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Research article

Artemia as a sustainably cultured live feed for ornamental fish in zoological institutions with immunostimulant properties when bioencapsulated with spirulina *Arthrospria platensis*

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Abstract
Sustainable in-house cultures of bio-enriched live feeds for ornamental fish can enhance development, nutrition and welfare of the animal by improving immunity while further reducing economical costs to zoological institutions. *Artemia* is an easily harvestable feed with its nutritional profile highly dependent on its diet which is easily manipulated through bioencapsulation. This study evaluates the effects of two types of commonly used feeds for *Artemia*: spirulina and yeast, and how this affects the growth, feed conversion ratio (FCR), survival rate, immunity and colour intensity of ornamental fish, compared to a commercial pellet (control) diet. A total of 198 mature, mixed sex serpae tetra *Hyphessobrycon eques* were subjected to the three different diet treatments conducted in duplicate. There was no significant difference in growth weight, FCR, lysozyme, myeloperoxidase and alkaline phosphatase between treatments. Protease index of activity was significantly lower in fish fed with *Artemia* bio-enriched with spirulina (71.48%) and highest in the control diet (95.48%). The colour intensity and redness of the fish also significantly increased when fed *Artemia* bio-enriched with spirulina versus *Artemia* bio-enriched with yeast or a control diet.

Introduction

Satisfying the nutrient requirements of ornamental fish is essential to their health and welfare. Optimal nutrition, on the other hand, may go beyond simply meeting requirements and has been shown to improve immune function and reproduction, and to support the performance of natural behaviours (Craig and Helfich 2002; Cordoba 2011). A complete commercial diet should thus supply all essential nutrients in adequate proportions to reach an optimal state of health. Recent advancements in fish nutrition have led to the production of diets fortified with specific functional ingredients that may have various beneficial effects, such as probiotics, prebiotics and immunostimulants (Oliva-Teles 2012). This is important as evaluating the health of the fish solely through visual observations can be limited and subjective, while immunological parameters may provide quantifiable results (Oliveria et al. 2001; Goncalves et al. 2008). Parameters include lysozyme and the protease index of activity as well as carotenoids. Lysozymes possess bactericidal properties and are an important defence molecule of the innate immune system which protects the fish against microbial invasion (Saurabh and Sahoo 2008). Proteases are enzymes that catalyse proteolysis, the breakdown of proteins into smaller polypeptides. Proteolysis is a critical process required for many aspects of pathogenesis and often observed by obligate intracellular pathogens which have

highly regulated life cycles inside host cell environments (Li et al. 2012). Host protease inhibitors will modulate protease activities and control various critical protease-mediated processes which include resistance to infectious agents. Carotenoid pigments are biologically active compounds that have beneficial effects on the metabolism of fish, providing protection against several stressors (Carvalho and Caramujo 2017). Additionally, carotenoid-based body colouration has been positively correlated with reproductive success and social dominance (Maan and Sefc 2013), where fish use carotenoid-driven colouration cues to evaluate the health and vigour of prospective mates. Aquatic animals conspicuously accumulate carotenoids in their gonads, which are assumed to be essential for reproduction and the successful development of eggs and early larval stages (Maan and Sefc, 2013). He et al. (1999) showed that the addition of spirulina to feed can enhance the colour of crucian carp *Carassius carassius*. Nya and Austin (2009) also found that adding garlic to feeds for rainbow trout *Oncorhynchus mykiss* resulted in stimulation of erythrocytes and leukocytes, significantly boosting haematocrit, enhancing phagocytic activity, respiratory burst, lysozyme, anti-protease and bactericidal activities. However, such feeds must be custom made and can carry high maintenance costs for aquariums. A sustainable culture of feed that can be bio-enriched with different feeds may be more economical and physiologically appropriate to meet exhibit fish needs.

