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RESEARCH ARTICLE

Effects of environmental enrichment on forebrain neural plasticity and survival success of stocked Atlantic salmon

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ABSTRACT

Fish reared for stocking programmes are severely stimulus deprived compared with their wild conspecifics raised under natural conditions. This leads to reduced behavioural plasticity and low post-release survival of stocked fish. Environmental enrichment can have positive effects on important life skills, such as predator avoidance and foraging behaviour, but the neural mechanisms underpinning these behavioural changes are still largely unknown. In this study, juvenile Atlantic salmon (*Salmo salar*) were reared in an enriched hatchery environment for 7 weeks, after which neurobiological characteristics and post-release survival were compared with those of fish reared under normal hatchery conditions. Using *in situ* hybridization and qPCR, we quantified the expression of *brain-derived neurotrophic factor* (*bdnf*) and the neural activity marker *cfos* in telencephalic subregions associated with relational memory, emotional learning and stress reactivity. Aside from lower expression of *bdnf* in the Dlv (a region associated with relational memory) of enriched salmon, we observed no other significant effects of enrichment in the studied regions. Exposure to an enriched environment increased post-release survival during a 5 month residence in a natural river by 51%. Thus, we demonstrate that environmental enrichment can improve stocking success of Atlantic salmon parr and that environmental enrichment is associated with changes in *bdnf* expression in the fish's hippocampus-equivalent structure.

KEY WORDS: *bdnf*, *cfos*, Cognition, Neuroanatomy

INTRODUCTION

Hatchery and aquaculture rearing environments have a profound impact on fish development and behavioural responses. Compared with fish in the wild, hatchery-reared fish are kept at unnaturally high densities in a uniform environment and are severely stimulus deprived in terms of feeding variability, predator exposure and fluctuations in abiotic factors (Johnsson et al., 2014). As a result, current commercial hatchery procedures result in the production of fish that deviate from their wild conspecifics in behavioural (Johnsson et al., 2014; Olla et al., 1998) and neural (Mes et al., 2018) characteristics. This has implications for production and welfare aspects of fish rearing, in particular within the context of compensatory stocking programmes (Brown and Day, 2002). Millions of hatchery-reared salmonids, primarily Atlantic salmon (*Salmo salar* L.), are released into natural waters in Northern Europe yearly (ICES, 2015). Unfortunately, salmonid stocking (i.e. all forms of stocking from fingerlings, to parr, to smolts) is currently characterized by high post-release mortality rates and reared salmon show lower survival compared with wild conspecifics (Jonsson et al., 2003; Tatara et al., 2009; Thorstad et al., 2011). From both an ethical and a financial perspective, it is imperative to improve survival rates of hatchery-reared fish by providing rearing conditions that allow for optimal development of neural and behavioural plasticity, thus producing fish that resemble the 'wild-type' phenotype more closely.

To improve stocking success, efforts have been directed towards enrichment of the hatchery environment to create more wild-like rearing conditions. These hatchery modifications typically encompass structural enrichment such as rocks, plants and shelter (Näslund and Johnsson, 2016). Structural environmental enrichment (EE) has been shown to improve the learning capacity (Ahlbeck Bergendahl et al., 2016; Salvanes et al., 2013), exploratory behaviour (Lee and Berejikian, 2008), prey capture and handling skills (Brown et al., 2003; Rodewald et al., 2011; Sundström and Johnsson, 2001) and, ultimately, post-release survival of hatchery-reared fish in some (Hyvärinen and Rodewald, 2013; Maynard et al., 1995; Roberts et al., 2014) but not all studies (Brockmark et al., 2007; Tatara et al., 2009; Fast et al., 2008). While the effects of EE on behavioural characteristics of hatchery-reared fish have thus been mapped to some extent, the brain, which underlies these behaviours, remains understudied. Some studies have demonstrated that EE can increase brain size (Kihslinger and Nevitt, 2006; Näslund et al., 2012), but it is difficult to interpret how a larger brain size relates to increased processing capacity and, ultimately, changes in behaviour (Johnsson et al., 2014; Ebbesson and Braithwaite, 2012). Notably, a few studies have reported altered expression of neurogenesis markers in the whole telencephalon after EE rearing. For example, Salvanes et al. (2013) reported that EE rearing increased telencephalic *neurogenic differentiation factor 1* (*neurod*; a marker for the number of cell divisions within the brain) transcript abundance in Atlantic salmon parr and von Krogh et al. (2010) observed higher numbers of proliferating cell nuclear antigen (Pcna)-expressing cells (a marker for the number of newborn cells within the brain) in EE-reared zebrafish (*Danio rerio*), while Manuel et al. (2015) reported lower telencephalic *neurod* and *pcna* expression in zebrafish reared in an enriched environment. However, changes in the neuroplasticity marker *brain-derived neurotrophic factor* (*bdnf*) and the neural activity marker *cfos*, both associated with brain processing capacity (de Azua et al., 2013; Jee et al., 2008), due to EE have not been reported. Importantly, the telencephalon consists of a plethora of neuronal subpopulations, each

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driving distinct behavioural and cognitive processes. For example, the dorsolateral pallium (Dl) in the telencephalon is involved in relational memory and spatial orientation, while the dorsomedial pallium (Dm) is associated with emotional memory and stress reactivity (Portavella et al., 2004; O'Connell and Hofmann, 2011; Broglio et al., 2015). Furthermore, the ventral part of the ventral telencephalon (Vv) is believed to be involved in the regulation of goal-oriented and social behaviour (O'Connell and Hofmann, 2011; Vargas et al., 2009). Thus, these regions drive neural processes that may underlie the behaviours which are influenced by EE, and we hypothesize that exposure to EE will alter the expression of the neuroplasticity marker *bdnf* and the neural activity marker *cfos* in these regions. The protein Bdnf of the neurotrophin family promotes neural remodelling such as synaptic plasticity, long-term potentiation, neurogenesis and cell survival (Mattson et al., 2004; Pang et al., 2004; Gray et al., 2013). Cfos is a marker for recent neural activity. That is, increased expression of Cfos indicates increased neuronal signalling (Okuno, 2011). Notably, we have previously reported lower telencephalic *bdnf* and *cfos* expression in wild compared with hatchery-reared fish (Mes et al., 2018), and we expect that enriched fish will present a profile similar to that of wild individuals.

We studied the effects of a 7 week period of EE on *bdnf* and *cfos* expression in the Dl, Dm and Vv telencephalic region-specific areas by means of *in situ* hybridization and qPCR, as well as post-release survival success in juvenile Atlantic salmon. Post-release survival was assessed by capturing downstream migrating salmon, after 5 months of residence in a natural river. Our study confirms that EE can increase post-release survival of stocked fish, but we found few effects on the expression of *bdnf* or *cfos* in the selected telencephalic regions after 7 weeks of enrichment.

MATERIALS AND METHODS

Ethical permit

This experiment was performed in accordance with current Norwegian law for experimentation and procedures on live animals, and was approved by the Norwegian Food Safety Authority (Mattilsynet) through FOTS application ID 10034.

