How to improve foraging efficiency for restocking measures of juvenile Baltic sturgeon (*Acipenser oxyrinchus*)

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ABSTRACT

Atlantic sturgeon (*Acipenser oxyrinchus*), also known as Baltic sturgeon, is considered missing or extinct in German waters. Current conservation efforts focus on re-stocking activities, but classical hatchery rearing may reduce the fitness of the respective juveniles. In this study, we evaluated if foraging efficiency can be improved by short term training. Over a period of 14 d, we kept individuals of the training group in a raceway and fed them chironomids buried in a small sand spot to stimulate benthic feeding behavior while fish of the control group were fed in tanks without substrate. Thereafter, each fish was transferred to a raceway entirely covered with sand. For feeding, chironomids were randomly buried in the sand. During the first 7 days, trained fish recovered the feed significantly faster than untrained fish of the control group. Gene expression revealed an up-regulation in *neurod1* in all brain regions after 14 d of training. Thus, this study suggests that foraging efficiency can be improved through short-time training thus improve fitness upon restocking into the wild.

Table of Abbreviations

d- days

TL- total length

Key words: *Acipenser oxyrinchus, fitness, foraging, conservation, neuroD1, pcna, bdnf, restocking*

1. Introduction

Sturgeons (Acipenseridae) were once native in all major rivers of the Northern Hemisphere, but over the last 100 years, they have shown a drastic decline due to fishing, habitat destruction and hydro-construction as well as pollution. In general, sturgeons exhibit an unusual combination of behavior and life history characteristics, particularly their late onset of maturity, making them highly vulnerable to anthropogenic impacts (Rochard et al., 1990). Nowadays, sturgeons are among the most endangered fish species worldwide (IUCN, 2018) and several restoration programs have been implemented. Baltic sturgeon (*Acipenser oxyrinchus)* has been indigenous to the Baltic region for the last 8000 years, but is now considered extinct in German waters. Restocking programs have been established as a part of the ongoing recovery efforts to reintroduce Baltic sturgeons into their natural habitats and, thereby, initiate self-sustaining populations (Gessner et al. 2011).

However, there are major concerns regarding early life experiences in artificial environments that may not produce fish prepared to face life in the wild (Johnsson et al., 2014). Studies have shown that classical hatchery rearing negatively affects fish fitness and eventually the performance and survival of the fish upon release into the wild (Sulak et al., 2014). It is known that in the natural environment, sturgeons experience stimuli that shape and influence brain plasticity, cognition and behavioral phenotype. In contrast, modern hatchery practices, including high stocking densities, predictable feeding regimes and uniform stimulus-poor rearing conditions, can result in impaired cognition and behavioural responses in sturgeons reared in captivity, leading to reduced fitness and survival (Ebbesson and Braithwaite, 2012).

In contrast to mammals, fishes display remarkable plasticity in brain neurogenesis, which remains active throughout adult life. As a consequence, fish are sensitive too and respond to changes in both social and environmental conditions (Ebbesson and Braithwaite, 2012). In their natural habitats, most fishes experience environmental challenges and are able to adapt

their physiology and behavior in order to cope more effectively. Much of this flexibility is supported and influenced by cognition and neural plasticity. Furthermore, current literature on fish cognition indicates that many fish species are capable of learning and integrating multiple pieces of information that require more complex processes than associative learning (Ebbesson and Braithwaite, 2012).

