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Article

Neuromorphological and behavioural effects of early developmental exposure to alarm cue on captive-reared Atlantic salmon (*Salmo salar*)

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Abstract

Behavioural plasticity plays an important role in an organism's ability to adapt to captive settings, but a lack of perceived predation risk during early development in captivity can lead to diminished anti-predator behaviours. Here, we used Atlantic salmon (*Salmo salar*) to test whether early developmental exposure to alarm cues (pre-exposure) led to (1) a developmentally plastic response to alarm cue in yearling and (2) an observable change in neural investment. We exposed fry to either a conspecific alarm cue (pre-exposed fish) or control water (non-exposed fish) and measured activity related to anti-predator behaviour such as time spent motionless, number of aggressive acts, and time spent associated with shelter. We found no indication of a developmentally plastic response to early alarm cue exposure, but we found that pre-exposed fish developed relatively smaller olfactory bulbs compared to non-exposed fish. Our results demonstrate the importance of and ability to exploit plastic responses in captive-reared Atlantic salmon and highlight the need to link behaviour with neuromorphological changes.

Key words: behaviour, environmental effects, fishery management, salmon

Introduction

The capacity of an organism to alter its behaviour in response to environmental conditions is referred to as behavioural plasticity (Stamps 2016). Behavioural plasticity can be beneficial to organisms that are reared in captivity because it allows for those organisms to adjust to novel features of the captive setting (reviewed in Johnsson et al. 2004).However, captive breeding conservation programs tend to produce domesticated, behaviourally compromised animals that are less fit, in natural settings, compared to their wild conspecifics due, in part, to differences in ecological conditions, such as an absence of predation and predator cues (e.g., Fritts et al. 2007; Salvanes 2017; Solberg et al. 2020). Traits that are necessary for survival and reproduction but costly in the wild, such as anti-predator behaviours, are lost or wane if they provide no current utility within the environment to which they are exposed, often via plastic responses (Pigliucci et al. 2006; Johnsson et al. 2004). This is especially problematic for reintroduction efforts, particularly those efforts using hatchery-reared fishes, because predation is a leading cause of reintroduction failure (Tetzlaff et al. 2019). For example, for domesticated Atlantic salmon (*Salmo salar*), directional selection for increased growth rate in hatcheries presents a trade-off with susceptibility to predation (Solberg et al. 2020). In other words, while Atlantic salmon grow at a faster rate in hatcheries, they exhibit lower survival rates when exposed to live predators (brown trout (*Salmo trutta*)) in an artificial stream compared to wild conspecifics (Solberg et al. 2020). The authors suggest that domestication is accompanied by a reduction in predator recognition and anti-predator-related behaviour, leading to increased predation susceptibility. Numerous studies of hatchery-reared salmonids (reviewed in Huntingford 2004) and particularly in Atlantic salmon (e.g., Houde et al. 2010) echo these results and report reduced anti-predator responses when exposed to predators.

Efforts to study and improve anti-predator behaviour in hatchery settings often utilize alarm cues as signals of predation risk in lieu of live predators (reviewed in Jackson and Brown 2011). The presence of conspecific alarm cues, alarm chemicals released by mechanical damage to the skin of many aquatic taxa (reviewed in Chivers and Smith 1998), elicits innate anti-predator behaviour (alarm reaction) in the absence of live predators (Brown and Smith 1997; Kopack et al. 2015; Brown et al. 2016) and strengthens the response and increases survival rates of hatchery-reared fishes under direct threat from live predators (Berejikian et al. 1999; Gazdewich and Chivers 2002; Mirza and Chivers 2003). The alarm response is generally characterized by decreased activity (i.e., decreased movement and increased shelter use; Chivers and Smith 1998). The response to alarm cue (alarm

response), however, is significantly reduced in conventionally reared hatchery fish compared to wild conspecifics (Jackson and Brown 2011). Behavioural differences between hatchery-reared and wild salmon seem to be partially genetically based (Houde et al. 2010; Jackson and Brown 2011); however, anti-predator behaviour has been shown to be behaviourally plastic (Vilhunen 2006; Poisson et al. 2017).