Artemia, known colloquially as brine shrimp, is a genus of crustacean that can be found in marine and saline lake environments in many parts of the world. Due to multiple factors including nutritional profile, ease and convenience of propagation, and ready acceptance by a wide range of aquarium and aquaculture species, *Artemia* has been used as feed for the culture of many different species of commercially important fish and crustaceans, including sea bream *Pagrus pagrus*, sea bass *Centropristis striata*, wolf fish *Anarhichas lupus*, and cod *Gadus morhua* (Stottrup and McEvoy 2003). *Artemia* biomass can be harvested through aquaculture or natural salt ponds or lakes (Baert et al. 1996) before being frozen, flaked, dried, or incorporated into compound diets. Larval *Artemia* (*Artemia* nauplii) grow and differentiate through 15 different moults, with the first moult occurring just 8 hr after hatching (Stottrup and McEvoy 2003). Adulthood is reached between 2–3 weeks depending on food availability, but it can be as quick as 8 days under optimal conditions (Baert et al. 1996). Adults, under the right conditions, can live for several months and reproduce at a rate of up to 300 nauplii every 4 days per adult (Baert et al. 1996). However, when environmental factors deteriorate, such as decreases in temperature or when food is scarce, the females release dormant cysts, which are embryos protected in a shell and arrested in development until conditions ameliorate. The presence of cysts thus generally indicates poor water quality for reproduction. These traits promote *Artemia* as an easy and possibly sustainable harvestable feed culture. The ease of transporting, storing and hatching *Artemia* cysts, together with the small size and active motion of *Artemia* nauplii, render it a convenient food source for freshly hatched fry of many freshwater and marine species. As live feed, it can provide a more enriching feeding experience to fish compared to commercial pellets and has a lesser risk of carrying diseases compared to other commonly used live feed choices (such as water fleas, *Moina* sp.) while still possessing comparable nutritional values (Lim et al. 2001). Stottrup and McEvoy (2003) performed a proximate analysis of *Artemia* and found that adults had 9.2 to 20.2% higher protein and energy content compared to freshly hatched nauplii. Additionally, their nutritional content can be easily manipulated through bioencapsulation (or gut loading), allowing certain nutrients to be passed on to the fish more readily. Being a non-selective filter feeder with a relatively high ratio of gut content to body volume, the composition of its nutritional profile can be highly dependent on its diet (Baert et al. 1996).

Spirulina and yeast are two commonly used feeds for *Artemia*. Both are ideal feeds as the particle size composition are less than 50 µm (Storrtup and McEvoy, 2003). Spirulina is known for being growth-promoting, immunomodulatory, and for providing high antioxidant support in fish. It can also improve and intensify pigmentation in some fish (Guroy et al. 2012). Yeast is a cheaper alternative and can be administered for relatively long periods without causing obvious immunosuppression. Studies also showed that fish fed with brewer's yeast were found to have significantly higher feed efficiency (Li and Gatlin 2003).

This study aims to evaluate the effects of two different types of bio-enriched *Artemia* as ornamental fish feed by assessing and comparing the health and condition of the fish. Growth, feed conversion ratio, survival rate, immunity markers and colour intensity in the fish serve as indicators for health. Positive effects may provide evidence for breeders and aquariums to replace commercially purchased feed with a more economical and sustainable option in the form of bio-enriched *Artemia* colonies, sustainably bred in-house.

Methods

Harvesting of Artemia

An air-water lift system with valve was constructed at the River Safari (Singapore) and attached to a conical 6.0 l bottle filled with 5.0 l of saltwater, adjusted to the optimum conditions for hatching *Artemia* described by Kumar and Babu (2015) (25 ppt, pH 8.0, 25–28°C). Strong illumination was added (>2000 lux) and cysts were stocked at a density of 1.0 g/l. Cysts hatched between 18–36 hr. The hatched nauplii were separated from hatching waste, with a fine mesh screen filter (<150 µm) and placed into a 1 l bottle of saltwater with aeration. Number of nauplii present was extrapolated from 1 ml of water, viewed under a microscope. Nauplii were then separated into two 40.0 l saltwater tubs, where salinity has been raised to the median optimal value for growth suggested by Baert, Bosteels and Sorgeloos (1996) (40 ppt, pH 8.0, 27°C). Water changes (20%) were performed weekly, with water quality monitored and maintained at normal parameters (ammonium <0.19 mg/l, nitrite <5.0 mg/l, nitrate <50.0 mg/l, pH >7.5). Cultures in each tub were fed exclusively either 0.25 g of powdered spirulina *Arthrospira platensis* (Brine Shrimp Direct, USA) or 0.25 g of yeast *Saccharomyces cerevisiae* (Origins Healthfood, Singapore) once daily, during the study. *Artemia* life stages and lengths were measured three times a week and population tracking was recorded.