Neuroscientists have payed special attention to the molecular mechanisms of neural plasticity associated with memory. This work has resulted in markers related to neural plasticity. Recent studies have indicated that proneural gene neurogenic differentiation 1 factor (*neurod1*) is a reliable measure of neurogenesis in fish and a useful indicator of the neural plastic changes associated with memory and learning (Rossi et al.,2006; Grassie et al., 2013). Moreover, brain-derived neurotrophic factor (*bdnf*) has an important role in neural plasticity through sculpting and refinement of synapses and through promoting neurogenesis and cell survival (Castrén and Rantamäki, 2009). Though not specific to the brain, proliferating cell nuclear antigen (*pcna*) is a marker for cell proliferation in the respective organ (Leung et al., 2005). Taking into account the functions of the genes previously mentioned (*neurod1*, *bdnf* and *pcna)*, they were of interest for this present study.In the wild, Baltic sturgeon is a benthic feeder which shows a digging behavior with help of the rostrum, preying on worms, shrimps and other invertebrates and making use of a powerful suction feeding mechanism (Carroll and Wainwright, 2003). Since most of their time is spent in waters with low visibility, feeding is performed by using a combination of olfactory, taste, tactile chemosensory cues and electroreceptors rather than vision (Mclean et al., 2013; Miller, 2004).The reduced importance of vision in feeding in sturgeons is supported by the observation that these fishes have relatively small eyes in relation to body size. In captivity, Baltic sturgeons are fed with artemia and thereafter with deep-frozen chironomids until they can be weaned on dry feed.

98 Taking into account that efficient foraging behavior is a key determinant of juvenile
99 survival, the aim of this study was to determine whether natural foraging behaviour could be
100 improved in hatchery-reared Baltic sturgeon following a short training period. In addition, this
101 study also investigated whether this training resulted in positive changes in brain plasticity
102 and cognition. To that end, following training, brains were sampled for gene expression
103 analysis.

2. Materials and Methods

2.1. Experimental design

The experiments were performed at the experimental facilities at the Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB, Berlin, Germany) using fish from the sturgeon stock kept at the IGB. The experiment was conducted successively with 4 trained and 4 non-trained one year old Baltic sturgeon (*Acipenser oxyrinchus*) (TL 19-25 cm, 34 – 37 g) randomly distributed between 8 experimental raceway units (2.40 m * 0.225 m * 0.1 m) at a natural photoperiod and acclimatized for 7 days. Dissolved oxygen (8.41 – 9.16 mg/L), and temperature (18 – 20 °C) were measured daily, nitrit-nitrogen (0.002 mg/L) and total ammonia (TAN, 0.021 mg/L) every three days. Furthermore, 10 sturgeons were transferred to a constructed river stretch (6 m length, 1 m width, 0.2 m water depth, supplied by a Pontec Pondomax Eco 8000 pump) simulating close to natural conditions. The pond group was reared in an outside river stretch in order to compare the results of the trained and non-trained experimental groups to a more naturalistic environment (positive control).

Three experimental groups were established: non-trained, trained and pond (8 fish per group). The 8 raceways were assigned to non-trained and trained experimental group. The trained group had a sandy bottom (10 cm depth) while for the non-trained group the four raceways were left bare (10 cm depth).After a 7 day acclimatization period, during which the fish were fed chronomids, the training school started. The training was specifically designed to improve foraging behavior. Therefore, trained fish were fed chironomids hidden below a sand spot (< 10 cm), while the fish in the remaining bare raceways received chironomids on the bare tank bottom (non-trained). Before feeding in the respective raceway, fish were isolated by introducing a wall which was removed after feed had been introduced in the remaining part of the raceway. In the pond group, chironomids were hidden in the sandy substrate.

After 14 days, two sturgeon of each group were sampled for the gene expression, the remaining two fish were transferred to a raceway covered with sand (behavioral assessment). . For the assessment, chironomids were hidden in the sand and time until successful foraging was recorded for trained as well as for untrained fish (n=8). If chronomids were not successfully found within 120 min, food was removed. This assessment of foraging behaviour was repeated for seven consecutive days.

For the gene expression analysis, fish were euthanized with MS222 (300 ppm) followed by cutting through the spinal cord. Brains from 8 Baltic sturgeons per group (trained, non-trained, pond) were dissected and divided into three parts representing the three main brain regions (forebrain, midbrain and hindbrain). Samples were stored in RNA later at -80 °C for later gene expression analysis.