One approach to improving the alarm-related behaviour of hatchery-reared fish that has gained recent attention is to increase hatchery background predation risk via alarm cue exposure during early development (Tetzlaff et al. 2019). This approach has been shown to increase survival during manipulated predator interactions and strengthen baseline alarm reaction and anti-predator behaviour (e.g., Ferrari et al. 2015; Brown et al. 2016; Joyce et al. 2016). Animals exposed to cues that simulate an increase in perceived predation risk (via alarm cue exposure) may develop distinct anti-predator behavioural phenotypes in as little as 4 days (Ferrari 2014; Ferrari et al. 2015). For example, juvenile convict cichlids (*Amatitlania nigrofasciata*) exposed to alarm cue for 5 days exhibited higher levels of anti-predator behaviours when exposed to either a predator model or a novel predator smell (Brown et al. 2016). Furthermore, Poisson et al. (2017) provide evidence that embryonic exposure to alarm cue generates a plastic response in anti-predator-related behaviour in rainbow trout (*Onchorhynchus mykiss)*. It is, however, unclear if the plastic predator-related response is retained to an ecologically relevant stage and whether the exposed fish exhibit a difference in sensitivity to the alarm cue itself. The retention of learned predator-related cues varies widely and can be diminished through a process of adaptive forgetting (Ferrari et al. 2010; Brown et al. 2013). It is thus important to investigate whether alarm cue exposure can produce long-term plastic changes in the developmental trajectory of captive-reared animals—referred to as "developmental plasticity". A single study, to our knowledge, has demonstrated a developmentally plastic response to alarm cue exposure (Poisson et al. 2017). Embryonic rainbow trout exposed to alarm cue during a sensitive period of neural development (during the alevin stage) develop differential behavioural phenotypes as fry. Rainbow trout fry in that study displayed developmentally plastic variation in several behavioural measures linked to anti-predatory behaviour and cognitive ability. However, behavioural measures in that study were observed between 5 and 90 days after exposure to alarm cue, raising the question of whether these responses correspond to short-term, reversible changes (referred to as "flexibility"; Stamps 2016) or developmental plasticity.

Behavioural plasticity is influenced, in no small part, by variation in neural investment (reviewed in Ebbesson and Braithwaite 2012). Teleost fish exhibit a high degree of neurogenesis and cell proliferation occurring continuously throughout life (Zupanc 2008), which sets the stage for high levels of plasticity in brain morphology (Eifert et al. 2015). Predation is a key factor in shaping the brain through evolution via natural selection (Walsh et al. 2016; Samuk et al. 2018), but given the close link between brain and behaviour, the role of plasticity on brain morphology is gaining attention (Gonda et al. 2013; Reddon et al. 2018). Laboratory studies manipulating perceived predation risk demonstrate variation in overall brain size and investment to specific region size that corresponds to anti-predator-related behaviour (Gonda et al. 2012; Reddon et al. 2018; Joyce and Brown 2020). For example, in a study on nine-spined stickleback (*Pungitius pungitius*), Gonda et al. (2012) found that the presence of predators in housing tanks affected the volume of the olfactory bulb (OB), as well as the hypothalamus—a significant increase in the relative (to body size) OB size and a decrease in relative hypothalamus size when exposed to increased predation risk. In a semi-natural experiment, Atlantic salmon and redbelly dace (*Phoxinus eos*), exposed to alarm cue for a two-week period, showed changes in brain structure (Joyce and Brown 2020). Atlantic salmon developed an overall different brain shape, including a smaller optic tectum compared to non-exposed conspecifics, and northern redbelly dace (*Phoxinus eos*) developed larger brains, accounted for by larger OBs and optic tecta.

