cm; mean trunk length (TrL) of 3.9856 cm and

3.1330 cm; mean tail length (TL) of 7.3012 cm and 5.1790 cm; and a mean standard length (SL) of 13.8602 cm and11.1274 cm, respectively.

Fig. 2 shows the mean length (cm) and standard deviation (SD) for male and female individuals across different microalgae-enrichment type. Among the three enrichment types, both individuals under *Chlorella*-enriched *Artemia* have the highest body length of 15.436 cm and 13.704 cm, male and female respectively. Both female individuals under the control and *Scenedesmus*-enriched set-ups had the lowest body length of 11.195 cm and 11.127 cm, respectively.

Table 3. One-way analysis of variance (ANOVA) for growth of *H. kuda*, in terms of length under different microalgae-enriched *Artemia*.

	Sum of sqrs	df	Mean square	F	p
Between groups:	49.427	2	24.713	8.251	**0.002**
Within groups:	77.875	26	2.995		
Total:	127.302	28			

*p<0.05 is significant.

Table 4. Post-hoc test for multiple comparisons (Scheffe's test) on the length of *H. kuda* under different microalgae-enriched *Artemia*.

(I) Group	(J) Group	Mean Difference (I – J)	p – value	Decision	Interpretation
Control	*Chlorella*-enriched	- 3.157**	0.002	Reject H_o	Significant difference
	Scenedesmus-enriched	- 1.081	0.410	Accept H_o	Insignificant Difference
Chlorella-enriched	*Scenedesmus*-enriched	2.076**	0.042	Reject H_o	Significant difference

**p < 0.05 level of significance.

In Table 3 there is a significant difference in the growth of *H. kuda* in terms of length both between groups and within groups across different microalgae-enrichment type (One-way ANOVA: F=8.251; DF=2; p=0.002). Post hoc test for Multiple Comparisons (Scheffe's Test) on the length of the seahorse *H. kuda* using microalgae-enriched *Artemia* (Table 4) revealed a significant difference between the individuals in the Control set-up and in the *Chlorella*-enriched set-up with p =0.002, and also between the individuals in the *Chlorella*-enriched treatment and in the *Scenedesmus*-enriched treatment (p=0.042).

Table 5. Test for significant difference in the growth between male and female *H. kuda* in terms of length across different microalgae-enriched *Artemia*.

	GROUPS	
	Male	Female
Mean	13.628	12.067
SD	1.894	2.138
Mean difference	1.561	
t– value	2.085*	
Degrees of freedom	27	
p– value	0.047	
Decision	Reject H_o	
Interpretation	Significant difference	

* Difference is significant at the 0.05 level (2-tailed).

Meanwhile, there is no significant difference between the Control and the *Scenedesmus*-enriched set-ups (p = 0.410). Moreover, Table 5 shows the test of significant difference in the growth between male and female *H. kuda* in terms of the average length across different microalgae-enriched *Artemia* at 0.05 level of significance using independent samples t - test. The table depicts that t - value = 2.085 and p – value = 0.047. Since p – value is less than the level of significance $\alpha = 0.05$, the null hypothesis is rejected inferring that there is a significant difference in the growth between male and female *H. kuda* in terms of the average length across different microalgae-enriched *Artemia*.

The result further shows that the male species of *H. kuda* exhibited greater rate of growth in terms of length compared to the female species. Herewith, the greater the average length the better effect of microalgae-enriched *Artemia*.

Table 6. One-way analysis of variance (ANOVA) for growth of *H. kuda*, in terms of weight under different microalgae-enriched *Artemia*.

	Sum of sqrs	df	Mean square	F	p
Between groups:	103.251	2	51.626	21.560	0.001
Within groups:	62.257	26	2.395		
Total:	165.508	28			

*p<0.05 is significant.

Table 7. Post-hoc test for multiple comparisons (Scheffe's test) on the weight of *H. kuda* under different microalgae-enriched *Artemia*.