All experiments were in compliance with EU Directive 2010/63/EU and approved by the national authorities (G0305/15, Landesamt für Gesundheit und Soziales, Berlin, Germany).

2.2.Gene expression

Total RNA was extracted with TRIzol as described by (Reiser et al., 2011), including a DNase I digestion. Total RNA concentration and purity were determined in duplicates with a Nanodrop® ND-1000 UV–Vis spectrophotometer. Purity was validated as the ratio of the absorbance at 260 and 280 nm (A260/280) ranging between 1.8 to 2.0. Moreover, integrity of the total RNA was checked by gel electrophoresis and, in 10% of all samples, on a RNA 6000 Nano chips with an Agilent 2100 Bioanalyzer. To eliminate potential DNA contamination, DNAse I digestion was performed in all samples prior to transcription. Next, mRNA was transcribed with MMLV Affinity reverse transcriptase (Agilent, 200 Units/µl) according to the manufacturer's instruction. In 10% of the samples, the enzyme was substituted by pure H_20, serving as a control (-RT) to monitor DNA contamination.

153 Species-specific primers targeting elongation factor 1α (*ef1a*), brain-derived neurotrophic
154 factor (*bdnf*), neurogenic differentiaton factor (*neurod1*) and proliferating cell nuclear antigen
155 (*pcna*) were designed using the sequence information available. Specificity of the assays was
156 confirmed by direct sequencing (SeqLab, Germany). Real-time PCR was carried out with
157 Mx3005p qPCR Cycler (Stratagene), monitoring specificity by melting curve analysis. Full
158 specifications of qPCR assays, including primer sequences are given in Table 1.

159 Briefly, 2 µL of the diluted sample (40 ng/µL) were used as template in 20 µL PCR mix
160 [SYBR-Green I (Invitrogen), 200 µM of each dNTPs (Qbiogene), 3 mM MgCl 2 and 1 U
161 Invitrogen Platinum Taq polymerase]. PCR conditions comprised an initial denaturation at 96
162 °C for 3 min, followed by 40 cycles of denaturation at 96 °C for 30 s, primer annealing (for
163 Ta, see Table 1) for 30 s and elongation at 72 °C for 30 s. PCR efficiencies were determined
164 experimentally with a dilution series of a calibrator corresponding to 200 ng/µl. PCR assays
165 for all individual samples were run in duplicate. Expression of target genes were calculated by
166 the comparative CT method (ΔΔCT) according to (Pfaffl, 2001), correcting for the assay
167 efficiencies and normalizing to elongation factor 1α (*ef1a*) as a housekeeping gene.
168 Expression data are presented as fold increase of the respective control.

169 **2.3.Data analysis and statistical methods**

170 Data are presented as mean ± standard deviation (SD). Prior to statistical analyses, all data
171 were tested for normality of distribution using the Kolmogorov-Smirnov/ Shapiro Wilk test
172 and for homogeneity using Levene test. Data on the behavior were analyzed using T-test. The
173 level of significance used was $P \leq 0.05$. All statistical analyses were performed with GraphPrism
174 statistical program.

175

176 ## 3. Results

177 ### 3.1.Behavioral assessment

178 In the behavioral assessment (Fig.1), significant differences were observed between non-
179 trained and trained fish. While the time taken to initiate feeding successfully progressively
180 decreased in both groups over the 7-day feeding study, this time was significantly less
181 ($p<0.05$) in the trained compared to the non-trained fish at all time points (Fig. 1). On day 1,
182 trained fish took 58±6 min to successful foraging whereas none of the non-trained fish
183 recovered the chironomids within 120 min. After 7 d, chironomids were recovered after 8±2
184 min and 18±9min in trained and non-trained fish, respectively.

