Thesis of the Ph.D. dissertation

IMPROVEMENT OF JUVENILE REARING AND FEEDING TECHNOLOGY OF BARRAMUNDI (*LATES CALCARIFER*, BLOCH, 1790) AND RED DRUM (*SCIAENOPS OCELLATUS*, L. 1766)

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CONTENT

1. INTRODUCTION AND MAIN AIMS OF THE WORK

Larval and juvenile rearing of barramundi (*Lates calcarifer*) and red drum (*Sciaenops ocellatus*) were investigated in this dissertation both can be considered as new fish species in the Hungarian aquaculture. In the experiments considerable attention were paid to assess the importance of trace minerals in fish nutrition, regarding both species.

Barramundi and red drum has wide range salinity tolerance, however, the adjustment of optimal concentration and ratio of dissolved solids in water are essential in the early life stages. Whether to use freshwater and thermal water with different salt concentrations as rearing media is a key factor regarding the economically sustainable production of both species in Hungary. Nevertheless, ensuring the micro and macro element requirement according to the life stages has great importance.

The role of essential trace minerals in biological systems is well known, however, less information is available considering fish nutrition. Cobalt is a naturally occurring element in the aquatic environment and is essential in trace amount for many living functions of vertebrates (Watanabe et al., 1997). Cobalt is necessary for the synthesis of B12 vitamin that plays an important role in the development of erythrocytes, in the metabolism of fatty acids as well as it is needed for the normal maturation to occur (Steffens, 1989; Kashiwada et al., 1970). Cobalt is essential in fish nutrition (Davis and Gatlin, 1991), still a lower number of papers are available considering it as a trace element in the nutrition of fish compared to other minerals such as zinc or copper (Blust, 2011). Limited data exists exploring cobalt supplementation combined with zinc and manganese, although both minerals are vital for the optimal growth and accordingly are investigated and applied diet supplements in fish rearing accordingly (Ovesen et al., 2001; Yamaguchi and Fukagawa, 2005; Lall, 2002). Manganese and zinc are necessary for optimal growth and skeletal development in trace amount as well as both play an important role in numerous physiological processes (Davis and Gatlin, 1991; Watanabe et al., 1997).

The main aim of the current dissertation is to establish the possibilities of cobalt, zinc and manganese enrichment in the early life stages of barramundi, through the trace mineral supplementation of live feed *Artemia nauplii* for the larvae, as well as micro element supplementation of artificial dry diet for juveniles. The physiological impacts of the most important micro and macro elements regarding red drum are investigated by the uptake from the ambient water.

Cobalt accumulation of *Artemia nauplii* is analysed after the 24 hours enrichment period with the addition of different doses of cobalt chloride in the first part of the current study. Further experiments are carried out with cobalt, zinc and manganese enrichment of live feed in combined treatments and special attention was paid to observe the interaction of trace minerals in the uptake of zooplankton. The accumulation and effects of the added minerals on the larval growth performance of barramundi were investigated, when the retention occurred through a nourishment organism. Commercially available dry feed was supplemented with the different combinations of trace minerals applied previously in the larval rearing trials in the third phase of the dissertation. Red drum is well known to be a less stress tolerant fish species, therefore, during and after transportation, significant losses can be taken into account. Thus in the experiments conducted with juveniles, the ion content of ambient water vital for the survival of species was analysed.

2. MATERIALS AND METHODS

1. Enrichment of *Artemia* nauplii with cobalt, zinc and manganese

Newly hatched *Artemia nauplii* were enriched for 24 hours with cobalt chloride, zinc sulphate and manganese chloride ($CoCl_2$, $ZnSO_3$, $MnCl_2$). Treatments were set in plastic tanks of 4L at a density of 100-150 nauplii mL^{-1}. Seawater was prepared by dissolving synthetic sea salt (Sera Marin Basic, Germany) in tap water at TDS of 20 ppt. The oxygen saturation in the tanks was maintained at 100 % by continuous aeration.