Experimental animals

This experiment was conducted at the Norwegian Institute for Nature Research (NINA) salmon hatchery in Ims, using hatchery-reared Atlantic salmon parr (i.e. juvenile salmon that live in fresh water before undergoing smoltification, the metamorphosis that prepares them for their migration into the ocean). The experimental fish were first-generation offspring from wild parents from the river Imsa, south-western Norway (58°50′N, 5°58′E). A total of 15 females and 10 males were paired. All eggs (10,880) were fertilized together and placed on an incubator where the temperature went from 9 to 2.7°C until hatching (approximately 2 months) and from 3.2 to 4.9°C until the start of feeding (approximately 2 months). At this point, 9000 fish were moved to a plastic tank (1×1×0.45 m) and were kept in constant light at temperatures between 9.4 and 12.4°C for approximately 3 months. Fish were then divided into two groups of 4400 individuals in two indoor tanks (1×1×0.45 m) and kept with constant light at temperatures between 12.5 and 20.4°C for 1 month. A total of 6847 fish were then moved to an outdoor cement tank of 72 m^2 (approximately 50 m^3) and were kept on a natural temperature and photoperiod until the start of the experiment. Throughout this time, fish were fed with commercial feed (Nutra Parr, Skretting, Stavanger, Norway) provided *ad libitum* throughout the day by automatic feeders, following the feeding tables for feed size recommended by the distributor.

Fish were transferred to the experimental tanks at 9 months post-hatching and brain sampling, and release into the wild took place at 11 months post-hatching. All fish were lightly anaesthetized in Benzoak vet [ACD Pharmaceuticals AS; 0.14 ‰ (v/v) in water], weighed, measured (total length) and individually marked by making a small incision with a #10 scalpel blade and implanting intraperitoneally a 12 mm passive integrated transponder (PIT) tag prior to the start of the experiment. At the end of the enrichment study (more than 2 months after inserting the PIT tags), we found that all tags had been retained. Note that when properly implanted, PIT tags have a retention rate >99%. Early sexually mature (precocious) males showed swollen and soft bellies, and upon touch they would release sperm. Once recognized, all mature males were excluded from the experiment.

Environmental enrichment

In the first week of October 2016, 9 month old Atlantic salmon parr (n=780; 152±25 mm total length, 41±18 g body mass, mean±s.d.) were randomly distributed between three control and three enriched tanks (n=130 fish per tank) for a duration of 7 weeks. The duration of enrichment was chosen based on previous research conducted by Salvanes et al. (2013) on salmon parr of approximately the same age as those used in this experiment. Furthermore, we chose to conduct the experiment on parr as fish were to be released into the river, where they would later smoltify and migrate into the sea. Control tanks were square holding tanks measuring 2×2×1.2 m (l×w×h), filled with approximately 3 m^3 of flow-through ambient river water and covered with a fibreglass lid, through which natural light could penetrate. Enriched tanks were identical to control tanks, except with the addition of 40 rocks (10–20 cm diameter), as well as three artificial plastic 'plants', composed of eight black fronds (9 cm wide and 100 cm long) each, which were weighted down with a small rock, following methodology described by Salvanes et al. (2013). Fish appeared to use both rocks and plants for shelter and fish in the enriched tanks showed less movement and schooling behaviour than control fish in reaction to disturbance, although none of these behavioural responses were quantified. All tanks were manually cleaned every third day by partially draining the water while scrubbing the bottom of the tank with a broom. The rocks and plants in the enriched tanks were rearranged weekly using a net and control tanks were disturbed with the same net for an equal amount of time to control for stress effects. Commercial feed (Nutra Parr, Skretting, Stavanger, Norway) was provided *ad libitum* throughout the day by automatic feeders. Water temperature was 11°C at the start of the experiment and decreased to 4°C at the end. After the 7 week enrichment period, all fish were again measured and weighed, and individual standardized specific growth rate (SGR; Ω, %) was calculated as follows:

$$\Omega = \frac{M_{\mathrm{f}}^{b} - M_{\mathrm{i}}^{b}}{b \times t} \times 100, \quad (1)$$

where M_{f} is the final body mass (in g) at the end of the enrichment period, M_{i} is the initial mass (in g) at the start of the experiment, b is the allometric mass exponent for the relationship between specific growth rate and body mass (estimated to be 0.31 for Atlantic salmon juveniles; Robertsen et al., 2013) and t is the experimental time in days. Fig. S1 depicts the time line for the experimental setup.

Brain sampling

After 7 weeks of enrichment, the brains of a subset of fish were sampled (by taking n=4 fish from each tank, n=12 per treatment),

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and processed for quantification of neuroplasticity markers in subregions of the telencephalon by either *in situ* hybridization (ISH) or qPCR. To this end, fish were randomly collected in pairs from each of the six holding tanks and anaesthetized in 0.75‰ (v/v) 2-phenoxyethanol (Sigma-Aldrich 77699), which rendered them unconscious within 30 s, after which total length and body mass were recorded. Brains were sampled in two ways. (i) To sample brains for ISH, fish were sampled as described by Mes et al. (2018). In short, anaesthetized fish were fixed by vascular perfusion in 2% paraformaldehyde and brains were then dissected and post-fixed overnight. After three washing steps, brains were cryopreserved overnight in 25% sucrose and subsequently embedded in Tissue-Tek OCT compound, and stored at −80°C until further processing. (ii) To sample brains for microdissection and subsequent qPCR analysis, anaesthetized fish were decapitated and the jaw and gills were trimmed away. The tissue was then sealed in a plastic bag, snap-frozen on dry ice and stored at −80°C until processing. Time from decapitation to freezing ranged between 1 and 2 min.

Post-release survival

After the enrichment period, enriched (n=314) and control (n=313) PIT-tagged 11 month old salmon parr were released into the river Imsa during the first week of December to assess their post-release survival and subsequent downstream smolt migration the following spring. To this end, salmon were collected from their tanks, anaesthetized in Benzoak vet [ACD Pharmaceuticals AS; 0.14‰ (v/v) in water] and subsequently measured, weighed and adipose fin-clipped for identification, following the standard stocking procedures of the hatchery. Thereafter, fish were allowed to recover for 3 days in their holding tanks, but without structural enrichment. The river Imsa is approximately 1 km long and it drains Lake Liavatn into the Høgsfjord (Jonsson et al., 1998). Salmon parr were released at the upstream limit of the river (58°32′N, 5°34′E), which is marked by a 2 m high barrier, preventing upstream migration of fish into Lake Liavatn. Stocked fish migrated downstream towards the sea in April and May 2017, approximately 5 months after release. All downstream migrating fish (i.e. both hatchery reared and indigenous wild fish) were intercepted by a Wolf-type fish trap (Wolf, 1951), located 100 m upstream from the estuary. The entire body of water from the river Imsa passes through this trap and therefore every migrating fish is intercepted. The trap was emptied at least twice daily and captured fish were PIT scanned, weighed and measured, after which they were released downstream of the trap so they could resume their ocean migration. The cumulative number of migrating fish per day (i.e. the number of fish that migrated each day added to the total number of fish that had migrated in the previous days) and the cumulative proportion per day of migrating fish (i.e. the cumulative number of migrated fish divided by the final total number of migrating fish) were calculated. Successful migration was used as a proxy for survival and we calculated the difference in survival success (%) by the following formula:

$$[(S_{EE} - S_C)/S_C] \times 100, \quad (2)$$

where S_{EE} is the number of EE fish that survived and S_C is the number of control fish that survived.

