Optimised Selenium Enrichment of Artemia sp. Feed to Improve Red Drum (Sciaenops ocellatus) Larvae Rearing

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ABSTRACT:

Selenium is an essential microelement for the normal functioning of life processes. Moreover, it is a component of enzymes with antioxidant effects. However it has the smallest window of any micronutrient between requirement and toxicity.

Selenium is a regularly used element in fish feeds; moreover, enriching zooplankton with selenium to rear larvae is also a well-known technology. It is recognized that the most common starter foods of fish larvae, natural rotifers contain the smallest dosage of selenium, but providing selenium enriched *Artemia* sp. instead could increase survival and growth rate of fish. However, no such references are available the red drum (*Sciaenops ocellatus*) larvae. Therefore, in this study, *Artemia* sp. was enriched with nano-selenium of verified low toxicity and easy availability in 5 treatments (1, 5, 10, 50, 100 mg/l Se), and then, fish larvae were fed with four of these enriched *Artemia* stocks (1, 5, 10, 50 mg/l Se) and a control group. At the end of the 9-day-long experiment, survival rate (S) and growth parameters (SL, W, K-factor, SGR) of fish larvae were calculated as well as their selenium retention and glutathione peroxidase enzyme activity were analysed. It was revealed that a moderate level of selenium enrichment (~4 mg/kg dry matter) of *Artemia* sp. positively influences the rearing efficiency (i.e. survival and growth) of fish larvae, but higher dosages of selenium could cause adverse effects.

Keywords: nano elemental selenium, red drum, *Artemia* sp., enrichment, optimum

INTRODUCTION

Selenium is an essential element for the normal functioning of life processes [20].The importance of this element was revealed in 1973 when [8] and [34] discovered that selenium was an essential component of the enzyme glutathione peroxidase. Four selenium-containing glutathione peroxidases are known, namely, the cytosolic (GPx1), gastrointestinal (GPx2), plasma (GPx3), and phospholipid hydroperoxidase (GPx4) [3]. Each of them is an individual selenoprotein, but all of them have antioxidant effects. Thus, selenium is a component of enzymes with antioxidant effects, unlike vitamin E and C, which are antioxidants that function independently of enzymes [4].

Selenium deficit in fish can easily lead to reduced growth rate [40], increased oxidative stress [9, 2] and increased mortality [9]. Although selenium is essential for vertebrates [17], it has the smallest window of any micronutrient between requirement and toxicity [5, 32, 26], however the chemical form affects both its bioavailability and potential toxicity [38]. Selenium from selenomethionine has a higher retention [25, 16, 35] and bioavailability in fish [40] than selenium from selenite. Selenium toxicity is caused mainly by selenium in its ionic forms [23], which may explain why selenomethionine, that contains inert selenium until its carbolization, is less toxic than selenite and selenate. Better availability of organic forms in contrast to inorganic ones was demonstrated in Atlantic salmon *(Salmo salar)* [1, 25] and channel catfish *(Ictalurus punctatus)* [40].

According to the latest research, red elemental selenium in nano-sized particles is similar in its efficiency to organic selenium forms, while its potential toxicity is lower [41]. The redox potential of elemental selenium is zero, it is not water-soluble and generally considered to be biologically inert, and thus its toxicity can be lower than that of other selenium forms [43]. Nano-sized elements – including selenium – can have new characteristics, just like an expanded surface and high reactivity [15], which may contribute to their further utilization.

While feeding with lipid-enriched *Artemia* sp. (Crustacea, Anostraca) has been demonstrated to increase larval rearing success for many fish species [22, 44, 7, 11, 18], the effects of mineral enriched *Artemia* sp. on larval rearing of fish is still poorly known. [12] demonstrated that one of the largest nutritional differences between planktonic crustaceans (copepods in that case) and rotifers lies in their mineral composition. The largest differences could be found in case of the selenium, because selenium levels in rotifers were more than 30 times lower than in copepods [12] and 3–8 times lower than the selenium requirement of juvenile fish, equalling 0.25–0.7 mg Se kg^{-1} DW [30]. Furthermore, it is generally known that the mineral content of the wild-reared saltwater zooplankton is higher than that of artificially reared zooplankton [28, 13] because of the nutrient-rich seawater. Consequently, using selenium enriched crustaceans (e.g. *Artemia* sp.) can be beneficial in the larval rearing of certain fish species.