(I) Group	(J) Group	Mean Difference (I – J)	p – value	Decision	Interpretation
Control	*Chlorella*-enriched	- 4.389**	0.001	Reject H_o	Significant difference
	Scenedesmus-enriched	- 0.956	0.471	Accept H_o	Insignificant difference
Chlorella-enriched	*Scenedesmus*-enriched	3.433**	0.001	Reject H_o	Significant difference

**p < 0.05 level of significance.

In this respect, inadequacies in the nutritional profile of the artificial diets and even live foods solely fed (e.g. inadequate levels of certain lipids, proteins or carbohydrates) in relation to the dietary requirements of *H. kuda* could be a causative factor in the typically lower survival and growth of the said species fed with these diets, thus enhancing it with microalgae proved to have a better effect on the growth and survival of the yellow seahorses. It seems that depending on the sex of the seahorse and of the type of microalgae enriched to *Artemia*, the *H. kuda* species sometimes had lower growth and survival rates.

Based, on this study, it was revealed that male possessed the highest rate of growth in terms of length across different treatment.

Table 8. Test for significant difference in the growth between male and female *H. kuda* in terms of weight across different microalgae-enriched *Artemia*.

	Groups	
	Male	Female
Mean	5.783	3.633
SD	2.562	1.757
Mean difference	2.150	
t– value	2.617*	
Degrees of freedom	27	
p– value	0.014	
Decision	Reject H_o	
Interpretation	Significant difference	

*Difference is significant at the 0.05 level (2-tailed).

Effect of microalgae-enriched Artemia on the weight of H. kuda

Fig. 3 depicts the mean weights and the standard deviation of the male and female individuals in different microalgae-enrichment type. Individuals under the treatment of *Chlorella*-enriched *Artemia* were the heaviest among the three (3) treatments with 8.792 g for the male and 5.79 g for the female. Male individual under the control set-up and the female individual exposed in *Scenedesmus*-enriched treatment were the least heavy individuals with 2.882 g and 2.04 g of weight, respectively.

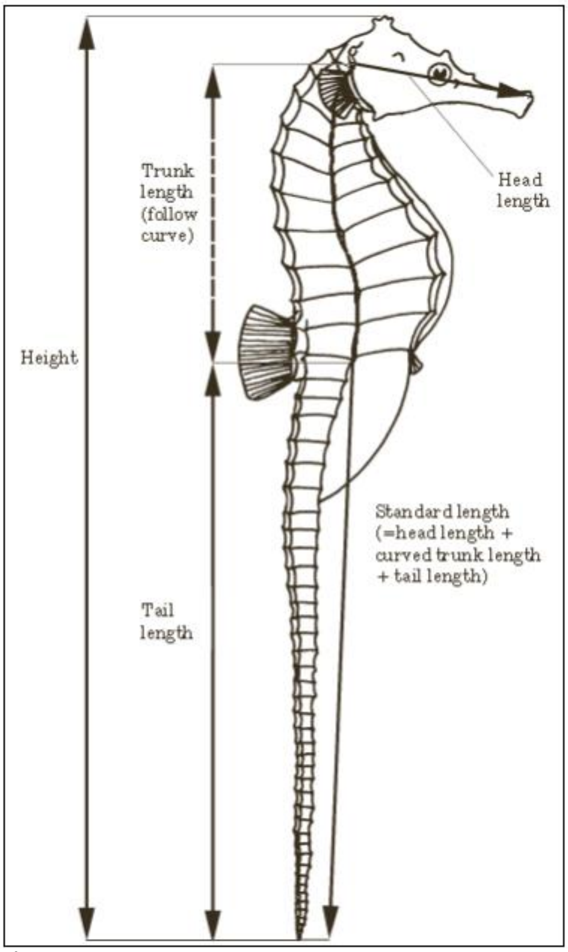

Fig. 1. Standard length (SL) as measured on a seahorse (figure by S. A. Lourie).

Table 6 illustrates the one-way analysis of variance (ANOVA) to test the hypothesis on the equality of mean weight of *H. kuda* as a measure of growth using the different microalgae-enriched *Artemia* at $\alpha = 0.05$ level of significance. Accordingly, the test yields an F – value = 21.560 with a p – value = 0.001 which emphasizes that the p – value is less than the level of significance $\alpha = 0.05$. Thus, the null hypothesis is rejected inferring that there is a significant difference in the average weight of *H. kuda,* as a measure of growth using the different microalgae-enriched *Artemia*.