ISH and quantification of labelled cells

ISH for *cfos* and *bdnf* transcripts (sense and antisense; for a representative example, see Fig. S2) was performed on parallel sections for n=7 fish per treatment. For each fish, the telencephalon was sectioned transversely onto one Superfrost Ultra Plus slide (Menzel-Gläser) using a cryostat (Leica CM 3050) at −24°C. Sections were 14 µm thick and spaced 90 µm apart. Slides were dried at 60°C for 10 min and subsequently stored at −80°C until further analysis. The ISH digoxigenin-labelled probes for *bdnf* and *cfos* were 485 and 906 nucleotides long and were cloned using the following primers: *bdnf* forward TCACAGACACGTTTGAGCA GGTGA, reverse ATGCCTCTTGTCTATTCCACGGCA; and *cfos* forward ACTCCGCTTTCAACACCGAC, reverse TGTAGAGA GGCTCCCAGTCC. The ISH protocol was conducted according to Ebbesson et al. (2011). In short, slides were mounted in 70% glycerol in 10 mmol l^{-1} Tris-HCl (pH 7.5), 1 mmol l^{-1} EDTA and 150 mmol l^{-1} NaCl. For both *bdnf* and *cfos*, all 28 slides were stained simultaneously in the same Coplin staining jars in random positions to avoid differences in coloration due to handling effects. After ISH, slides were photographed using an Axio Scan.Z1 slide scanner (Zeiss) at 20× magnification. Labelled *cfos* and *bdnf* cells were quantified using the Fiji platform (Schindelin et al., 2012) (RRID: SCR_002285) in ImageJ2 (Rueden et al., 2017) (RRID: SCR003070). Brain regions were identified using several salmonid stereotaxic atlases (Carruth et al., 2000; Navas et al., 1995; Northcutt, 2006) and transcript-positive cells were counted in the dorsolateral (both the dorsal and ventral subregions; Dld and Dlv, respectively) and dorsomedial (both the dorsal and ventral subregions; Dmd and Dmv, respectively) pallium, as well as in the ventral part of the ventral telencephalon (Vv; see Fig. 1 for an overview of the subregions). We used a previously developed Image J macro script (Mes et al., 2018) to semi-automate quantification of labelled cells. In short, images were converted into grayscale (8 bit), the area of interest was manually selected and the black and white threshold was adjusted within the range 145–190 to match the labelled cells in the original image. Then, all labelled cells that measured between 15 and 500 pixels were counted using the 'Analyze Particles' command. For each section, the total number of transcript-labelled cells was counted in the entire Dld, Dlv, Dmd, Dmv and Vv to elucidate subregion-specific expression patterns and to allow comparison with previous studies (e.g. Vindas et al., 2017). The number of labelled cells was quantified as described by Vindas et al. (2018) and Moltesen et al. (2016). In short, the number of

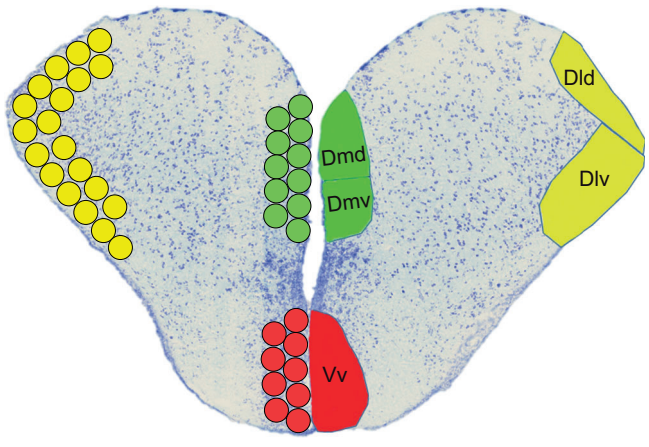

Fig. 1. Transverse view of the Atlantic salmon telencephalon. Toluidine Blue-stained telencephalon with a schematic representation of the right lobe depicting the location of the dorsal (Dld) and ventral (Dlv) dorsolateral pallium, the dorsal (Dmd) and ventral (Dmv) dorsomedial pallium, and the ventral part of the ventral telencephalon (Vv), and the left lobe depicting microdissected areas. Scale bar: 100 µm.

transcript-expressing cells was counted within each subregion for both lobes in each section (in which areas of interest were found). Labelled cells were counted in 11.2±2.8 (mean±s.d.) telencephalon sections per fish and because the number of brain sections differed per fish, we corrected for the number of counted sections by calculating the average number of labelled cells per section for each subregion; this number was used in the statistical analysis. Samples were quantified in random order and the experimenter did not know the identity of the samples at the time of quantification.

Microdissections

Frozen trimmed skulls of eight fish per treatment were sectioned (100 µm thick) transversely in a cryostat (Leica CM 3050) at −22°C. Sections were thaw-mounted on glass slides (VWR 631-151) and subsequently stored at −80°C. Microdissections of the Dld, Dlv, Dmd, Dmv and Vv (Fig. 1) were performed on frozen sections kept on a cooling plate (−14°C) as described by Vindas et al. (2017). On average, per individual, a total of 37, 37, 38, 36 and 13 punches were taken for the Dld, Dlv, Dmd, Dmv and Vv, respectively. Microdissected tissue was injected into RLT buffer (RNeasy Micro Kit, Qiagen 74004) and immediately frozen at −80°C until RNA extraction, which was conducted within 3 days of microdissection.

Relative transcript abundance

Relative transcript abundance of *cfos* and *bdnf* in microdissected areas was measured using real-time PCR (qPCR). Microdissected tissue was thawed, vortexed for 30 s, centrifuged at 13,400 ***g*** for 5 min and total RNA was subsequently extracted using the RNeasy Micro Kit (Qiagen 74004), which includes a DNase I treatment, according to the manufacturer's instructions. RNA concentration was measured using a BioTek Epoch microplate spectrophotometer and the quality of the extracted RNA was checked on a subset of samples using a Bioanalyzer RNA 6000 Pico Kit (Agilent 2100): RNA integrity number (RIN) was 9.8±0.3 (mean±s.d.) with all RIN being above 8.9. Reverse transcription was performed using an iScript cDNA Synthesis Kit (Bio-Rad 1708891) according to the manufacturer's instructions, using 36 ng of total RNA as template in a total reaction volume of 20 µl. Subsequently, cDNA was stored at −20°C.