In the first experiment, the cobalt accumulation of *Artemia nauplii* is analysed after the 24 hours enrichment period with the addition of different doses of cobalt chloride. In control groups no supplementation was used. The other treatments were designated as follows: in Co-50 groups 50 mg L^{-1}, in Co-100 100 mg L^{-1} while in Co-1000 1000 mg L^{-1} $CoCl_2$ supplementation was applied. In the next step, further experiments were carried out with cobalt, zinc and manganese enrichment of live feed in combined treatments. No supplementation was used in the control groups. Single treatments were designated as follows: in Co-1 groups 50 mg L^{-1} while in Co-2 groups 100 mg L^{-1} $CoCl_2$ supplementation was applied. In Mn-1 groups 50 mg L^{-1} while in Mn-2 groups 100 mg L^{-1} $MnCl_2$ supplementation was used. Combined treatments were the followings: in CoMn-1 groups 50 mg L^{-1} $CoCl_2$ and 50 mg L^{-1} $MnCl_2$ was used while in CoMn-2 groups the concentrations were doubled; in CoZn-1 groups 50 mg L^{-1} $CoCl_2$ and 50 mg L^{-1} $ZnSO_4$ was applied while in CoZn-2 groups the concentrations were doubled. Experiments were carried out in triplicate (n=3).

2. Feeding trials of barramundi larvae with Co, Zn and Mn enriched *Artemia*

Barramundi larvae of 12 days post hatch (dph) were purchased from artificial propagation of the MADAN-Kibbutz Ma'agan Michael, Israel. After 48 hours of acclimatization the nine treatments in duplicate were arranged in rectangular glass aquaria of 40 L, with 100 barramundi larvae in each (SL: 5.01±0.42 mm; W: 2.95±0.61 mg). Treatments were in a completely randomised block design. Seawater was prepared by dissolving synthetic sea salt (Sera Marin Basic, Germany) in tap water at TDS of 14 ppt. Oxygen saturation was maintained at 100% by aeration stones and temperature was controlled at 28.0±0.5 °C by heaters placed in the tanks. Daily water exchange was 30%. Water temperature, pH, total dissolved solids (TDS, Hanna HI98130), dissolved oxygen (DO, Hach HQ30d), NO_2^-, NO_3^- and NH_4^+ concentration (Aquamerck compact laboratory for water testing, Merck) were verified daily. Barramundi larvae were fed by enriched *Artemia* three times daily, at 8:00, 13:00 and 17:00. The amount of

nauplii introduced into the larval tanks was adjusted daily in order to obtain complete consumption and ranged from 250 to 300 nauplii per larva. Daylight fluorescence lights of 20 W (Sera Brilliant daylight, Germany) were placed above each aquaria and the photoperiod was kept light for 24 hours daily. The feeding trial lasted for 16 days (30 dph). Aquaria were checked daily for dead larvae.

3. Feeding trials of barramundi juvenile with Co, Zn and Mn supplemented artificial dry diet

In the 8 weeks experiment barramundi juveniles with initial body weight of 5.41±0.06 g were obtained from artificial propagation of the MADAN-Kibbutz Ma'agan Michael, Israel. A total of 600 fish were equally distributed into 6 treatments (Co-50, Mn-50, Zn-50, CoMn-50, CoZn-50 and control). The treatments were set in plastic tanks with volumes of 70 L (50 juveniles in each) that formed a freshwater recirculating system provided with aerated biofilter and UV lamp. Experiments were carried out in duplicate (n=2) as well as treatments were in a completely randomised block design. Oxygen saturation was maintained at 89±0.9% by aeration stones and temperature was controlled at 25.56±0.7 °C by electronic heaters. Water temperature, pH, total dissolved solids (TDS, Hanna HI98130), dissolved oxygen (DO, Hach HQ30d), NO_2^-, NO_3^- and NH_4^+ concentration (Aquamerck compact laboratory for water testing, Merck) were verified daily. Fish were fed by hand three times daily (08:00, 12:00 and 16:00), the feeding rates were 2.5% of total biomass and adjusted weekly. The feeding trial lasted for 56 days while fish were counted and weighed weekly.

A common practical diet was supplemented with cobalt chloride, zinc sulphate and manganese chloride ($CoCl_2$, $ZnSO_3$, $MnCl_2$). In control feed no supplementation was used. Single treatments were designated as follows: in Co-50 experimental diet 50 mg kg^{-1} $CoCl_2$ supplementation was applied. In Mn-50 diets 50 mg kg^{-1} MnCl2, as well as in Zn-50 diets 50 mg kg^{-1} $ZnSO_4$ supplementation was used. Combined treatments were the followings: in CoMn-50 diet 50 mg kg^{-1} $CoCl_2$ and 50 mg kg^{-1} $MnCl_2$ was used while in CoZn-50 diet 50 mg kg^{-1} $CoCl_2$ and 50 mg kg^{-1} $ZnSO_4$ was applied. The mineral and proximate composition of experimental feeds were confirmed by chemical analyses. The diets were pelleted after adding 10% water and then air dried. The formulated dry feed was stored at 4°C.