Experimental design

Six 45.0 l freshwater tanks with aeration, were each stocked with 33 mixed-sex serpae tetra *Hyphessobrycon eques*. Subsequently, 10 random fish were selected from each set up to provide an initial weight. During the experiment, water quality in each tank was monitored and maintained at normal parameters (Ammonium/ Nitrite/Nitrate <0.01 mg/l, pH=7.0). Three treatments, each with a duplicate, were tested. Fish were fed one of three treatments: adult *Artemia* enriched with spirulina, adult *Artemia* enriched with yeast, adult *Artemia* with the control diet (Tetra, TetraMin Pellets). Fish in each tank were hand-fed 0.50 g of feed, once daily with the trial lasting for 30 days.

Colour analysis and immune assays

After 30 days, five fish from each tank were weighed and euthanised by submerging them in a water bath containing tricaine methanesulfonate (MS-222) at a concentration of 3.0 g/l by veterinary staff. The tail fins were scanned with a CM-700d

Table 1. Growth performance of serpae tetra after 30 days of feeding with different diet treatments. FCR refers to Food Conversion Ratio.

Treatment	Weight gain	Survival rate	FCR
Control	0.71±0.05	100%	10
Spirulina	0.80±0.12	100%	4
Yeast	0.73±0.12	100%	7.2

Spectrophotometer (Konica Minolta Sensing Inc., Osaka, Japan) with an average of three readings for colour intensity, dL* and redness, da* recorded for each fish, at 450 nm (using methods of Yun, 2010). The reference points for desired colour intensity and redness were set at dL*, -54.05 and da*, 46.34, respectively, values determined from an identical scan of the red colour of the Singapore flag.

The fish were then homogenised, and the supernatants collected and stored at -19°C. During analysis, the homogenates were thawed on ice to prevent enzyme denaturation. The Infinite 200 Pro (Tecan, Switzerland) and Tecan i-control application (version 1.12.4.0) were used on the fish homogenates to assay various innate immune response parameters.

For protease, methods of Lazado et al. (2015) were used. Equal volumes of serum/skin mucus and 100 mM ammonium bicarbonate buffer (AB, pH 7.8) containing 0.7% azocasein (Sigma, Steinheim, Germany) were mixed and incubated for 20 hr at 28°C. The protein content of serum/skin mucus was determined using bovine serum albumin as a standard (Atlantis Bioscience, Singapore). The reaction was stopped with the addition of 4.6% tricholoroacetic acid and followed by centrifugation at 10,000 rpm for 10 min. The supernatants were pipetted to a plate with a flat bottom and mixed with 100 µl 0.5 N sodium hydroxide (NaOH) (per well). The optical density (OD) of the reaction mixture was determined at 450 nm. Buffer solution with 0% protease activity was used as the negative control.

For myeloperoxidase we used the methods of Mohanty et al. (2007). A volume of 10 µl of serum was diluted with 90 µl of Hank's balanced salt solution without Ca2+ or Mg2+ in a microtitre plate and mixed with 35 µl of 20 mM 3,3',5,5'- tetramethyl benzidine hydrochloride (Merck, Singapore) and 5 mM H2O2. The reaction was stopped by adding 35 µl of 4 M sulphuric acid, after 2 min. Optical density was read at 450 nm.

For alkaline phosphatase (ALP), the method of Apines-Amar et al. (2004) was employed. ALP activity was determined by adding 15 µl of the diluted sample to p-nitrophenyl phosphate (Merck, Singapore) and incubated at 20°C for 30 min, after which the reaction was stopped by adding 2 ml of 3 N NaOH. The change in absorbance at 405 nm was measured.

For lysozyme activity, the methods of Sitja-Bobadilla et al. (2008) were employed. The substrate for the serum was lyophilised *Micrococcus lysodeikticus* (0.3 mg ml^{-1}) (Merck, Singapore) and 0.05 M sodium phosphate buffer. Triplicates of test serum (diluted 1:8, 10 µl) were added to 200 µl of the *M. lysodeikticus* suspension solution and the reduction in absorbance at 450 nm was measured after 0.5 and 4.5 min.

Statistical analysis

The growth, feed conversion ratio (FCR) and survival rate of fish in each treatment tank were recorded. To determine if there was a significant difference between treatments, a Welch's ANOVA test was conducted along with Games-Howell post hoc tests, where appropriate. A significance level of 95% ($P < 0.05$) was used.