The target genes, as well as three reference genes [*elongation factor 1αa* (*ef1αa*), *ribosomal protein S20* (*S20*) and *hypoxanthine phosphoribosyltransferase 1 (hprt1)*], were selected for qPCR (Table 1). Previously published primer sequences were available for all genes, except for *cfos*, for which primers were designed in this study. The predicted sequence for *cfos* in Atlantic salmon (accession number: XM_014206157.1) was retrieved from the National Center for Biotechnology Information (NCBI: http://ncbi.nlm.nih.gov/nuccore) and primers were designed using the NCBI Primer-BLAST function. Two *cfos* primer pairs were designed at exon–exon junctions and the primer pair with the lowest Cq value and with a single melting peak was selected for further use (Table 1). Calibration curves were run for all primer pairs (Table S1) and qPCR products were sequenced to confirm the specificity of the primers. The stability of the three reference genes (*ef1αa*, *S20* and *hprt1*) was evaluated using the NormFinder (Andersen et al., 2004) and geNorm (Vandesompele et al., 2002) methods, after which *ef1αa* and *S20* were selected as most stable reference genes.

qPCR was carried out in duplicate using a Roche Light Cycler 96 (Roche Diagnostics, Penzberg, Germany) and accompanying software (version 1.1.0.1320). The reaction volume was 10 µl including 5 µl LightCycler® 480 SYBR® Green I Master (04887352001, Roche Diagnostics GmbH, Mannheim, Germany), 1 µl of each forward and reverse primer (1 nmol l^{-1} final concentration for each primer) and 3 µl of cDNA (diluted 1:5). Cycling conditions were 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 10 s at 60°C and 8 s at 72°C, followed by a melting curve analysis. The Cq values of all genes were <35 and thus were included in the analysis following qPCR analysis methodology of Bustin et al. (2009). A calibrator, made by pooling aliquots of cDNA of all samples, was included in triplicate in all plates to allow for comparison of Cq values between plates. Expression values were computed according to Vandesompele et al. (2002), and expression values were expressed as relative to the expression of the two reference genes (*ef1αa* and *S20*).

Statistical analyses

All data were analysed in JMP Pro 14.0.0 (SAS Institute Inc.). All values are given as means±s.e.m., unless stated otherwise, except for non-parametric data, for which median values with minimum and maximum values are given. A generalized linear model (GLM) with binomial distribution was employed to compare migration success between control and enriched fish. Body size and treatment, as well as an interaction between these two variables, were considered as explanatory variables. The most parsimonious model (with the lowest corrected Akaike information criterion score, AICc) was a model with only treatment (i.e. control versus enriched) as the explanatory variable, and this model was subsequently used to compare migration success between treatments (Fig. S3).

For morphometric data, normality and homogeneity of variance were assessed by Shapiro–Wilk's and Levene's tests. Because the data showed a bimodal distribution, Wilcoxon rank-sum tests were used to compare total length, body mass and specific growth rate (SGR) between control and enriched groups. To test whether there were differences in body mass between wild and hatchery-reared fish at the time of migration, an analysis of covariance (ANCOVA)

Table 1. Primer sequences for target and reference genes

Gene	Primer sequence 5′→3′	Accession no.	Reference
ef1αa	F: CCCCTCCAGGACGTTTACAAA R: CACACGGCCCACAGGTACA	BT059133.1	Ingerslev et al., 2006
S20	F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	NM_001140843.1	Olsvik et al., 2005
hprt1	F: CGTGGCTCTCTGCGTGCTCA R: TGGAGCGGTCGCTGTTACGG	BT043501.1	Andreassen et al., 2009
bdnf	F: ATGTCTGGGCAGACCGTTAC R: GTTGTCCTGCATTGGGAGTT	GU108576.1	Vindas et al., 2014
cfos	F: AATGGAACAGCTTTCGCCTGA R: TGTCGGTGAGTTCCTTTCGC	XM_014206157.1	This study

Target genes: *bdnf* (brain-derived neurotrophic factor), a neuroplasticity marker; *cfos*, a neural activity marker. Reference genes: *ef1αa*, elongation factor 1αa; *S20*, ribosomal protein S20; *hprt1*, hypoxanthine phosphoribosyltransferase 1.

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Table 2. Biometric measurements

	Median		Minimum		Maximum		
Biometrics	Control	Enriched	Control	Enriched	Control	Enriched	Statistics
Initial length (cm)	158	159	95	93	204	195	Z=−1.2, *P*=0.23
Initial mass (g)	42.3	43.7	8.4	8.1	92.9	85.3	Z=−1.2, *P*=0.25
Final length (cm)	162	164	96	95	213	201	Z=−1.5, *P*=0.13
Final mass (g)	39.9	42	7.7	7.5	95.5	79.1	Z=−1.4, *P*=0.15
SGR	−0.46	−0.41	−1.41	−1.42	1.5	1.57	Z=−1.1, *P*=0.26

Median, minimum and maximum values for initial and final length and mass, and specific growth rate (SGR) for control and enriched fish. Non-parametric Wilcoxon statistics are provided.

was used, with body mass as a dependent variable, fish origin [hatchery (pooled enriched and control) versus wild] as an independent variable and migration date as a continuous covariate.

ISH cell counts were compared using Student's *t*-tests assuming equal variances (homogeneity of variance was verified using Levene's tests). Cell counts of one fish from the enriched treatment were excluded because slides were of insufficient quality. Cell counts in the brain areas of the left and right telencephalic lobes were pooled after assessing (by means of a Spearman's correlation test) that there were no significant lateralization differences, with the exception of the cell counts of *cfos* within the Dlv. The fact that the number of *cfos* cells in the Dlv between the right and left lobe was not correlated is likely to be due to the low number of stained cells in this area (0.45 cells per section). Because the number of labelled cells showed a significant bilateral correlation in all other brain areas in the current study, as well as for *cfos* in the Dlv in our previous work (Mes et al., 2018), *cfos* cell counts in the left and right lobe of the Dlv were also pooled. To adhere to Gaussian distribution, values were $\log_{10}$ transformed for *bdnf* (only in the Dlv). For qPCR data, gene expression levels of *cfos* and *bdnf* were compared between control and enriched groups using Wilcoxon rank-sum tests. All data used for statistical analyses can be found in Dataset 1.

RESULTS

Growth during enrichment

There were no statistical differences in length, mass or SGR between enrichment and control groups, both before the start of the experiment and after the 7 week experimental period (Table 2). For frequency distribution histograms of end mass for all fish per tank, please refer to Fig. S4.

Post-release growth, survival and timing of downstream migration

A significantly higher number of fish from the enriched environment (50 out of 314 released fish: 15.9%) migrated downstream the following spring compared with control fish (33 out of 313: 10.5%, binomial GLM, *P*=0.04; Fig. 2A). Because no experimental fish were observed to migrate downstream in the following year, and 3 year smolts are rare in the river Imsa (Jonsson et al., 1998), we assumed that the observed migration rates equated to survival of the hatchery-reared fish. In other words, exposure to EE increased migration success and therefore post-release survival during a 5 month residence in a natural river by 51%. In the same spring (2017), a total of 316 wild smolts also migrated downstream in the river Imsa. The timing of downstream migration seemed to differ between wild and hatchery-reared fish (both enriched and control groups), with the wild fish migrating 1.5 weeks later (Fig. 2B). That is, while 95% of the wild fish had migrated downstream by 17 July, 95% of the hatchery-raised fish had already migrated downstream by 7 July.

Upon downstream interception of hatchery-reared fish, it was noticeable that released parr in the largest size range (>170 mm length) had lower survival rates compared with those of the intermediate-sized fish (110–169 mm length). That is, large and intermediate-sized fish showed migration success rates of 8% (range 6–9%) and 18% (range 16–22%), respectively (Fig. 3A).