The extremely plastic nature of the fish brain and behaviour (Gonda et al. 2013; Joyce and Brown 2020) and the ability for fish olfactory systems to function at early embryonic stages (Hara and Zielinski 1989; Dittman et al. 2015) allow for the testing of hypotheses pertaining to developmental plasticity and perceived risk of predation. In this study, we test the hypothesis that early developmental exposure to alarm cue (i.e., increased background predator risk perception) leads to a developmentally plastic response in anti-predator behaviour in yearling Atlantic salmon. First, we test the assumption that there is an innate behavioural response to alarm cue exposure, and then we test two predictions that follow from the hypothesis—(1) if early developmental exposure to alarm cue (pre-exposure) influences anti-predator behaviour in a developmentally plastic manner, then anti-predator-related behaviours should differ between developmentally exposed fish (henceforth referred to as "pre-exposed") and non-exposed fish (referred to as "non-exposed") at a later developmental stage; and (2) pre-exposure should affect the behavioural response to acute exposure to alarm cue (a change in environment) as yearling. Behaviour was measured at the yearling stage to provide an ecologically relevant assessment of anti-predator behaviour, given that the fingerling/yearling stages are the predominant life stages that Atlantic salmon are reintroduced to by hatcheries (e.g., Ontario Ministry of Natural Resources and Forestry 2020). Finally, to better understand the link between perceived predation risk and neural investment, we examine the relative size of the whole brain and five brain regions related to anti-predator behaviour and survival in the wild (OB, telencephalon, optic tectum, hypothalamus, and cerebellum) (reviewed in Ebbesson and Braithwaite 2012) between pre-exposed and non-exposed fish (at the fry stage). Relative investment in brain development was measured at the fry stage to increase our chance of detecting effects of pre-exposure, given that environmental effects on brain morphology are known to disappear over time and after transfer to environments lacking a given stimulus (Näslund et al. 2012).

Materials and methods

Alarm cue preparation

The alarm cue was extracted from 2-year-old Atlantic salmon, reared at the Freshwater Restoration Ecology Centre, located in LaSalle, ON, Canada. The alarm cue extraction followed the protocol of a previous study (Brown and Smith 1997). Briefly, fish for skin extraction were administered a lethal dose of anaesthetic (buffered tricaine methanesulfonate, MS222), skin was removed, subsequently homogenized using a mortar and pestle, and filtered through a cotton filter. Dechlorinated water was added to the homogenate to produce a final skin homogenate (i.e., alarm cue) stock concentration of 487.5 $cm^2 \cdot L^{-1}$. The alarm cue was divided into 20 mL aliquots and then frozen. Dechlorinated water was also frozen in 20 mL aliquots and eventually (see below) served as the control for the untreated group.

Experimental crosses

Atlantic salmon gametes (eggs and sperm) were collected from hatchery-reared fish, of the Sebago strain, maintained at the Ontario Ministry of Natural Resources and Forestry (OMNRF) Harwood Fish Culture Station since 2006. This strain is being used for reintroduction efforts to Lake Ontario (Ontario Ministry of Natural Resources and Forestry 2020). Original lines were collected in 2006 from Sebago Lake, ME, and reared at Harwood Fish Culture station. Gametes were collected from fish that had been reared in captivity for two generations (parents collected from the wild but raised exclusively in captivity). Eggs from a haphazardly chosen female were fertilized using one haphazardly chosen male. Batches of fertilized eggs were divided in half to produce duplicate family replicates, and each replicate was reared in a separate recirculating vertical incubator that used dechlorinated municipal water that was aerated and maintained at 7–8 °C throughout the experiment. Alarm cue exposure began 51 days post-fertilization (dpf) during early post-hatch (when ~50% hatch was occurring for each of the family replicates) and continued until the fry stage, and 103 dpf (when ~50% of the fish had absorbed the yolk-sac). The timing of alarm cue administration followed Poisson et al. (2017) to induce developmentally plastic responses and is supported by evidence suggesting that the olfactory system is functional immediately after hatch (Hara and Zeilinski 1989). The alarm cue was administered to the "pre-exposed" incubation stack by adding a single frozen 20 mL aliquot to the recirculation reservoir once every 3 days until the end of the administration period—a total of 16 alarm cue administrations took place during this period. The final concentration of skin (alarm cue) that pre-exposed fish were exposed to, per administration, was 0.032 $cm^2 \cdot L^{-1}$. This concentration is in line with developmental exposures necessary to increase perceived predation risk and elicits a response in salmonids (Mirza and Chivers 2003; Brown et al. 2011). Non-exposed fish received 20 mL administrations of frozen dechlorinated water only, with the same method and timeframe as the pre-exposed group. Non-exposed fish, therefore, experience a relatively lower level of perceived predation risk compared to the pre-exposed fish. For post-hatch rearing, replicate groups of each family were transferred to and housed separately in 35 L tanks connected to a recirculating system using dechlorinated municipal water (absence of alarm cue) that was aerated, filtered, and kept between 10 and 16 °C to mimic wild river temperatures.