Table 7 portrays the post hoc test for multiple comparisons in the average weight of *H. kuda* as a measure of the growth using the different microalgae-enriched *Artemia*. Scheffe's Test enables to determine which of the three different treatments differ significantly in the mean weight of *H. kuda*. Results show that the control group does not differ significantly with *Scenedesmus*-enriched treatment in their effects to the weight of *H. kuda* as apparently shown in the resulting p – value of 0.471. Nevertheless, the control group entails a significant difference with the *Chlorella*-enriched treatment as revealed in the p – value of 0.001. Similarly, the *Chlorella*-enriched treatment posted a significant difference with the *Scenedesmus*-enriched treatment in their effects to the weight of *H. kuda* as apparently shown in the resulting p – value of 0.001 which is lower than $\alpha = 0.05$ level of significance.

Table 8 illustrates the test of significant difference in the growth between male and female *H. kuda* in terms of the average weight across different microalgae-enriched *Artemia* at 0.05 level of significance using independent samples t - test. The table depicts that t - value = 2.617 and p – value = 0.014. Since p – value is less than the level of significance $\alpha = 0.05$, the null hypothesis is rejected inferring that there is a significant difference in the growth between male and female *H. kuda* in terms of the average weight across different microalgae-enriched *Artemia*. The result further shows that the male species of *H. kuda* exhibited a greater rate of growth in terms of weight compared to the female species as confirmed in the mean difference of 2.150. Thus, the greater the averages weight the better effect of microalgae-enriched *Artemia*. Hereby, the male species obtained maximum growth under *Chlorella*-enriched *Artemia*.

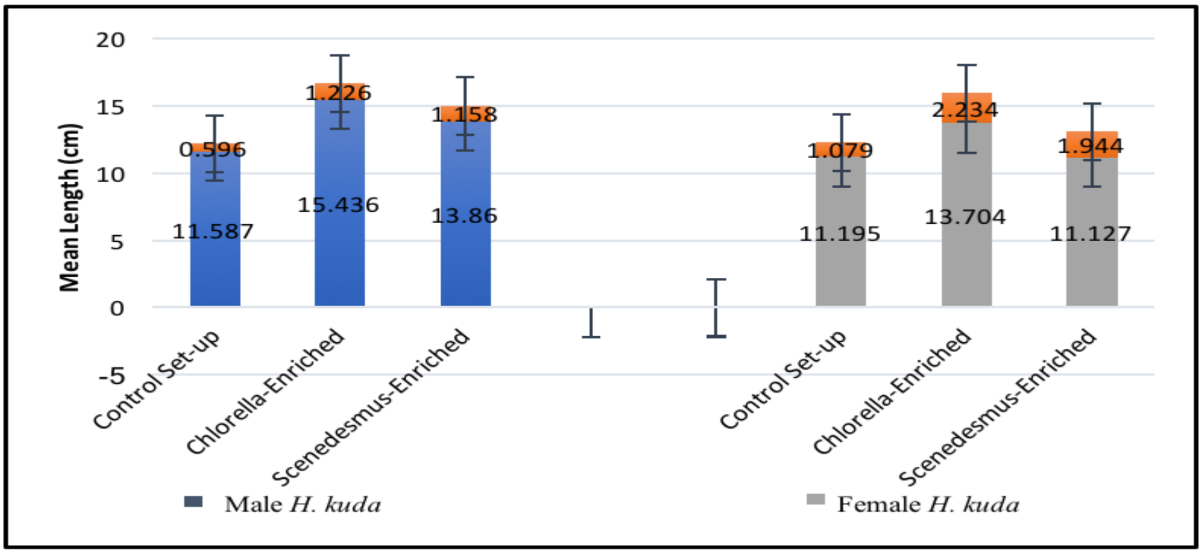

Fig. 2. Mean length (cm) and the standard deviation of the male and female individuals of *H. kuda* in different types of microalgae-enrichment.