185 ### 3.2.Brain plasticity and cognition

186 Selected genes related to brain plasticity and cognition (*neurod1, bdnf, pcna*) were
187 analyzed in all three brain areas of Baltic sturgeon. Regarding the forebrain region (Fig. 2),
188 significant differences were observed in the expression of *neurod1*. In particular, there was an
189 up-regulation in the trained and pond groups with respect to the non-trained group in Baltic
190 sturgeon after 14 d of training (Fig 2A). Furthermore, *pcna* expression showed an up-
191 regulation in the pond group in comparison to both trained and non-trained groups in the
192 forebrain of Baltic sturgeon (Fig. 2B). Similar results were observed in both midbrain and
193 hindbrain region (Figs. 3B & 4B) of Baltic sturgeon in which *neurod1* showed an up-
194 regulation in both the trained and pond groups compared to the non-trained group after 14 d
195 of training.

196

197 **Discussion**

198 Stocking still remains an important conservation tool to combat the continuing global
199 decline in fish biodiversity (Pikitch et al., 2005). Hatcheries are a key element in the recovery
200 plan for sturgeon and have been regarded as a temporary measure until more aggressive
201 habitat restoration programs are established. In fact, hatcheries are currently the only viable
202 option to increase sturgeon populations. Hatchery programs for sturgeon have demonstrated
203 considerable success in collecting or developing brood-stock, spawning, and rearing juveniles.
204 However, the success of sturgeon hatcheries for conservation will ultimately depend on how
205 effectively the hatchery-reared sturgeon can adapt to the natural habitat following release
206 (Brown and Day, 2002).

207 In general, when released directly into the rivers, the survival of hatchery reared
208 juveniles from different fish species is only approximately 1-3% after a few months due to a
209 combination of predation, starvation and other factors (Brown and Day, 2002; Chebanov et
210 al., 2011). Furthermore, it is widely accepted that post-release survival rates of hatchery-
211 reared fish are lower compared to their wild conspecifics (Campton et al., 1991; Svasand and
212 Kristiansen, 1990). Fisheries scientists are increasingly convinced that the uniform stimulus-
213 poor environment experienced during hatchery rearing is one of the main contributing factors
214 for this reduction in fitness and post-release survival (Ellis et al.,1997; Masuda and
215 Tsukamoto, 1998). Thus, research to improve the post-release survival of hatchery-reared
216 juveniles through behavioral performance is needed in order to continue successful recovery
217 plans.

218 Suggested methods for improving the survival of hatchery fishes include
219 supplementary feeding with live foods, the provision of under-water feeders, sub-aquatic
220 structure, natural substratum, etc (Maynard and Flagg, 1994) In the wild, Baltic sturgeon is a
221 benthic feeder which shows a digging behavior in order to find worms, shrimps and other

222 invertebrates from the substrate (Miller, 2004). Like all fish behavior, foraging relies on
223 experience. The foraging skills of fishes become adjusted to ecological conditions through
224 learning (Hughes et al., 1992; Warburton, 2003). Fish are able to learn to recognize prey, how
225 to handle them and where they are likely to be located (Warburton, 2003). Results from the
226 behavioral assessment of this study suggest that a short training period can improve the
227 foraging ability of Baltic sturgeon by reducing the amount of time taken to successfully
228 forage. This significant improvement was seen in the first 7 days, which can be critical when
229 released into their natural habitat. This could be an important approach since when recaptured
230 after release into the wild, hatchery fishes are often found to have empty stomachs (O'Grady,
231 1983; Johnsen and Ugedal, 1989).

232 The understanding of fish cognition and the role played by different brain regions has
233 improved significantly in recent years. Fish brain remains plastic throughout their entire life
234 and continues to be sensitive to both social and environmental changes. Most fishes
235 experience challenges in their environmental and are able to adjust and adapt their physiology
236 and behavior to help them cope more effectively. Much of this flexibility is supported by
237 cognition and neural plasticity. Neural plasticity allows for the development and function of
238 cognitive processes (Ebbesson and Braithwaite, 2012; Knudsen, 2004), and thus has a large
239 role in the adaptation to changing environments. Current literature on fish cognition indicates
240 that many fish species are capable of learning and integrating multiple pieces of information
241 that require more complex processes than just associative learning (Ebbesson and Braithwaite,
242 2012). The most important brain area for these complex neural processes in fish is the
243 dorsolateral telencephalon (Dl) (Durán et al., 2010; Rodríguez et al., 2002; Wullimann and
244 Mueller, 2004), and has been recognized as the functional homologue of the mammalian
245 hippocampus (Mueller et al., 2011; Mueller and Wullimann, 2009).