4. Development of juvenile rearing technology of red drum

Red drum juveniles were obtained from artificial propagation of the MADAN-Kibbutz Ma'agan Michael, Israel and treatments were set in rectangular glass aquaria of 40 L. Water was prepared

by dissolving synthetic sea salt (BlueTreasure, Qingdao Sea-Salt Aquarium Technology Co) in tap water at TDS of 5 ppt. Oxygen saturation was maintained at 100% by aeration stones and temperature was controlled at 24 °C by heaters placed in the tanks. Water temperature, pH, total dissolved solids (TDS, Hanna HI98130), dissolved oxygen (DO, Hach HQ30d), NO^{2-}, NO^{3-} and NH_4 concentration (Aquamerck compact laboratory for water testing, Merck) were verified daily. Water samples from aquaria were collected and the main cations were analysed.

Thereafter, juveniles were transferred into plastic tanks with volumes of 70 L that formed a brackish water recirculating system at TDS of 5 ppt. provided with aerated biofilter and UV lamp. Specific growth rate and feed conversion ratio was calculated at the end of the experiments with red drum.

5. Determination of survival, growth performance and feed conversion ratio

Percent survival of barramundi larvae and juvenile were calculated for each treatment by dividing the number of survivors by the initial number times 100.

Living individuals were counted and the rate of cannibalism was calculated by the following formula: (number of missing fish-number of observed dead fish)/initial number of fish *100 (Arockiaraj-Appelbaum, 2011).

$K = W/L^3 \times 100$, where W is the wet weight (g) and L is the standard length (mm). Growing performance was estimated by calculating the specific growth rates (SGR, %/day) for every larva using the following formula:

$SGR\ (\%) = (\ln W_f - \ln W_i) / t \times 100$, where W_f is the wet final body weight (g), W_i is the wet initial body weight (g) and t is the time (day).

WG (%) = (Wf – Wi)/Wi x 100%, where Wf is the wet final body weight (g) and Wi is the wet initial body weight (g)

FCR (g g-1) = F/(Wf-Wi), where F is the feed intake, Wf is the wet final body weight (g) and Wi is the wet initial body weight (g)

6. Chemical analyses

Artemia were sampled for the determination of moisture content and elemental concentration at the end of the enrichment period. *Artemia* were collected from each tanks by plankton net (mesh size of 150 μm) and samples were washed thoroughly with ultrapure water. Samples were then centrifuged and supernatant was discarded. The moisture content was determined by gravimetric method where 1.000±0.003 g of exactly known weight samples were taken into the

drying cabinet in beakers and were dried until constant weight at 105 ºC. Then 0.5 mg of dry samples were digested prior to elemental analysis in a microwave assisted system (MLS Mega 1200) with the mixture of 2 mL 68 % (m/m) HNO_3 and 0.2 mL 30 % (m/m) H_2O_2. After cooling the containers for 20 min. under circulating tap water samples were transferred without loss into calibrated plastic tubes and were diluted up to 10 mL with 0.1 M HNO_3 prepared in ultrapure water. Blanks were set to verify the purity of applied reagents. The concentration of Co, Zn and Mn were determined from diluted samples by atomic absorption (AAS, Varian spectra) spectrometry.

At the end of the feeding trials a total of 360 larvae (40 larvae/treatments) and 96 juveniles (16 juvenile/treatments) were sampled and starved for 24 hours prior to analysis. Individual wet body weight (W) of each sample was measured on analytical balance (Precisa 240A). Individual standard body length (SL) of each larvae was measured by a stereomicroscope equipped with digital camera (Olympus SZ51). Millimetre calibrated microscopic slide was used for the calibration and taken pictures were evaluated as well as SLs were calculated by a computer program (WinImag 1.0). Until further experiments fish samples were kept frozen at -30 ºC. For the elemental analysis, samples were thawed at room temperature, rinsed with ultrapure water and then taken into 50 mL Erlenmeyer flasks. Moisture content of larvae was determined by gravimetric analysis, drying the samples at 105 ºC. Moisture content of juvenile was determined after lyophilisation. After measuring their dry weights samples were wet digested in the same containers along with 5 mL of 68% (m/m) HNO_3 and 0,2 mL 30 % (m/m) H_2O_2 (reagent grade, VWR International) on an electric hot plate at atmospheric pressure. Samples were transferred into plastic tubes and diluted with 0.1 M HNO_3 prepared in ultrapure water at a final volume of 10 mL prior to analysis. Reagent purity was verified by blank samples. The Zn concentration of samples was determined by AAS, while the Mn and Co concentration was determined by graphite furnace atomic absorption (GF AAS, Perkin Elmer Analyst 600 equipped with Zeeman background correction system) spectrometry. The other micro and macro elements were determined by microwave plasma atomic emission spectrometry (MP-AS 4100, Agilent Technologies).