Results

Artemia *growth rate*

The culture set up saw *Artemia* nauplii reached adulthood (0.8 cm) within 12 to 15 days. There was no observable difference in growth rates between *Artemia* fed with spirulina or *Artemia* fed with yeast.

Fish health and colouration

Fish fed the experimental diet were found to have slightly higher growth and feed conversion ratio (FCR); however, these differences were not statistically significant (Table 1).

The Welch's ANOVA test performed on these variables showed that results for Protease ($FW(2,27)=4.827$, $P=0.036$), colour intensity, dL* ($FW(2,27)=2.361$, $P=0.001$) and redness, da* ($FW(2,27)=7.283$, $P=0.009$) were significantly different while lysozyme, myeloperoxidase and alkaline phosphatase were not (Figures 1 and 2).

Protease activity was significantly lower in fish fed spirulina-enriched *Artemia* (71.48±22.09%) compared to both fish fed yeast-enriched *Artemia* (80.40±19.84%, $P=0.026$) and the control diet (95.48±13.69%, $P<0.001$). Colour intensity, dL* and redness, da* were significantly higher, and closest to the reference points set, in fish fed the spirulina-enriched *Artemia* compared to those fed yeast-enriched *Artemia* or the control diet ($P<0.001$ for both).

Discussion

Artemia can be a successful and complete diet for ornamental tetras and depending on the functional food they were bioencapsulated with, can provide some form of immunomodulatory benefits as well as enhance pigmentation compared to fish fed a control diet of commercial fish feed. Bioencapsulated live feed also provides enrichment through visual and chemical stimulation. These benefits may allow *Artemia* to be an interesting supplement to commercially available pellets in improving the health of captive fish. Despite the minimal culture and harvest set up in this experiment, *Artemia* was readily harvested with high yield, as long as the right abiotic factors (e.g., temperature, salinity,