Only intermediate-sized fish, measuring between 120 and 149 mm at the time of release, showed on average an increase in body mass at the time of downstream capture following the 5 month period of river residency, compared with their body mass at the time of release. In contrast, both smaller (<119 mm) and larger fish (>150 mm) had lost mass during their river residency (Fig. 3B). Median values for growth rate of the surviving hatchery fish in the river were −0.2 (minimum −0.56 and maximum −0.51) and −0.2

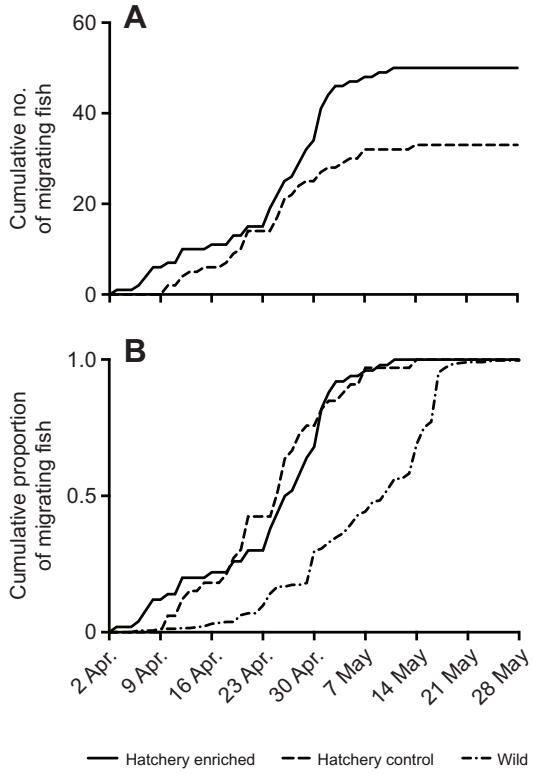

Fig. 2. Migration success and timing of hatchery-reared and wild Atlantic salmon. (A) The cumulative number of migrating hatchery-reared fish per day (i.e. the number of fish that migrated each day added to the total number of fish that had migrated in the previous days). (B) The cumulative proportion per day of migrating fish [i.e. the cumulative number of migrated fish divided by the total number of migrating fish, where a value of 1 indicates that the last individual(s) for this group has successfully migrated at this particular date]. Data are from April–May 2017.

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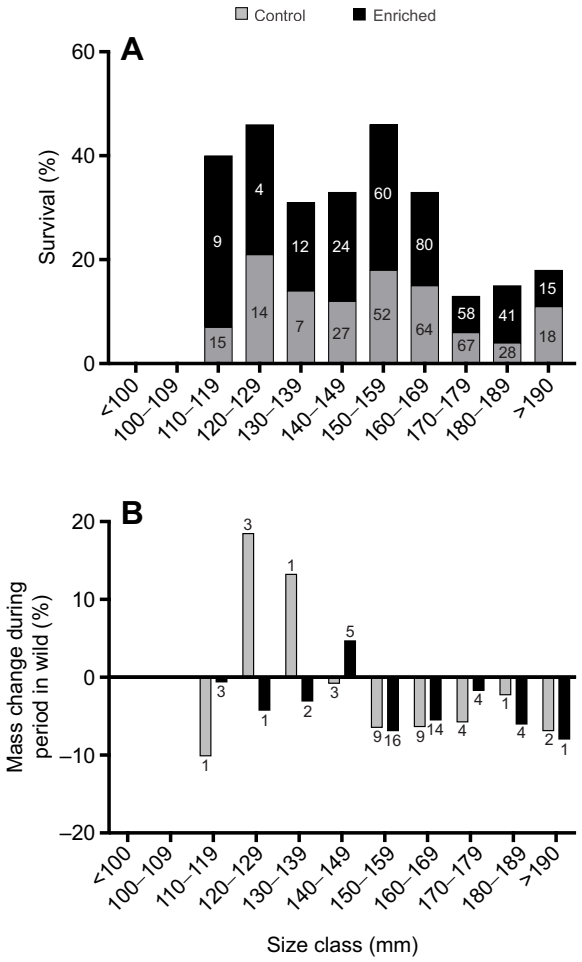

Fig. 3. Intermediate-sized stocked fish appear to show highest post-release survival and growth rates. (A) Stocked hatchery-reared control and enriched Atlantic salmon with a total length >170 mm show reduced survival compared with intermediate sizes, and fish <110 mm show no survival during a 5 month winter in the wild. Total number of released fish per size class per treatment is displayed in each bar. (B) On average, only hatchery-reared fish between 120 and 149 mm increase in body mass during river residency, while larger and smaller size classes lose mass in the wild. The total number of fish that survived per size class per treatment is displayed in each bar.

(minimum −0.83 and maximum −1.03) for control and enriched fish, respectively, which were not statistically different from each other (Wilcoxon test, Z=0.22, P=0.83). Body mass at the time of downstream migration was significantly affected by both fish origin (wild versus hatchery; $F_{1,394}$=31, P<0.001) and migration date ($F_{1,394}$=106, P<0.001), where wild fish had a higher body mass than hatchery-reared fish, and body mass decreased during the migration season (Fig. S5).

ISH

Quantification of ISH images revealed region-specific expression patterns of *cfos* and *bdnf* in the subregions of the telencephalon (Fig. 4). The number of *bdnf*-labelled cells was significantly lower in the Dlv of enriched fish compared with controls (Student's *t*-test, t_{13}=−2.08, P=0.03; Fig. 4A). In addition, there was a strong tendency for decreased labelling in the Dld of enriched compared with control fish (t_{13}=−1.63, P=0.06). No significant differences in the number of *cfos*- or *bdnf*-labelled cells between treatment groups were found in any of the other regions (Table 3). For representative examples of ISH images used for the quantification analysis, see Fig. 5.

Relative transcript abundance

No significant differences in relative mRNA abundance were found between control and enriched fish for *cfos* and *bdnf* in the Dld, Dlv, Dmd, Dmv or Vv (Fig. 6, Table 4).

DISCUSSION

In this study, we demonstrate that modification of the hatchery environment can have important effects on fish survival in stocking programs. A 7 week exposure to environmental enrichment in 9 month old fish increased post-release survival of hatchery-reared Atlantic salmon parr by 51% compared with fish reared under standard hatchery conditions. That is, 50 out of 314 released enriched individuals completed downstream migration compared with 33 out of 313 control fish. These results illustrate that current restocking practices are suboptimal (as the control procedures adopted in this experiment followed common practices, e.g. Brown and Day, 2002), but that survival success may be improved by adopting EE. Notably, it appeared that hatchery-reared stocked fish (both from an enriched environment and controls) of intermediate size (110–170 mm) showed higher survival rates than both larger and smaller size classes.

It has been reported that EE can affect a wide range of fish behavioural outputs, such as aggression (Berejikian, 2005; Rosengren et al., 2016), shelter seeking (Näslund et al., 2013; Roberts et al., 2014) and exploration behaviour (Manuel et al., 2015). These behaviours are mostly under control of the telencephalon, which contains neural subpopulations associated with processes such as relational and emotional memory, stress coping and goal-oriented behaviour (Broglio et al., 2005; Broglio et al., 2015; Rodríguez et al., 2007; O'Connell and Hofmann, 2011). Environmental enrichment affects the expression of the neurogenesis marker *pcna* and cell differentiation marker *neurod* in the whole telencephalon (Manuel et al., 2015; Salvanes et al., 2013; von Krogh et al., 2010). In agreement with these results, we also found that EE was associated with remodelling of the brain. That is, we found a significantly lower number of *bdnf*-labelled cells in the Dlv of fish exposed to EE. Interestingly, in a previous study, we found that wild-caught salmon parr show a significantly lower number of *bdnf*-expressing cells in the Dlv, Dmd, Dmv and Vv, compared with hatchery-reared parr with the same genetic background (Mes et al., 2018). In this respect, the lower *bdnf* expression seen in the Dlv of enriched fish in the current study resembles the wild phenotype more closely than that of control fish. We have previously hypothesized that higher *bdnf* expression in the Dlv of hatchery-reared salmon, compared with wild fish, may be linked to stressors in the hatchery environment (Mes et al., 2018). In this context, it is tempting to speculate that, as EE has been shown to reduce stress (Näslund et al., 2013), the decrease of *bdnf* expression in the Dlv of enriched fish may be a direct consequence of the stress-alleviating effects of EE.