Selenium requirement of fish species is different [6]; therefore, the purpose of the present study was to investigate the optimal selenium enrichment level of the diet in the widely cultured red drum (*Sciaenops ocellatus*) larvae, which has not been reported yet. In the present study, *Artemia* sp. was enriched with nano-selenium in 5 treatments compared to the control. Four of these enriched *Artemia* sp. stocks were then tested relative to the untreated control food in a 9-day-long feeding trial with red drum larvae, where survival and growth parameters (length, weight, K-factor) of fish were calculated, as well as the selenium retention and the activity of the enzyme glutathione peroxidase were analysed.

MATERIALS AND METHODS

Preparation of the Selenium Solution and *Artemia* Enrichment

The solution used to enrich *Artemia* sp. contained nano elemental selenium (60-80 nm). Its production was based on a new ascorbic acid reduction method: 2.5 litres of 2000 mg/l

selenite solution were mixed with the same amount of 10000 mg/l ascorbic acid solution and the reduction proceeded for 30 minutes. Five litres of 1000 mg/l nano-selenium stock-solution were diluted for the preparation of the different solutions for the treatments.

After a 24-hour incubation period [29], newly hatched *Artemia* sp. (Sera, Germany) were enriched with nano-selenium in 5 treatments (1 mg/l; 5mg/l; 10 mg/l; 50 mg/l; 100 mg/l) compared to the control, each treatment being set up in three replications (n=3). The experiment was carried out in plastic tanks (with 4 l water volume each) with a permanent light regime and 100% oxygen saturation. The salinity was 20 ppt (Sera Marin Basic sea salt, Germany) and the water temperature was constant (28 ± 1 ^{o}C, Sera water heater). 100-150 *Artemia* sp. larvae per ml were stocked into each tank. After 24 hours, the enriched *Artemia* sp. were filtered with 150 μm plankton net and stored separately until use.

Experimental Design

Red drum larvae (14 d.p.h.) were purchased from MADAN – Kibbutz (Israel) and were set for the trial after a 48 hours acclimation period. The feeding experiment was carried out in 15 separate tanks (each with 40 l water volume). Four treatment groups (1 mg/l, 5 mg/l, 10 mg/l, 50 mg/l Se) and controls were set up randomly in three replications (n=3). Since the treatment concentration of 100 mg/l selenium proved to be toxic even for the *Artemia* sp., this dosage was not tested in the feeding experiment.

The water was previously aerated and salted, then 70 fish larvae were placed in each tank (1050 larvae overall, SL: 9.3 ± 0.4 mm; W: 15.06 ± 4.77 mg). Filter pipes ensured permanent ventilation and filtration, while individual heaters (Sera) regulated the temperature (27 ± 0.8 ^{o}C) of the salted water (15 ppt Tetra Marin sea salt). 12-hour light periods were alternated by 12-hour dark periods during the experiment. The fish were fed *ad libitum* three times a day

(7 AM, 1 PM and 6 PM). Dead animals and solid wastes were removed every day using a suction tube. Mortality was registered every day.

To keep the experimental environment constant, the water temperature (27 ± 0.8 ^{o}C), pH (7,9-8,2), salinity (~15 ppt) (Hanna HI98130), oxygen saturation (Hach HQ30d), NO_3^- (<8 mg/l), NO_2^- (<0,1 mg/l) and NH^+ (<0,45 mg/l) (Aquamerc Compact Laboratory, Merc) were checked every day. The oxygen saturation in the tanks was maintained at 100 % by continuous aeration.

The 9-day-long feeding trial finished with a 24-hour-long starvation period before the fish were sampled for further analysis.

Growth and Survival Analysis

Six larvae from each tank (18 larvae per treatment) were separated randomly for body measurements (W, SL). A digital camera (Olympus SZ51) fixed to a microscope was used to measure the standard length of larvae on a 0.1 00 stage micrometer microscope slide.

Pictures were analysed and the length was measured with the software WinImag 1.0. Wet weight was measured with a digital scale (Precisia 240A) with the accuracy of 0.001 g.

Survival and growth parameters were calculated as follows:

- Survival (S; %) = (harvested individuals/stocked individuals) x 100;
- Specific Growth Rate (SGR; %/day) = (ln W_f – ln W_i)/ t × 100, where W_f is the mean weight of the harvested individuals (g), W_i is the mean weight of the stocked individuals (g) and t is the duration of the experiment in days;
- K-factor [39]: K = W × 100 / SL^3, where W is the wet weight (g) and SL is the standard length (cm) of fish.

Chemical Analysis

Seven larvae from each tank (21 larvae per treatment) were taken for enzyme activity analysis. They were placed into liquid nitrogen immediately to stop vital processes. The method of [37] was used to analyse the activity of the enzyme glutathione peroxidase.