Accordingly, in the study of Vincent (1994), male seahorses compete more intensely than females for access to mates, on both the first and final days of courtship and even through their feeding behavior. Competing males are more active than competing females in those courtship and competitive behaviors common to both sexes, and only males exhibit uniquely competitive behaviors (wrestling and snapping) (Vincent, 1994). Also, males which succeeded in copulating are heavier than their rivals and copulating seahorses of both sexes generally are more active in courtship and competition than are their unsuccessful rivals (Vincent, 1994).

Through this, it can be inferred that male seahorses are more competitive by nature, not only in courtship but also in their feeding behaviors which proved to survive longer in the wild (Olivotto, 2001).

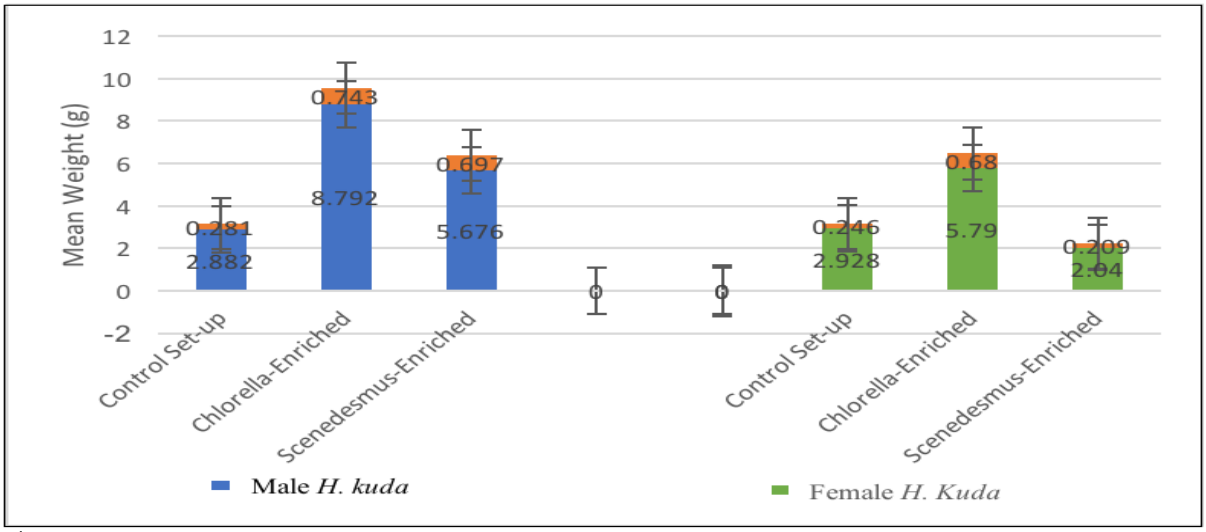

Fig. 3. Mean weight (g) and the standard deviation of the male and female individuals of *H. kuda* in different types of microalgae-enrichment.

Noteworthy, in this study *H. kuda* individuals exposed to *Chlorella*-enriched *Artemia* treatment had the highest growth in terms of length and weight and also in survival rate compared to the *Scenedesmus*-enriched treatment and to the individuals fed with *Artemia* nauplii alone. Herewith, *Artemia* nauplii proved to be an incomplete food source because of the low levels of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6u-3) generally thought to be required for successful of crustacean larvae (Sorgeloos, 2001). Moreover, in a study of Leema et.al (2002) in phyllosoma larvae, it suggests that future *Artemia* enrichments should combine bactericidalmicroalgae with other enrichments like vitamin enhanced oil emulsions for enhancing the survival rate of phyllosoma. Antibacterial activity of the microalgae should be examined in future *Artemia* enrichment experiments. Research showed that combination of different algal species provides better balanced nutrition and improves animal growth better than a diet composed of only one algal species (Spolaore *et al.*, 2006).