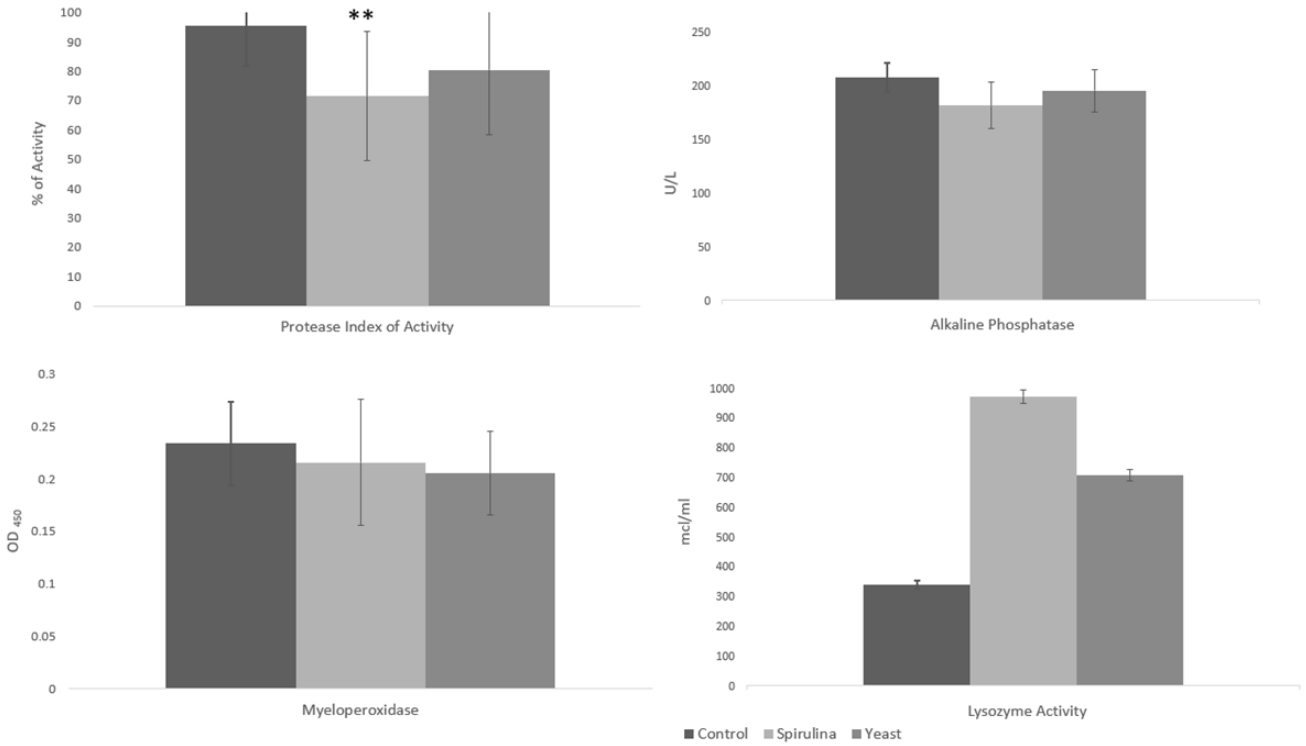

Figure 1. Immunological parameters of Serpae tetra fed either *Artemia* bioencapsulated with yeast, spirulina or a control commercial fish feed (Tetra brand) with standard deviation bars. ** shows significant difference between the treatments at P<0.05.

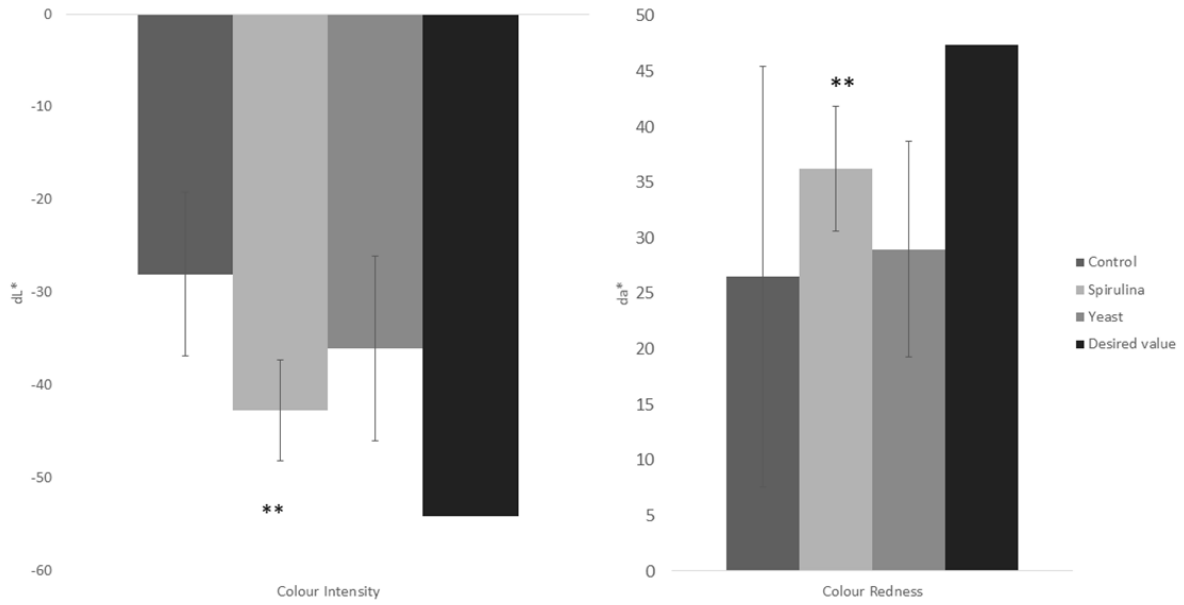

Figure 2. Colour intensity and redness of Serpae Tetra fed three different diets (*Artemia* bioencapsulated with yeast or spirulina or a control diet of a commercial fish feed, Tetra Brand), measured against the reference point (desired value) set with standard deviation bars. ** shows significant difference between the treatments at P<0.05.

pH, dissolved oxygen and other water quality parameters) were satisfactorily met (Stottrup and McEvoy 2003). This is particularly advantageous for systems set up in tropical environments or in climate-controlled facilities where the temperature can be kept consistent. Furthermore, in zoological institutions or aquaculture facilities where purchase and maintenance costs for feeds can be high, along with higher costs associated with providing medical care, maintaining a sustainable breeding culture of invertebrates as feed can potentially be more cost-effective.