7. Statistical analyses

Calculations were performed using the SPSS/PC + statistical software package and Canoco for Windows. The Gaussian population distribution curves of treatments were calculated and plotted according to Arockiaraj-Appelbaum (2011) by OriginPro 8.6 programme. Homogeneity of variances was tested by Levene's test and considered homogenous when *P* value was higher

than 0.05. One way ANOVA was carried out to compare the moisture content, Co, Mn and Zn concentration of *Artemia* groups to the control. The effect of treatments and replicates as well as their interaction effect on the results of W, SL, *K* factor, SGR, moisture content and Co, Mn and Zn concentration in barramundi larvae, as well as W, WG, SGR, FCR and Co, Mn and Zn concentration in juveniles was studied by GLM two-fold nested design ANOVA. Significance of differences were studied by Tukey's Multiple Comparison test, values were considered significant at $P<0.05$. Redundancy analysis (RDA) was used to study the interaction between the Co, Mn and Zn concentration of *Artemia* and barramundi larvae.

3. NEW SCIENTIFIC ACHIEVEMENTS

1. The results showed that newly hatched *Artemia nauplii* effectively accumulated high concentration of cobalt during the 24 hours enrichment period. All treatments had a significant effect on the uptake of live feed organism and the level of this essential trace element increased in parallel with the dose of supplementations (**table 1.**). Even the addition of the highest (1000 mg L^{-1}) cobalt chloride dose did not prove to be toxic for the zooplankton.

Table 1. Co levels in *Artemia nauplii* after the 24 hours of cobalt chloride enrichment period

Treatments	Co (mg g^{-1})
Control	0.0000 ± 0.0000^{a}
Co-50	0.0694 ± 0.0006^{b}
Co-100	0.2006 ± 0.0004^{c}
Co-1000	2.6700 ± 0.0018^{d}

2. The enrichment of *Artemia nauplii* with the trace element salts of cobalt, zinc and manganese in different concentrations and combinations resulted in significantly elevated level of these elements in the harvested brine shrimp samples (**table 2).**

Table 2. Co, Zn and Mn levels in *Artemia nauplii* after the 24 hours of enrichment period

Treatments	Moisture (%)	Co (mg g^{-1})	Mn (mg g^{-1})	Zn (mg g^{-1})
Control	95.4 ± 0.7^{b}	0.01 ± 0.0^{a}	0.02 ± 0.0^{a}	0.09 ± 0.0^{b}
Co-1	94.7 ± 0.5^{ab}	0.64 ± 0.04^{d}	0.02 ± 0.0^{a}	0.16 ± 0.0^{c}
Co-2	94.2 ± 0.2^{a}	5.26 ± 0.07^{e}	0.02 ± 0.0^{a}	0.23 ± 0.01^{d}
Mn-1	93.8 ± 0.3^{a}	0.04 ± 0.0^{a}	0.32 ± 0.0^{c}	0.34 ± 0.01^{e}
Mn-2	95.7 ± 0.4^{b}	0.02 ± 0.0^{a}	1.58 ± 0.01^{d}	0.02 ± 0.00^{a}
CoZn-1	95.8 ± 0.4^{b}	0.24 ± 0.05^{b}	0.02 ± 0.0^{a}	3.46 ± 0.12^{f}
CoZn-2	94.7 ± 0.3^{ab}	0.48 ± 0.07^{c}	0.01 ± 0.0^{a}	5.43 ± 0.11^{g}
CoMn-1	95.0 ± 0.4^{ab}	0.36 ± 0.06^{bc}	0.22 ± 0.0^{b}	0.25 ± 0.01^{d}
CoMn-2	94.7 ± 0.4^{ab}	6.51 ± 0.09^{f}	3.35 ± 0.02^{e}	0.12 ± 0.01^{bc}

Linear relationship was found between the applied concentrations of the added trace elements and the measured concentrations of the *Artemia* samples, while interactions were not observed in the mineral retention of live feed organism in this experiment.

3. The results of the feeding trial showed that the use of trace element enriched *Artemia nauplii* fed to barramundi larvae resulted in significantly higher larval growth performance compared to the control (**figure 1**.).