Neuromorphology

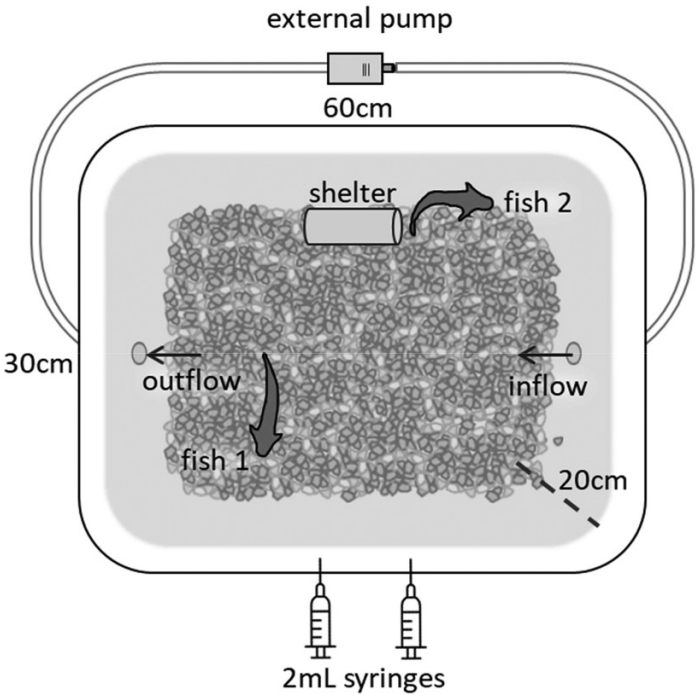
In January of 2018, once fish had absorbed their yolk sacs (~103 dpf) and before transfer into separate rearing tanks (see above), 60 fish ($n = 30$ pre-exposed and $n = 30$ non-exposed, see above), intended for our brain measure study, were euthanized in 100 $mg \cdot L^{-1}$ of MS-222 and subsequently weighed (to 0.01 g). Following body mass measurements, fish heads were removed and drop-fixed in 4% neural buffered paraformaldehyde for 24 h. Brains were then transferred into vials containing phosphate buffer solution and refrigerated for later analysis. Brains of the fish were excised from the skull, and the optic nerves and brain stem were severed at a standard position at the brain stem (at the entrance of the vertebral column) (Fraser et al. 2012). Once removed, the whole brain was weighed (±0.001 g) and placed on a wax dissection tray such that all hemispheres of the brain were proportionate (Fraser et al. 2012). Photographs of the dorsal, ventral, and lateral views of the brain were taken using a digital microscope (following Pollen et al. 2007). For the left and right lateral photographs, the brain was sectioned along the mid-sagittal plane before positioning on the dissection tray. Samples were hydrated with PBS every minute and immediately before photographs were taken. Samples that were damaged during dissection were noted and photographed but excluded from the photographic analysis, for a total of 56 brains that were analyzed (28 pre-exposed and 28 non-exposed). All individuals were identified by a haphazard identification number, and thus dissections and image analyses were performed blindly. The methods of Pollen et al. (2007) and Gonda et al. (2013) were used to determine the volume of the various brain regions. Briefly, the photographs taken were imported into ImageJ, and the length, width, and height of the OB, telencephalon, optic tectum, cerebellum, and hypothalamus were measured, as well as the total length, width, and height of each brain. The width of a structure was defined as the greatest distance enclosed by the structure perpendicular to the midline of the brain. The widths of the OB, telencephalon, optic tectum, and cerebellum were taken from dorsal images, and the width of the hypothalamus was taken from the ventral image. In accordance with Gonda et al. (2012), for paired structures (OB, telencephalon, optic tectum, and hypothalamus), the width of the two sides were measured together. The length of all regions was taken from lateral images, with the exception of the hypothalamus. The length of the hypothalamus was taken from the ventral image due to difficulties viewing the horizontal boundaries of this structure in the lateral image. The length of the OB, telencephalon, cerebellum, and hypothalamus were defined as the greatest distance enclosed by the given structure parallel to the estimated projection of the brain. For the optic tectum, the length was defined as the greatest distanced enclosed by this structure (Gonda et