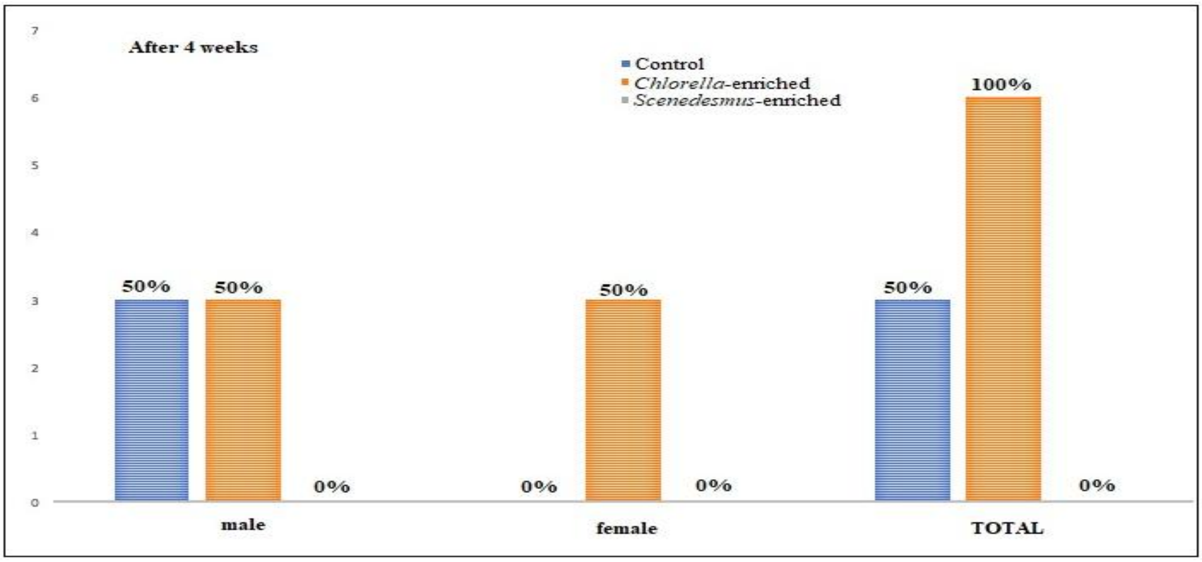

Fig. 4. Survival rate of male and female individuals of *H. kuda* in different types of microalgae-enrichment.

Effect on the Survival Rate of H. kuda among the different Microalgae-enriched Artemia

In addition, it was observed that individuals under the *Chlorella*-enriched treatment showed a 100% survival rate compared to other treatments. Samples exposed in the unenriched treatment, yield a 50% survival rate (Fig.4).

Selection of microalgae species for enrichment purposes can be vital for rearing seahorses. The high production cost of microalgae remains a constraint to many hatcheries. Improvements in alternative diets may continue but production costs of microalgae may also decrease due to the uptake of new technology by hatcheries. A good selection of microalgal species is also available to support the aquaculture industries (Elı´as *et al.,* 2003).

In this study, *Chlorella* proved to be effective. This microalgae species is an advantage because it is easily found in the wild. It can grow in waters high in nitrate and phosphate levels under direct sunlight. Availability and sustainability will not be a problem. In the aquaria, it can create green and opaque water problems. However, it is good for enrichment of *Artemia* since it is high in protein and other essential nutrients. It is easily cultured and mass-production methods are now being used in artificial media and large artificial circular ponds.

Conclusion

Among the three (3) treatments observed in this study, *H. kuda* individuals showed maximum growth in terms of length and weight when fed with *Artemia* enriched with *Chlorella*. Results yield, that the male species exhibited a greater rate of growth in terms of weight and length compared to the female species. Herewith, the greater the average weight and length the better effect of microalgae-enriched *Artemia*. Individuals under the *Chlorella*-enriched treatment showed a 100% survival rate compared to other set-ups. *Chlorella* proved to be effective.

This microalgae species is an advantage because it is easily found in the wild. It can grow in waters high in nitrate and phosphate levels under direct sunlight. Availability and sustainability will not be a problem. It proved to be high in protein and other essential nutrients and it is easily cultured. Hence, the ability to utilized live foods like *Artemia*, enhanced with microalgae in rearing seahorse species and even other marine organisms has important implications for the commercial culture. The use of this technique can dramatically reduce material and labor costs, which in turn can potentially increase the viability of commercial operation.

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