Neuronal activation can be mapped by the visualization of immediate early genes, such as *cfos*. The *cfos* gene is expressed at low levels under basal conditions but it is highly expressed in response to several extracellular signals, including ions, neurotransmitters and drugs. However, this effect is transient and *cfos* mRNA expression reaches a peak at about 30 min post-activation (Kovács, 2008). We found that under basal conditions there were no significant differences in *cfos* expression between EE and control groups. These results suggest that at the time of sampling, there were no major differences in the way individuals were perceiving external signals. Notably, *cfos* is highly expressed in response to acute stressful stimuli, such as confinement stress

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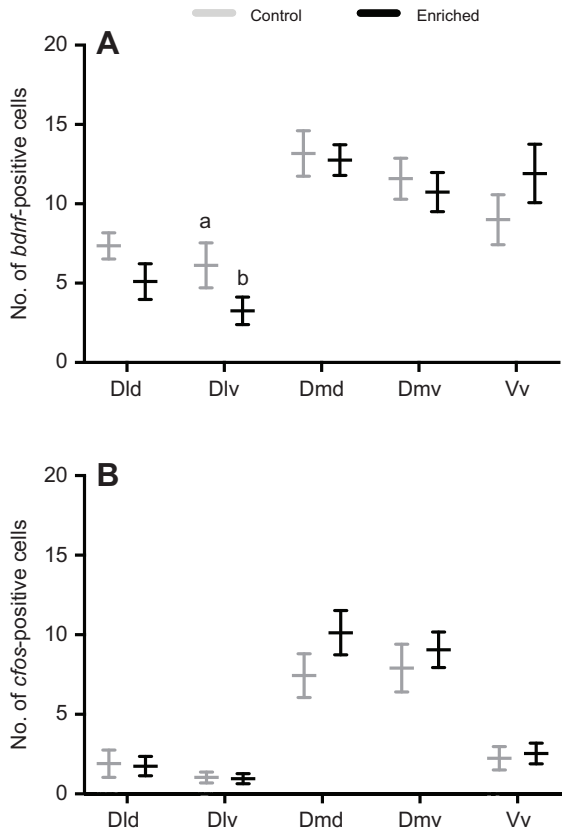

Fig. 4. *In situ* hybridization of the neuroplasticity marker *bdnf* and the neuronal activation marker *cfos* labelled cells in telencephalic subregions. Mean±s.e.m. number of (A) *bdnf*-positive cells and (B) *cfos*-positive cells in the dorsal (Dld) and ventral (Dlv) dorsolateral pallium, the dorsal (Dmd) and ventral (Dmv) dorsomedial pallium, and in the ventral part of the ventral telencephalon (Vv) in Atlantic salmon parr after 7 weeks in a control (grey bars) or enriched environment (black bars). Lowercase letters symbolize significant differences between treatment groups (Student's *t*-test, P=0.03). n=8 per brain area for control, n=7 per area for enriched.

(Okuno, 2011), and therefore differences in neuronal activation may be more pronounced when studying the short-term response to stimuli, such as acute stress. We previously found that wild fish showed less *cfos* activation in response to stress in the Dlv, compared with hatchery-reared fish (Mes et al., 2018). It would therefore be interesting in future studies to quantify *cfos* expression in response to acute stress in EE and control groups.

Interestingly, differences in *bdnf* expression found by ISH were not corroborated by the qPCR analysis in microdissected Dlv tissue. Although a contradictory finding at first sight, it is important to consider that quantification of ISH-labelled cells is a binary process (i.e. cells are counted as either expressing or non-expressing and no quantitative measure per cell is included); therefore, it is possible that even though there are fewer ISH-labelled *bdnf* cells in the Dlv of enriched animals, these cells could, on average, have a higher abundance of *bdnf* mRNA, masking the effects seen in the ISH analyses (quantified on the cell level) in the qPCR analysis (which quantifies whole-tissue transcript abundance). Additionally, ISH image analysis allows for precise identification of entire subregions, while mechanical microdissections with a needle may not be as precise and thus might fail to include all relevant cells within the target neural population, which may underlie the discrepancies found between ISH and qPCR results. To exclude this confounding factor, laser microdissections should be considered for future studies. Furthermore, it was surprising that we did not find more pronounced effects on *bdnf* and *cfos* expression between enriched and control groups. There are two possible reasons for this that should be investigated further. First, the longevity of the exposure to EE may be an important factor, as both neural (Manuel et al., 2015) and behavioural (Ahlbeck Bergendahl et al., 2016) effects of EE have been shown to vary with exposure duration and age of the animals, although we based our protocol on a salmon study conducted by Salvanes et al. (2013), in which telencephalic gene expression differences in neuroplasticity were found under the same EE regime. Second, it is also possible that differences might occur in brain regions not included in the current study, and therefore these differences would have escaped detection. Specifically, it would be interesting to include brain areas which are associated with social behaviour and food consumption (e.g. areas within the hypothalamus; O'Connell and Hofmann, 2011), as well as other areas also associated with navigation and learning (such as those within the cerebellum; Rodríguez et al., 2005). However, the telencephalic areas Dl and Dm are important drivers of the behavioural parameters that have been reported to be affected by EE, such as stress coping and spatial memory (Rosengren et al., 2016; Salvanes et al., 2013), and were thus the areas we chose to focus on in this experiment. Importantly, we note that even though *bdnf* mRNA expression is commonly used as a marker for neuroplasticity (for reviews, see Zupanc and Lamprecht, 2000; Sørensen et al., 2013), it is important to consider that mRNA gene expression does not always mirror protein levels and future studies should include protein analysis in the study of EE-associated changes in neuroplasticity.