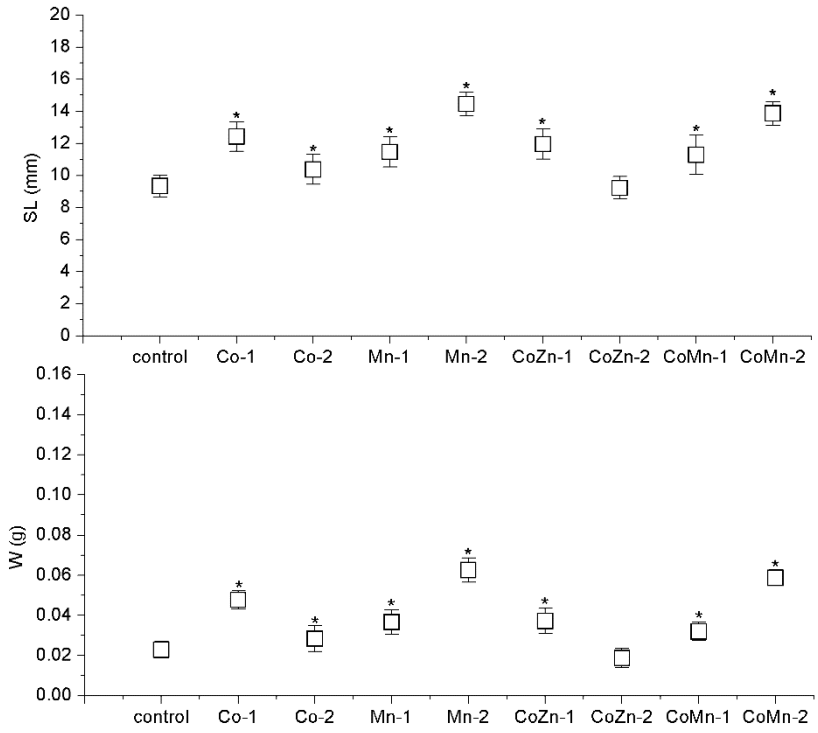

Figure 1. Standard length (SL) and wet weight (W) results of barramundi larvae at the end of the feeding trial

However, increased mortality and rate of cannibalism were observed in cobalt-manganese treatments, since the combination of these elements might be resulted in the production of a less uniform larvae population in which potential predators could appear (**figure 2.**).

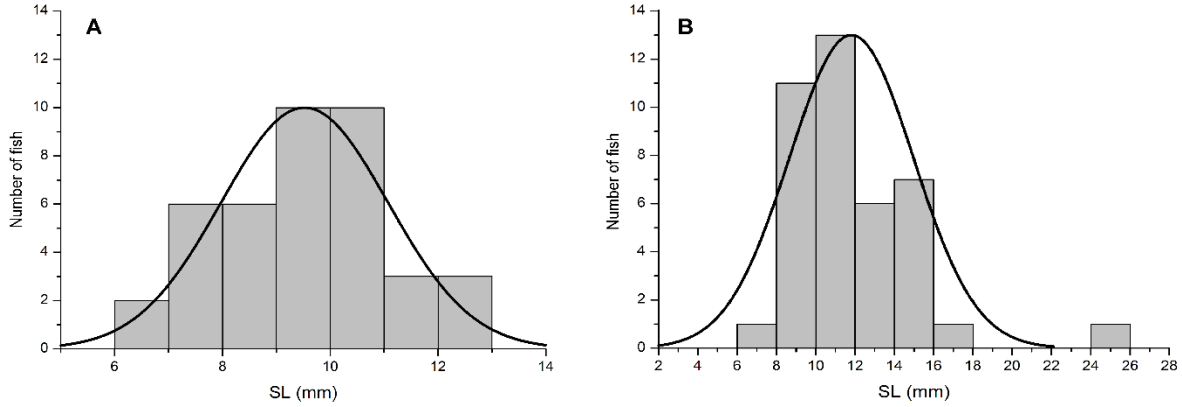

Figure 2. Normal Gaussian population distribution curve of control (A) and deviated distribution curve of CoMn-1 (B) barramundi larvae group

The use of enriched live feed had significant effect on the trace mineral retention of barramundi larvae (**figure 3.**).