246 Studies using intermediate early genes (IEG) make possible to investigate which brain
247 regions are activated during a particular cognitive process. In this study, three main genes
248 were studied: neurogenic differentiation factor (*neurod1*), brain-derived neutrophic factor
249 (*bdnf*) and proliferating cell nuclear antigen (*pcna*). Neurogenic differentiation factor
250 (*neurod1*), is a member of a family of pro-neural genes, which is involved in the initiation and
251 regulation of neural differentiation (Kiefer, 2005). Recent studies have shown that expression
252 levels of *neurod1* mRNA is a reliable measure of neurogenesis in fish and a useful indicator
253 of the neural plastic changes associated with memory and learning (Grassie et al., 2013;
254 Salvanes et al., 2013). Brain-derived neurotrophic factor (*bdnf*) is the most abundantly
255 expressed member of the nerve growth factor family, neurotrophins, and has an important role
256 in neural plasticity through sculpting and refinement of synapses and through promoting
257 neurogenesis and cell survival (Castrén and Rantamäki, 2009). It has recently been shown that
258 environmental challenges alter *bdnf* expression in the telencephalon of Atlantic salmon
259 (Vindas et al., 2017). Regarding proliferating cell nuclear antigen (*pcna*), it can be used as a
260 marker for cell proliferation (Leung et al., 2005).

261 In this study, *neurod1* was up-regulated in all brain regions in both the trained-group
262 and fish raised in the semi-natural pond, in comparison to the non-trained fish. This might be
263 an indication of the stimulation of cognitive processes such as learning and memory as a
264 result of the training method. Thus, it indicates that the fish from the trained group generally
265 learnt to locate the prey. These results are in agreement with the results found in the
266 behavioral assessment.

267 Currently, the main limitation of stocking programs for most fish species is the high
268 level of post-release mortality. The most critical period seems to be the immediate days
269 following release as hatchery-reared fish generally display reduce life fitness traits such as
270 foraging and anti-predator behaviour.. In our study, it was demonstrated that a short time

period of training could potentially help Baltic sturgeon in their process of learning to successfully forage, which could be a first approach to improve restocking practices. Since rearing conditions are highly important in stocking for conservation, hatcheries should aim to produce juveniles that are morphologically, genetically, behaviorally and physiologically similar to the stock they pretend to enhance and recover. Furthermore, restoration programs require a variety of information on sturgeon and thus, it is interesting to produce and keep up to date extensive reviews of the literature. Further work is needed in order to determine the survival of sturgeon reared under alternative hatchery-rearing practices taking into account other key factors in sturgeon survival.

Conclusion

To our knowledge, this is the first study that looks into foraging training in Baltic sturgeon to improve fitness for re-stocking purposes. We observed that both behavioural and physiological parameters were improved by a short-term training period. This improvement could significantly help Baltic sturgeon survive in the wild, since the highest percentage of mortality happens during the first days post-release.

Declaration of Interest

There are no conflicts to declare

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Author contributions

The experiment was conducted by C.E.S. The laboratory analysis was carried out by M.C.R. M.C.R. wrote the first draft of the manuscript. S.V. supervised the project. The manuscript was revised by all co-authors.

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Figure legends

Figure 1. Behavioural assessment. Time taken by Baltic sturgeon (*A. oxyrinchus*) of the non-trained, trained and pond group to successfully forage. Significant differences are indicated by asterisk (Tukey's, $P < 0.05$, $n = 8$)

Figure 2. Gene expression, determined by qPCR, in the forebrain of Baltic sturgeon (*A. oxyrinchus*) of the non-trained, trained and pond group after 14 days of training. Data are expressed as fold relative. Groups with different subscripts are significantly different ($P < 0.05$, $n = 8$). Statistic test used (Dunn's or Tukey's) is indicated below the respective graph.