The effect of EE on post-release survival of salmonids is inconsistent among studies: in agreement with our results, it is positive in some studies (Hyvärinen and Rodewald, 2013; Maynard et al., 1995; Roberts et al., 2014), but others find no effects (Brockmark et al., 2007, 2010; Tatara et al., 2009; Fast et al., 2008), or even a negative effect (Berejikian et al., 1999; Rosengren et al., 2016). A problem with comparing enrichment studies is the large variation in methodology, most notably with regard to the type and duration of the enrichment, age of the fish, release date and duration, recapture methods and the characteristics of the studied waterway (Näslund and Johnsson, 2016). Because we did not find EE-associated changes in the studied neuroplasticity markers, we can only speculate as to what caused increased post-release survival rates in fish subjected to EE. EE is known to reduce stress responsivity in captivity (Näslund et al., 2013), and stress is known to have adverse effects on cognitive performance (Gaikwad et al., 2011) and post-release survival (Teixeira et al., 2007), which could imply that a higher allostatic load during hatchery rearing leads to reduced fitness of control individuals. Thus, it is possible that

Table 3. *In situ* hybridization statistics (Student's *t*-tests)

	cfos		*bdnf*	
Brain region	*t*-statistic	*P*-value	*t*-statistic	*P*-value
Dld	0.07	0.53	−1.63	0.063
Dlv	0.32	0.62	−2.08	0.029
Dmd	1.36	0.90	−0.23	0.41
Dmv	0.60	0.72	−0.47	0.33
Vv	0.30	0.62	1.21	0.88

Dld, dorsal part of the dorsolateral pallium; Dlv, ventral part of the dorsolateral pallium; Dmd, dorsal part of the dorsomedial pallium; Dmv, ventral part of the dorsomedial palllium; Vv, ventral part of the ventral telencephalon. For all tests, d.f.=13.

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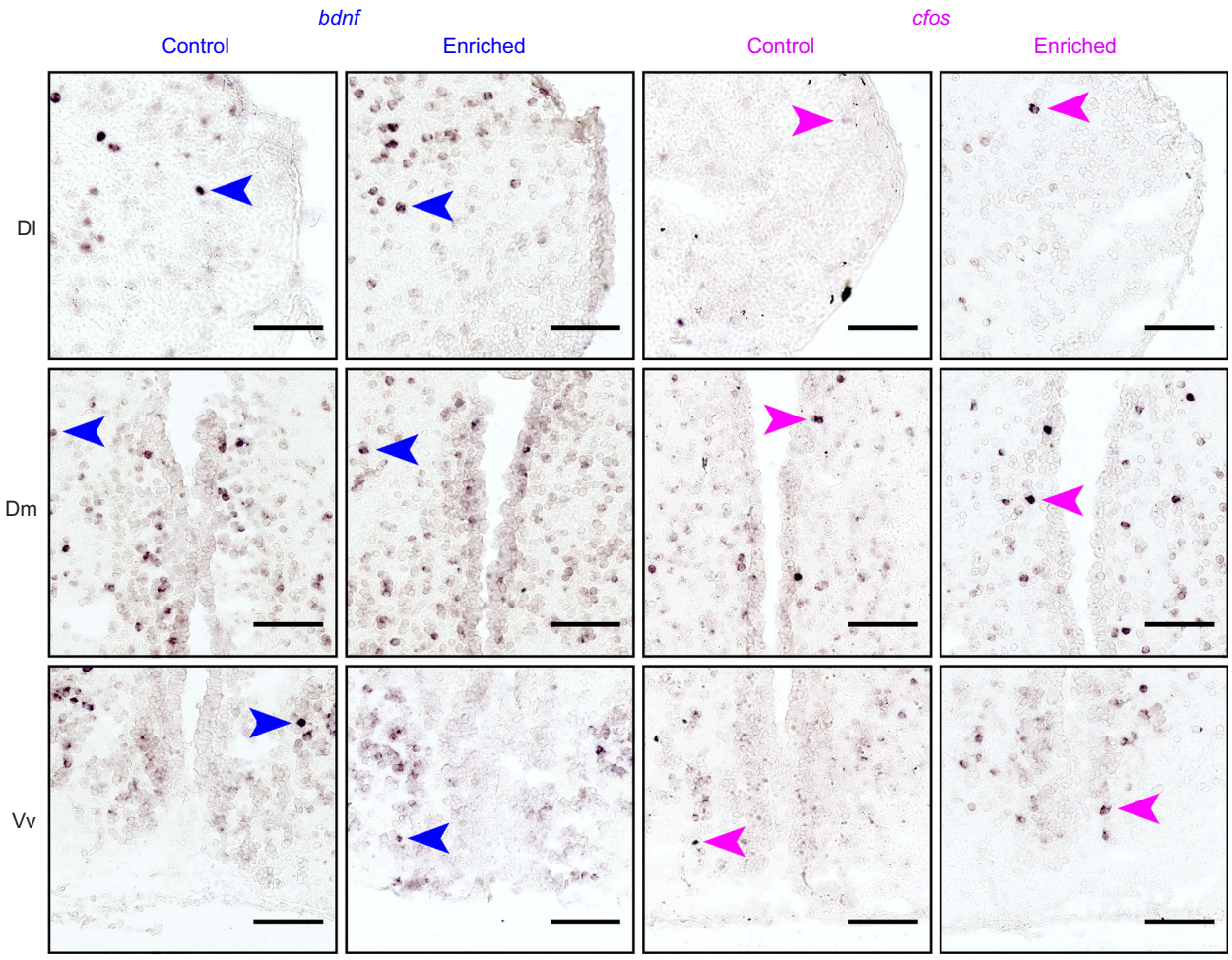

Fig. 5. Transcript abundance of the neuroplasticity marker *bdnf* and the neuronal activation marker *cfos* in treatment groups. Representative images of the expression of *bdnf* and *cfos* transcripts in the dorsolateral pallium (Dl), dorsomedial pallium (Dm) and the ventral part of the ventral telencephalon (Vv) of control and enriched hatchery-reared Atlantic salmon parr. Arrowheads indicate examples of transcript-labelled cells (blue for *bdnf* and magenta for *cfos*). Scale bars: 100 µm.

environmental enrichment partly alleviates the allostatic load on the fish and thus produces a more robust animal, which is better equipped to deal with stressors and changes in the environment. Another interesting observation is that exposure to EE can improve cryptic coloration (i.e. camouflage pattern) of reared fish (Maynard et al., 1995), which is linked to reduced predation susceptibility (Donnelly and Whoriskey, 1993). Moreover, coloration is known to play a role in maintaining dominance hierarchies (Höglund et al., 2000), allowing fish to occupy more favourable habitats. However, we did not test for these parameters in this study, and we suggest that future work should include plasma cortisol analyses for basal and post-handling conditions, as well as a body coloration assessment.

In this study, hatchery fish migrated 1.5 weeks earlier than wild fish, and while enrichment increased post-release survival, it did not alter the timing of downstream migration. Asynchrony in migration timing between wild and hatchery-reared salmonids has been reported previously, in some cases with hatchery-reared fish migrating earlier (Stich et al., 2015) or later (Urke et al., 2013; Chittenden et al., 2008) than wild conspecifics. The implications of sub-optimal migration timing may be particularly severe for long-river populations, where late-migrating fish run the risk of missing their capacity to survive in seawater if they stay too long in the river (McCormick et al., 1998), and may therefore not be able to survive in seawater. Additionally, early marine feeding is an important driver of smolt survival and sub-optimal smolt migration timing may lead to a mismatch with peak marine prey abundance (Thorstad et al., 2012). Interestingly, several studies report increased post-release survival with increased body size at the time of release in hatchery-reared salmon (Brown and Day, 2002; Kallio-nyberg et al., 2004; Rosengren et al., 2016). Although in this study, the smallest released parr did not survive in the wild, survival rates of the largest parr (>170 mm) were noticeably lower than those of intermediate-sized parr (110–170 mm). A possible explanation for the lower survival rates of the largest stocked parr could be related to starvation effects, as we observed that fish of larger size classes were unable to maintain their body mass after release into the wild, indicating insufficient food acquisition. Although supporting evidence is lacking, it is also possible that larger parr are more vulnerable to predator-related mortality. Notably, in agreement with previous reports from the river Imsa (e.g. Hansen and Jonsson, 1985), we found that bigger fish migrate earlier than smaller size classes (Fig. S2). This means that bigger fish spend less time in the river during spring (i.e. when there is higher food availability). This could explain the loss of mass experienced by bigger fish, compared with intermediate classes that spent up to 3 weeks longer in the river and could therefore take advantage of greater food availability during this period. It is a common stocking practice to release fish of large size to increase survival probability – a strategy that seems