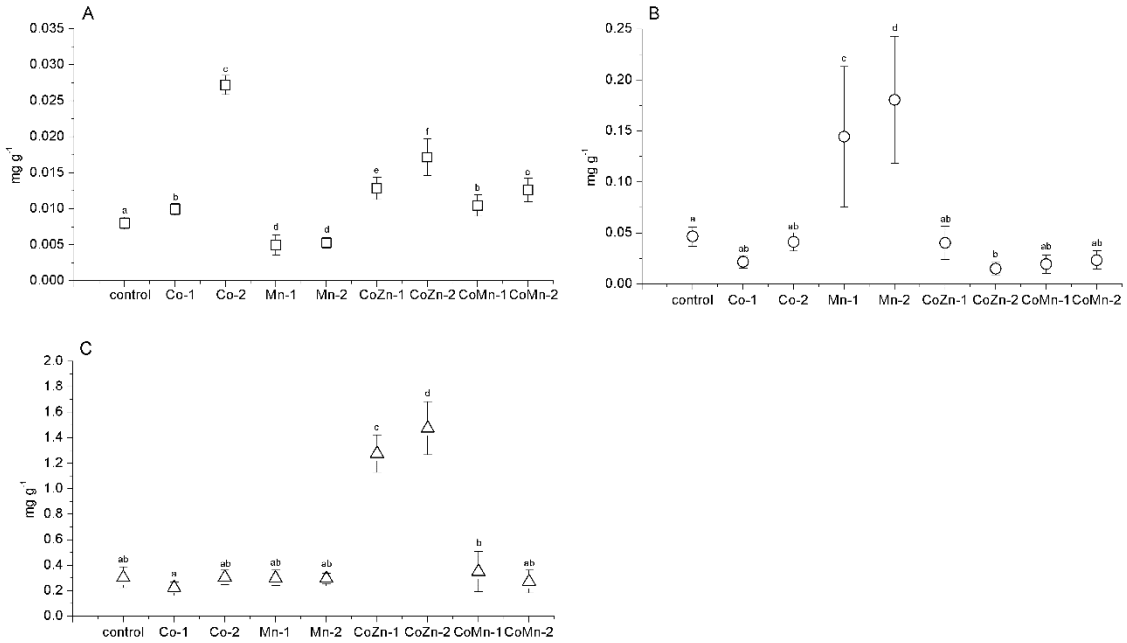

Figure 3. Co (A), Mn (B) and Zn (C) concentration (dry-matter basis) of barramundi larvae

Redundancy analysis showed a strong correlation between the cobalt and manganese concentration of *Artemia* and fish samples, as well as a total agreement between the zinc concentration of the zooplankton and the larvae (**figure 4.**).

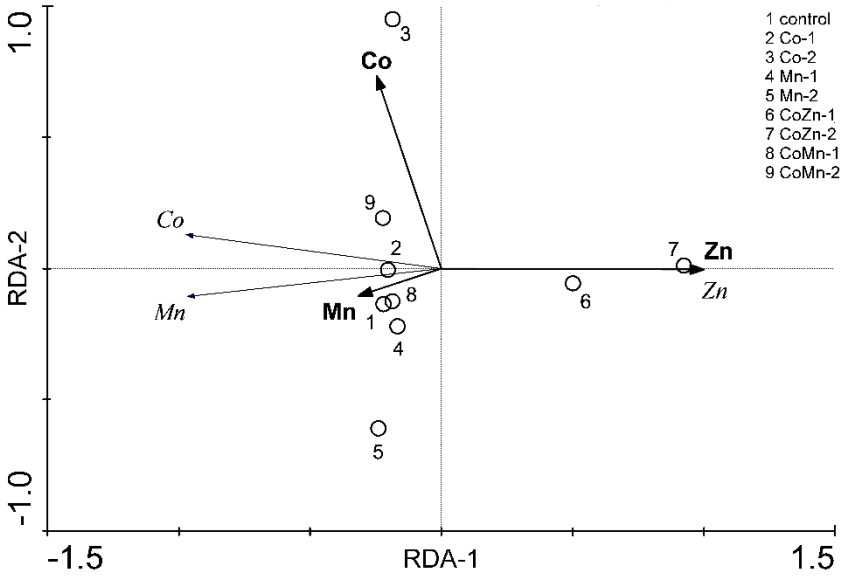

Figure 4. Redundancy biplot to study the interaction between Co, Zn and Mn concentration of *Artemia* and barramundi samples

4. No interaction occurred between the retention of zinc and cobalt, but a competitive type of interaction is assumed between the deposition of cobalt and manganese in barramundi larvae. One possible reason for the significantly increased mortality and rate of cannibalism observed in CoMn-1 and CoMn-2 groups is the use of these elements in combined treatments. Although no toxic effect by the enrichment on *Artemia* survival was observed, the vitality and active motion of live food organisms might have affected larval appetite and therefore, food consumption resulting in the extent of size heterogeneity.