Another six larvae from each tank (18 larvae per treatment) were separated and frozen for later selenium content analysis. The samples of enriched *Artemia* sp. were washed with distilled water and also kept frozen for selenium content analysis.

For measuring the moisture content, samples were dried in an oven (at a temperature of 80 ^{o}C) until they were oven-dry. Then, Hydride Generation Atomic Fluorescence Spectrometry (HG-AFS) was used to analyse the selenium content. Samples were prepared for measurement using wet digestion according to [21]: 5 ml of 65% HNO_3 was added to 1 g sample and digested for 1 hour at 60 ^{o}C, then for 240 minutes at 120 ^{o}C. After that, 3 ml of 30% H_2O_2 was added. The digested samples were diluted to 15 ml using 3 M HCl and then filtered. Selenium content was measured using a Millennium Merlin atomic fluorescence spectrometer with following parameters: argon gases flush with the flow rate of 15 litres/minute, 40 seconds/measure, 40 seconds of washing time. The device was calibrated to the Charlau-standard before measurement, which was repeated after each 5 measurements to maintain accuracy. 3 M HCl was used for the hydride reaction, while the reductive agent was 1.4 m/V% $NaBH_4$ dissolved in 0.1 M NaOH. The reagents were of analytical purity.

Statistical Analysis

Data were analysed with Microsoft Excel 2013 and SPSS for Windows 20.0 software.

SGR, survival, standard length, weight, K-factor and selenium retention were analysed with single factor ANOVA. The homogenty of each data collection was tested with Levene's test ($p>0.05$) and Tukey's post-hoc test ($p<0.05$) was used to analyse pairwise differences among treatment concentrations and the control in case of a significant factor effect.

The results of the single factor ANOVA were not appropriate to estimate the optimal selenium requirement of fish because of the limited number of treatments. Thus, second degree polynomial functions were defined and regression was used to calculate optimal selenium concentration for red drum larvae [42]. During data analysis, those parameters were used, where significant result was found and also important in case of the intensive aquaculture (e.g. survival, growth). Second-degree polynoms were set using the real weight, standard length and SGR data to calculate the optimum selenium requirement of the larvae. The selenium concentration at the peak point was calculated on the basis of trend equations. The „x" values belonging to highest „y" were searched, which was calculated by derivation of the second-degree equation. The theoretical treatments for the highest „y" value were considered as optimum treatments. Regression analysis was carried out excluding the 50 mg/l treatment where toxicity was proven by the analysis of the weight, standard length and SGR.

Pearson correlation (r) was used to investigate relationship between the selenium retention (i.e. the level of enrichment) of *Artemia sp*. and the red drum larvae fed on it.

RESULTS

Artemia sp. enrichment

The selenium retention of *Artemia* sp. was significant in each treatment compared to the control group (**Control** - 0.0002^{a} ± 0.000 mg/kg dry matter; **1 mg/l** - 0.670^{b} ± 0.002 mg/kg dry matter; **5 mg/l** - 3.788^{c} ± 0.055 mg/kg dry matter; **10 mg/l**- 6.340^{d} ± 0.037 mg/kg dry matter; **50 mg/l** - 26.914^{e} ± 0.153 mg/kg dry matter; **100 mg/l** - 4.740^{f} ± 0.066 mg/kg dry matter). Selenium concentration of *Artemia* sp. increased considerably with selenium concentration of the water was held in up to 50 mg/l selenium in the water, but it dropped markedly at the largest treatment concentration (100 mg/l Se) indicating some inhibition on

normal life functions of *Artemia* sp. For this reason, the 100 mg/l selenium treatment was not used in feeding experiments (Figure 1).

Survival, Growth, K-factor and Enzyme Activity of fish larvae

The survival rate of the control red drum larvae group was 57%. Slightly increased selenium content of *Artemia* sp. seemed to improve the vitality of fish larvae; however, only the 10 mg/l Se treatment showed a significant difference at 69% survival rate. Higher nano-selenium content had no positive effect on health conditions of larvae and the survival rate (56%) in the 50 mg/l treatment equalled that of the control group (*Table 2*).

Nano elemental selenium had a slight but generally not significant positive influence on growth parameters of fish as at concentrations up to 10 mg/l as well. The average weight of the control group was 0.095 g. Mean fish weight increased slightly to the 5 mg/l treatment level (average weight: 0.140 g) but not significantly. On the other hand, the 50 mg/l selenium treatment clearly resulted in a decreased fish growth (average weight: 0.058 g) and indicating a toxic effect. Similar results were obtained for standard length, K-factor and SGR, with highest index values observed in 5-10 mg/l Se treatments and lowest values in the 50 mg/l selenium treatment (*Tables 1 and 2*).