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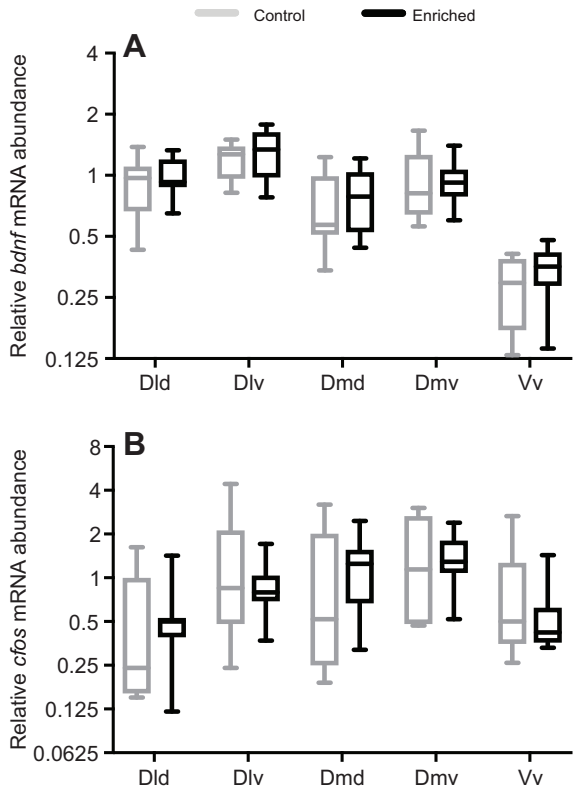

Fig. 6. Effect of environmental enrichment on expression of the neuroplasticity marker *bdnf* and the neuronal activation marker *cfos* in telencephalic subregions. Box plots depicting relative transcript abundance of (A) *bdnf* and (B) *cfos* in five microdissected areas of the telencephalon: the dorsal (Dld) and ventral (Dlv) part of the dorsolateral pallium, the dorsal (Dmd) and ventral (Dmv) part of the dorsomedial pallium, and the ventral part of the ventral telencephalon (Vv). Centre lines within the box plots represent the median, boxes reflect quartiles, and whiskers depict minimum and maximum expression values. Transcript abundance is relative to the expression of the reference genes *ef1αa* and *S20*. *n*=8 per brain area.

effective when salmonids are released as smolts directly before migration (Berejikian et al., 1999; Hyvärinen and Rodewald, 2013; Rosengren et al., 2016). However, when fish will remain in the river for a longer period before embarking on their migration, our data suggest that stocked fish should be of intermediate size at the time of release. Finally, as we observed that large fish migrate earlier than smaller fish, selection and stocking of intermediate-sized parr might also reduce the difference in migration timing between wild and stocked individuals.

Few studies on teleosts use an experimental approach to determine the optimal conditions of EE, but there are indications that the duration of exposure to EE (Ahlbeck Bergendahl et al., 2016; Manuel et al., 2015), age of the fish (Manuel et al., 2015) and the type of enrichment (in mice: Lambert et al., 2005) can affect the efficacy of EE in altering animal behaviour and neuroplasticity. In this study, we found that a 7 week enrichment period on 11 month salmon parr had a significant effect on migration success, compared with control fish. Interestingly, Salvanes et al. (2013), reported that the same enrichment period and type increased the cognitive capacity of salmon and that this was associated with increased expression of the cell division marker *neurod*. Together, these results indicate that this enrichment protocol may be enough to increase the quality of stocked salmon. However, further empirical testing of optimal EE conditions may shed light on inconsistencies between current enrichment studies and contribute to optimization of hatchery protocols. It is also important to note that we used lower densities in our study than are normally used at the Ims hatchery (Rosengren et al., 2016). However, this does not negate our results, as control fish were also kept at the same density and we still found differences between treatments, which suggests that density is not the driving parameter behind the increased survival in EE fish. However, we cannot deny that there may be an interaction effect between structural enrichment and density. Further research should therefore be conducted on the effect of EE on more hatchery-standard densities in order to confirm our current results. As hatchery managers are hesitant to implement EE because of hygiene and increased labour concerns, it is also important to investigate alternative innovative hatchery protocols to structural enrichment. For example, reduced rearing density has been shown to improve post-release survival of salmonids (Larsen et al., 2016; Brockmark et al., 2010), while environmental variability and unpredictability promote behavioural flexibility in Atlantic cod (*Gadus morhua*; Braithwaite and Salvanes, 2005) and predator conditioning using visual and/or olfactory cues can improve predator avoidance (Berejikian et al., 2003; D'Anna et al., 2012). Additionally, swimming exercise could potentially be an effective method to improve the behavioural responses and post-release performance of fish, as mammalian studies indicate that exercise, not structural EE, is the main driver of environmentally induced neurogenesis and neuroplasticity (van Praag et al., 1999; Voss et al., 2013), and several studies report increased post-release survival for exercised salmonids compared with sedentary individuals (Cresswell and Williams, 1983; Burrows, 1969).

In conclusion, we here report increased post-stocking success for EE-reared fish compared with control fish. Furthermore, we found that EE is associated with decreased *bdnf* expression in the Dlv. We suggest that future studies should empirically elucidate the optimal conditions of EE and compare its efficacy with that of other innovative hatchery protocols. Neuroplasticity markers should be studied in other brain areas and protein analysis should help elucidate EE-associated effects. However, even though the neural mechanisms have not been entirely uncovered, our results suggest that future Atlantic salmon hatchery strategies should provide EE and aim to produce or select intermediate size classes of fish for stocking, to improve the post-release survival of stocked Atlantic salmon parr in short rivers.

Table 4. qPCR statistics (Wilcoxon tests)

	cfos		*bdnf*	
Brain region	*Z*	*P*-value	*Z*	*P*-value
Dld	−0.17	0.86	0.47	0.63
Dlv	0.17	0.86	0.68	0.49
Dmd	−0.4	0.69	−0.35	0.73
Dmv	0.16	0.87	0.63	0.53
Vv	0.35	0.73	0.84	0.40

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.M., I.M., M.A.V.; Methodology: D.M., L.O.E.E., B.F., M.A.V.; Formal analysis: D.M., R.v.O.; Investigation: D.M., R.v.O., M.G., M.A.V.; Resources: L.O.E.E., B.F., I.M., M.A.V.; Data curation: D.M., M.A.V.; Writing - original draft: D.M.,

M.A.V.; Writing - review & editing: D.M., R.v.O., M.G., L.O.E.E., B.F., I.M., M.A.V.; Supervision: M.G., L.O.E.E., B.F., I.M., M.A.V.; Project administration: I.M.; Funding acquisition: I.M.

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Supplementary information
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