5. The trace mineral supplementation of artificial dry diet did not have an impact on the growth performance, survival and feed conversion ratio of barramundi juveniles (**figure 5.**), as well as the addition of cobalt, zinc and manganese neither alone, nor in combination affected the size distribution of barramundi juveniles (**figure 6.**).

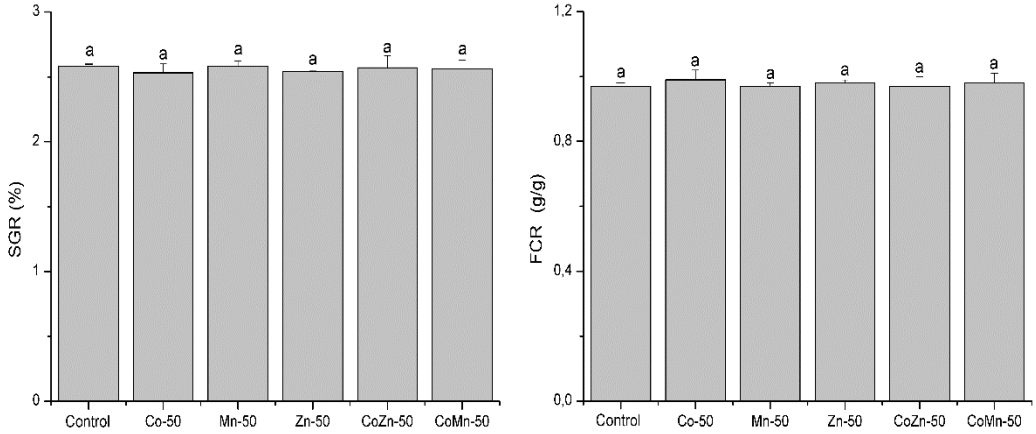

Figure 5. Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) results of barramundi juvenile at the end of the feeding trial

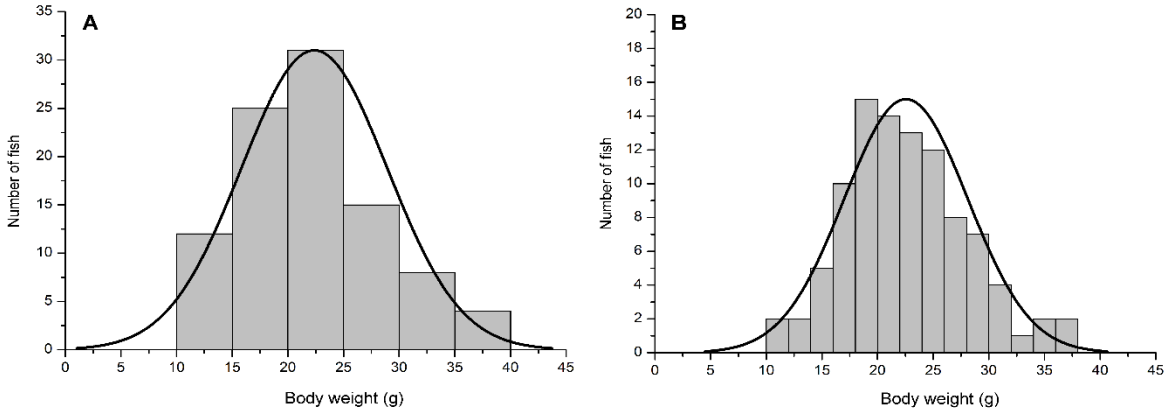

Figure 6. Gaussian population distribution curves of control (A) and CoMn-50 (B) groups

In addition, mortality originated from cannibalistic behaviour did not occur during the experiment. Since no significant differences were found in the zinc levels of juveniles between the treatments, it can be stated that the zinc concentration of the applied basal diet meets the dietary requirements of this age in itself (**figure 7.**). The growth performance and survival were not affected by the cobalt and manganese enrichment. However, the concentration of the same trace minerals in fish was elevated significantly with the supplementation dose of dry feed, assuming that the dietary cobalt and manganese demand of juvenile barramundi is higher than the levels of these trace minerals found in basal diet.