The selenium treatment had a slight, non-significant negative impact on the activity of the enzyme glutathione peroxidase up to 10 mg/l selenium concentration and a more expressed inhibitor effect at 50 mg/l selenium treatment concentration (*Table 2*).

Selenium Accumulation of Fish Larvae

The selenium retention of red drum larvae increased significantly from 2.19 mg/kg (control) to 39.24 mg/kg (50 mg/l Se) depending on the treatment concentration and proving the uptake of selenium by fish from the enriched *Artemia* sp. feed (*Figures 1 and 2*).

Assessment of the optimal selenium treatment concentration

The selenium requirement of red drum larvae was calculated on the basis the parameters, for which significant result was found. Based on these functions, significant statistical relationships were found between the considered growth parameters and the selenium treatment concentration (*Figure 3.*; weight: $R^2 = 0.9789$; standard length: $R^2 = 0.9911$; SGR: $R^2 = 0.9694$). The functions reached their maximum at 5.25 mg/l in case of weight, 5.22 mg/l in case standard length, and at 5.69 mg/l (in case of SGR).

The 50 mg/l treatment was also taken into account during the calculation of the regression because the optimal selenium concentration could range between 10 and 50 mg/l in case of survival rate. The polynomial functions revealed significant statistical relationship between the survival rate and the selenium treatment dosages ($R^2 = 0.8646$). The peak point was reached at 23.57 mg/l selenium treatment concentration, which is a substantially higher value than that of derived based on the growth parameters. To define selenium optimum of the red drum larvae, the lower values were taken into account, having regard to the suspicion of toxicity in higher selenium concentrations.

DISCUSSION

In aquaculture fish larvae are still very often reared on natural diet like rotifers and small planktonic crustaceans. However, these food organisms generally contain a suboptimal

amount of selenium, and therefore, rearing efficiency (i.e. survival and growth rates) of fish larvae can be lower than required. In this study, we tested whether *Artemia* sp. could be effectively enriched with selenium by keeping them in selenium solution of different concentrations, and whether these treated preys could improve the survival and growth rate of red drum larvae. Our results revealed that *Artemia* sp. very effectively accumulated selenium very effectively from water and accumulation rate was proportional to the selenium concentration of the water. Furthermore, results also proved some positive influence of this enriched food for the survival and growth rate of fish larvae but only up to moderate selenium concentrations.

Contrary to *Artemia* sp., there are several examples for the influence of rotifers enriched with selenium on fish development under experimental conditions. For instance, [12] did an experiment with Atlantic cod (*Gadus morhua,* L.) and observed a 32% higher survival rate in fish fed with rotifers enriched with 7 mg/l sodium selenite and 400 mg/l sodium iodide compared to the control group, but cod larvae showed a slight decrease in growth parameters. In our study, red drum larvae kept on selenium enriched *Artemia* sp. diet showed also a slight improvement in their survival, especially in the 10 mg/l treatment. On the other hand feeding trials of [34] with senegalese sole (*Solea senegalensis,* Kaup) and of [24] with malabar grouper (*Epinephelus malabaricus,* Bloch and Schneider) larvae did not show any significant selenium related difference in their survival rate. Growth parameters (i.e. increase in SL and W, and SGR) of red drum larvae were the best when they were fed with 5 mg/l selenium treated *Artemia* sp. that accumulated 3.788 mg/kg (dry matter) selenium. Similarly, [19] noticed an improved growth rate in red seabream (*Pagrus major,* Temminck and Schlegel) when they were provided enriched rotifers of 2.2 mg/kg (dry matter) selenium content.

In accordance with the results of [12], selenium treatments did not have significant impact on the activity of the enzyme glutathione peroxidase, except for the inhibitor effect observed at

the highest applied dosage of 50 mg/l. [34] had similar no-effect results analysing the impacts of zooplankton enriched with selenium on enzymatic activity of senegalese sole.