Figure 3. Gene expression, determined by qPCR, in the midbrain of Baltic sturgeon (*A. oxyrinchus*) of the non-trained, trained and pond group after 14 days of training. Data are expressed as fold relative. Groups with different subscripts are significantly different ($P < 0.05$, $n = 8$). Statistic test used (Dunn's or Tukey's) is indicated below the respective graph.

Figure 4. Gene expression, determined by qPCR, in the hindbrain of Baltic sturgeon (*A. oxyrinchus*) of the non-trained, trained and pond group after 14 days of training. Data are expressed as fold relative. Groups with different subscripts are significantly different ($P < 0.05$, $n = 8$). Statistic test used (Dunn's or Tukey's) is indicated below the respective graph.

Tables

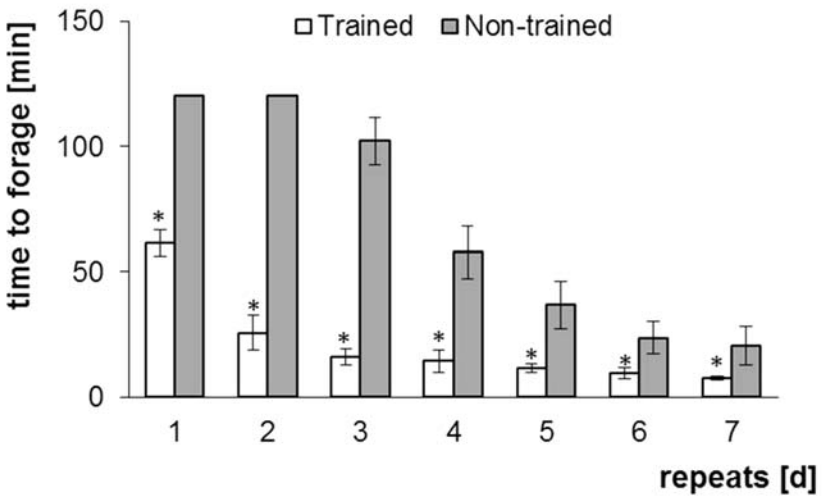
Table 1. Specifications of qPCR assays including primer sequences, annealing temperature (Ta), amplicon length [bp], PCR efficiency (Eff) and NCBI accession number of the respective housekeeping (ef) and target genes: ef- elongation factor 1 a, neurod1- neurogenic differentiation factor, bdnf - brain-derived neurotrophic factor, pcna- proliferating cell nuclear antigen-

gene	primer	5'-3' sequence	Ta [°C]	length [bp]	Eff.[1] [%]	GeneBank #
efa	f	TCAgggAgAAgATTgACCgT	65	239	97	2160436
	r	AgACTTggTgACTTTgCCTg				
neuroD	f	TATCATTCCCCTggTCTgCC	65	175	98	2160451
	r	CATTAACgCTCAgTggTggg				
pcna	f	gAAgAAggTTTTggAggCg	65	187	92.5	2160452

	r	CCTgCTCAgATTgACCCC				
bdnf	f	gACggCCgTAgACAAgAAgA	65	188	84.5	2160439
	r	TggTCCgACACTgTgAATTg				