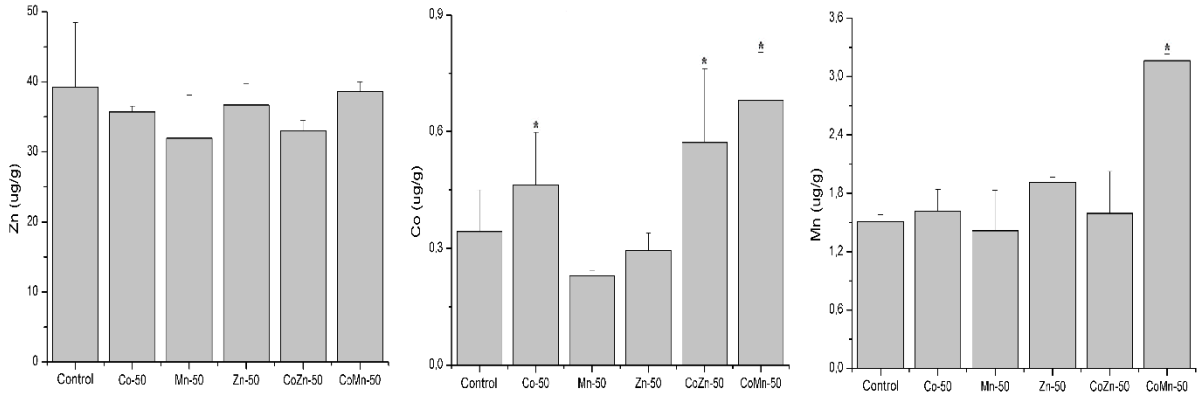

Figure 7. Zn, Co and Mn concentration (dry-matter basis) of barramundi juveniles

6. According to the results of the red drum experiments, it can be stated, that the optimisation of the calcium-magnesium ratio in ambient water is essential for the appropriate survival of juveniles, particularly right after the transportation. After it was established that the water hardness in aquaria was optimal for the species, the mortality caused by osmotic stress was reduced significantly by the magnesium-chloride supplementation. In further experiments, appropriate growth performance and feed conversion was achieved in comparison with available scientific papers. According to these results a technological description was established serving essential information regarding the introduction of red drum to Hungary.

4. POSSIBLE APPLICATIONS OF THE RESULTS

Barramundi *(Lates calcarifer)* and red drum (*Sciaenops ocellatus*) are euryhaline predatory fish species native in Southeast Asia and Gulf of Mexico. Both can be considered as new fish species in the Hungarian aquaculture and based on our geothermal potential these warm-water fish can be produced successfully. The results of this dissertation concerning the development of larval and juvenile rearing technology, as well as nutrition of barramundi and red drum serve essential information regarding the introduction of these species to Hungarian aquaculture.

Living nourishment organisms such as *Artemia nauplii* play an essential role in the larval rearing of marine fish species. However, zooplanktons in natural aquatic environments contain minerals in a higher concentration than the usually fed newly hatched *Artemia*. Therefore the aim of recent study was to investigate the effects of trace mineral supplementation on the larval growth of barramundi, when the retention occurs through a nourishment organism. The results showed that growth performance of larvae was positively affected by the cobalt, zinc and manganese enrichment of live feed. Since these micro elements are essential in trace amount for many living functions of fish, it can be stated, that these findings significantly contribute to the improvement of larval nutrition.

The impacts of different trace minerals on fish growth and health conditions are widely investigated during juvenile rearing, since adequate nutritional profile of the first formulated dry feeds are vital in the early life stages of marine fish species. However, less information is available concerning the cobalt, zinc and manganese requirement of barramundi, as well as the effects of dry diet supplementation with these micro nutrient on the growth performance of fingerlings are poorly documented. The results of the experiments indicated, while the main production parameters of juveniles were not affected by the trace mineral enrichment, the concentration of cobalt and manganese in fish was elevated significantly with the supplementation dose of dry feed. Nevertheless, these findings may contribute to further research regarding the cobalt and manganese requirement of barramundi in the juvenile stage.

Red drum is well known to be a less stress tolerant fish species. Therefore, during and after transportation, significant losses can be taken into account. Thus in the dissertation conducted with juveniles, the ion content of ambient water vital for the survival of species was analysed. The results showed, that the mortality caused by the osmotic stress can be reduced significantly by the optimisation of the calcium-magnesium ratio in ambient water with the addition of

magnesium-chloride salt. Based on the findings about the optimal ion content of water, as well as growth performance and feed conversion of red drum juveniles, a technological description was established serving essential information regarding the introduction of red drum to the Hungarian aquaculture.

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