Growth

No mortality or illness were observed during the study. Thus, it can be assumed that all treatments at least met the minimum nutrient requirements of the fish. Descriptive statistics within the study suggests that the spirulina treatment provided the greatest number of positive effects compared to the other two treatments. However, no significant differences were observed in growth weight between treatments. The feed conversion ratio (FCR), a mathematical relationship between the input of the feed fed and the weight gain of a population, was also not significant between treatments. This was a limitation of the study since the feeds were fed as is, and the moisture content varies greatly between each diet treatment. The higher moisture content of *Artemia* (95.4%) (Feher et al. 2013) compared to the control diet (8%) (Tetra, TetraMin Pellets) may illustrate the efficiency of the bio-enriched live feed since no significant differences were observed. Alternatively, this could be due to the limited growth potential of the fish since those involved in the study were already at sub-adult life stages or older. Red sea bream *Pagrus major* and giant prawn *Macrobrachium rosenbergii* have also been found to show pronounced effects and elevated growth performance and feed efficiency when fed spirulina (Mustafa et al. 1994; Nakagawa and Gomez-Diaz 1995).

Health

In fish fed with spirulina-enriched *Artemia*, the protease index of activity was significantly lower. This suggests an enhanced immune function compared to the yeast-enriched *Artemia* and the control diet, further supported by studies highlighting the common use of spirulina as an immunostimulant in aquaculture (Zhang et al. 2019). A lower protease index of activity indicates reduced proteolysis by harmful pathogens (Li et al. 2012), while a higher lysozyme activity in the blood indicates enhanced innate immunity in fish (Grinde et al. 1988). High activity can be particularly desirable in aquaculture or aquarium settings where fish are kept in high-density living situations with generally higher bacterial loads (Grinde et al. 1988). Lysozyme activity was also found to be higher in Nile tilap*ia Oreochromis niloticus* at the inclusion of spirulina in their diet (Ragap et al. 2012; Ibrahem et al. 2013). All manner of non-specific immune cell efficiency seems to be affected by spirulina, such as increasing the phagocytosis activity of *O. niloticus* (Abdel-Tawwab and Ahmad 2009) or increasing the count of immune cells such as more white blood cells in carp *Labeo rohita* fingerlings (Andrews et al. 2011). These beneficial physiologic changes may be attributable to the concentrated carotenoids found in spirulina, which stimulate lymphocytes and increase the protection of macrophages against inhibition from other substances (Chew et al. 1997). Spirulina is also a potent antioxidant, able to reduce the toxic effects of subacute deltamethrin (DLM) intoxication, and contains high concentrations of vitamins such as B6 and E, which support biochemical reactions in the immune system (Abdelkhalek et al. 2014). Taken together with these results, this body of research indicates that spirulina is likely to be a more useful bio-enrichment feed for *Artemia* than yeast as it has more reported health benefits. Although the financial costs of obtaining *Artemia* bio-enriched with spirulina may not be lower than a pellet diet (if the diet cannot be sourced in-house), the reduced veterinary costs could result in net savings overall.

Colouration

Colour intensity, dL* and redness, da* were also significantly higher in fish fed with spirulina-enriched *Artemia*. These values were also closer to the reference points set (the red colour of the Singapore flag). This result was expected due to the increase in diet carotenoid concentrations from spirulina algae (Guroy et al. 2012) compared to yeast, which does not contain any carotenoids (Conlon et al. 2013).

While the descriptive results were interesting, results from the statistical analyses were smaller than expected, perhaps due to large deviations within the sample data (Lim et al. 2015). A larger sample size or the implementation of immune assays with greater specificity can be used in future studies to overcome these limitations. Use of mucosal immunity assays would also allow larger sample sizes without requiring euthanasia of the animals (Adel et al. 2016).

Conclusion

Better understanding the effects of bio-enriched live feed for captive ornamental fish can improve their health and development. This study shows that the bioencapsulation of *Artemia* with spirulina to be used as omnivorous/carnivorous fish feed has the potential to be beneficial. Nutrients present in feed ingested by *Artemia* can be passed on efficiently to fish that consume it in a highly bioavailable format (Baert et al. 1996). This allows aquarists to manipulate the nutritional composition of aquarium feed while perhaps increasing some aspects of immunity and colouration of fish and overcoming certain economic challenges, since custom-made commercially available pellets are often expensive. Therefore, sustainable, in-house, bio-enriched live feed cultures may supplement commercial fish feeds providing the animals with a comprehensive diet at reduced financial costs to zoological institutions.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal have been adhered to and received approval from Singapore's Animal Welfare and Ethics Committee (AWEC) to which WRS subscribes. A project proposal was submitted from WRS to AWEC directly via e-mail and received ethical approval. Authors confirm this is consistent with the EU standards for the protection of animals used for scientific purposes.

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