Several authors [14, 10, 24] calculated GSH-px enzyme activity as a key index to define selenium requirement of fish. In these experiments, the maximum of enzyme activity was considered as the sign of the optimal level of selenium requirement. In our study, however, the maximum enzyme activity was observed in the control group which pattern contradicted results of the survival and growth analyses. Accordingly, we assessed optimal selenium treatment concentration of *Artemia* sp. for red drum larvae based on the maximum of survival rate and growth parameters. We revealed that the optimal treatment dosage for *Artemia* sp. enrichment ranged between 5.22 mg/l (based on SL), 5.25 (based on W increments) and 5.69 mg/l selenium in the water (based on SGR). These treatment levels corresponded to 3.95 and 4.30 mg/kg (dry matter) accumulated selenium in the zooplankton for the red drum, which substantially exceed the 0.25-0.7 mg/kg (dry matter) dosage range recommended by other authors for different fish species [30, 24, 33]. On the other hand, [31] estimated nearly as high selenium dosage optimum, ranging between 1.4 and 3 mg/kg (dry matter) for the Atlantic cod larvae in zooplankton than our values, which level is three times than of those that are generally available in hatcheries' practice. The estimated optimum of 4 mg/kg (dry matter) selenium content of *Artemia* sp. is similar to the natural selenium content of copepods as well [12].

Regarding all the production parameters that were investigated in the experiment declared that the selenium intake from the enriched zooplankton (26.90 mg/kg Se in DW) in group 50 mg/l caused toxic effect. The reduced growth is the sign of excessive intake of selenium [14, 24, 16], which was confirmed by the results obtained in group 50 mg/l as the lowest length and weight as well as the worst condition of fish larvae observed. In the group 10 mg/l reduced growth was also experienced, which suggests the high intake of the microelements. Regarding

the accumulated selenium in the body of the red drum larvae, significant differences were found between each treatment. Close relationship was found by Pearson correlation (r=0,765) between the level of the accumulated selenium in the zooplankton and the selenium content of the fish larvae which suggests that selenium has been successfully incorporated into the body of fish. Based on the foregoing, it can be clearly determinated that the nano sized elemental selenium can be toxic to the fish, like the organic and inorganic forms [27].

In conclusion, based on the results of the feeding trial, *Artemia* sp. enriched with nano-selenium had a positive impact on the efficiency of red drum larvae rearing. According to our estimates, the optimal selenium content of the feed could be 4 mg/kg (dry matter) for the red drum, which requires an enrichment treatment of *Artemia* sp. in 5 mg/l selenium solution for one day.

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Table 1: Influence of *Artemia* sp. enriched with nano-selenium on the growth and condition (K-factor) of red drum larvae *(Sciaenops ocellatus)*

Treatment	Weight (W, g)	Standard length (SL, mm)	K-factor
control	0.095 ± 0.005^{ab}	16.7 ± 0.5^{ab}	2.03 ± 0.29^{NS}
1 mg/l	0.117 ± 0.007^{a}	17.3 ± 0.1^{ab}	2.30 ± 0.20^{NS}
5 mg/l	0.140 ± 0.018^{a}	18.9 ± 0.8^{a}	2.07 ± 0.12^{NS}
10 mg/l	0.106 ± 0.034^{ab}	17.0 ± 2.1^{ab}	2.12 ± 0.11^{NS}
50 mg/l	0.058 ± 0.006^{b}	14.6 ± 0.2^{b}	1.78 ± 0.26^{NS}

Different letters mark significant differences within each column (SD, $p < 0.05$); NS = not significant.

Table 2: Influence of nano-selenium enriched *Artemia* sp. on the survival and specific growth rate (SGR) of red drum larvae *(Sciaenops ocellatus)* and on the activity of the enzyme glutathione peroxidase (GSH-px)

Treatment	Survival (%)	SGR (%/day)	GSH-px enzyme activity (µmol/g/min)
control	57 ± 2^{a}	20.22 ± 0.64^{a}	28.3 ± 1.5^{a}
1 mg/l	64 ± 1^{ab}	22.29 ± 0.86^{ab}	23.6 ± 2.5^{ab}
5 mg/l	66 ± 6^{ab}	24.52 ± 1.56^{b}	24.8 ± 3.0^{ab}
10 mg/l	69 ± 4^{b}	22.34 ± 1.98^{ab}	26.1 ± 1.8^{ab}
50 mg/l	56 ± 4^{a}	15.77 ± 1.24^{c}	17.3 ± 1.9^{b}

Different letters mark significant differences within each column (SD, $p < 0.05$).

Figure 1: Selenium retention of selenium treated *Artemia sp*.

Different letters mark significant differences (SD, $p < 0.05$).

Figure 2: Selenium retention of red drum *(Sciaenops ocellatus)* larvae

Different letters mark significant differences (SD, $p < 0.05$).

Figure 3: Response of red drum larvae to *Artemia* sp. feed enriched at different selenium concentrations.