[1]Efficiency was determined from serial dilution series

432



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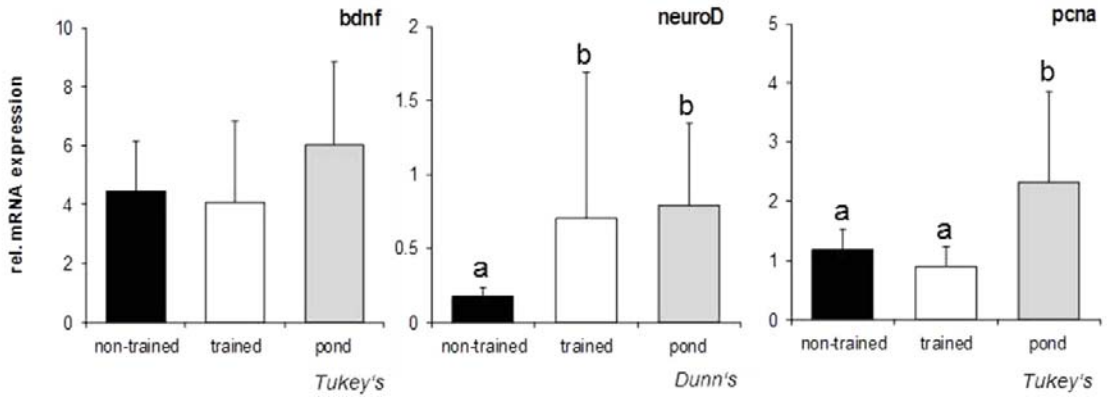

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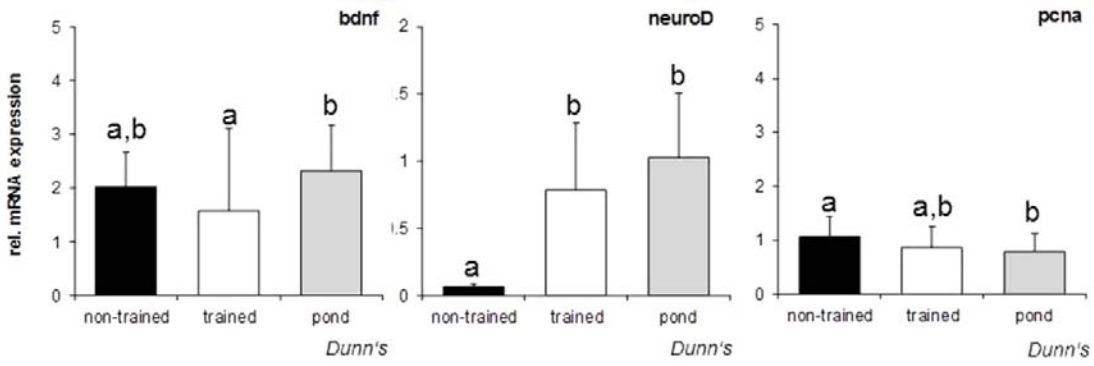

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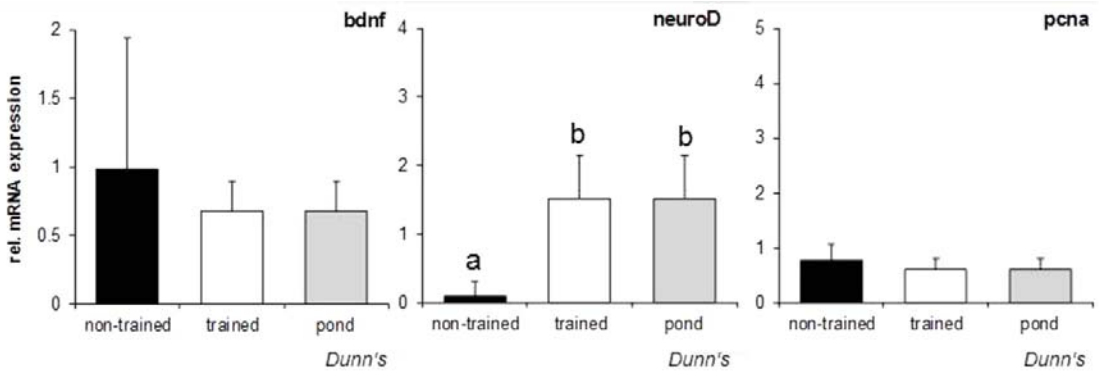

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