al. 2012). The heights of all regions were taken from lateral images. The height of a structure was defined as the greatest distance enclosed by the structure perpendicular to the estimated projection of the brain, with the exception of the optic tectum. The height of the optic tectum was defined as the greatest distance enclosed by this structure perpendicular to the length measurement (Gonda et al. 2012). Finally, the volume (V) of each brain structure was calculated according to the ellipsoid mode: $V = (L \times W \times H) \times \pi/6$ (van Staaden et al. 1995). Total brain volume was determined by adding the volume of the five major subregions (Fong et al. 2019). To assess the potential for observer bias, a subsample ($n = 20$) of brains was measured and coded (for all five brain regions mentioned above) blindly by two separate individuals. A two-way mixed model intraclass correlation found high reliability between the two observers for total brain volume measures (ICC = 0.95, $p < 0.001$).

Behavioural trials

A total of 120 individuals ($n = 60$ pre-exposed and $n = 60$ non-exposed) were used for the behavioural experiment. Behavioural trials were conducted on yearling Atlantic salmon when parr marks were visible, between 31 December 2018 and 14 January 2019 (approximately 1 year after pre-exposure; see above). The mean mass of pre-exposed fish was (4.65 ± 2.19 g), and the mean mass of non-exposed fish was (4.50 ± 2.63 g). Behavioural trials were conducted in a test-tank that was a 43 L plastic bin (30 cm × 40 cm × 60 cm) filled with approximately 20 L of water from the home tanks (approximately 20 cm depth), with a single layer of gravel on the bottom of the bin and a 10 cm-long PVC tube (used as shelter) with an internal diameter of 1.27 cm (referred to henceforth as test-tank; see Fig. 1). Each test-tank was fitted with an external pump to create a recirculating low flow (~0.3 $m \cdot s^{-1}$ at the inflow and 0.05 $m \cdot s^{-1}$ at the outflow) into the testing area—this would allow for circulation of the alarm cue within the test-tank. Dye tests were conducted between trials to ensure that the alarm cue would distribute throughout the entire testing arena. Fish were not fed for 24 h prior to the start of the experiment as part of an unrelated experiment and to control for variability in behaviour related to hunger (Näslund et al. 2017). A previous experiment found that food deprivation prior to behavioural trials did not affect the alarm response in Atlantic salmon (Lau et al. 2021). At the beginning of each trial, two fish were placed into a test-tank (to provide social ecological context)—resulting in a total of 60 trials with 120 unique individuals—and left to acclimate for 30 min. The behaviour of the fish was recorded for the final 5 min of acclimation to establish a baseline rate for the focal behaviours (referred to as baseline measures). Following the baseline recordings, 2 mL of distilled water was injected into each tank immediately followed by either another 2 mL of distilled water or 2 mL of alarm cue using 2 mL syringes fixed to the side of the test-tank above the water level (depending on the treatment, see below; referred to as post-stimulus measures). Juveniles in the post-stimulus trials were either exposed to 0.032 $cm^2 \cdot L^{-1}$ alarm cue (matching the pre-exposure concentration) or distilled water (control),

Fig. 1. Schematic top-down view of behavioural test-tank with two fish per trial. Overall dimensions of the tank are 60 cm length, 30 cm width, and 40 cm height (filled to a height of 20 cm). The bottom of the test-tank was covered by a single layer of substrate. The shelter was constructed of a 10 cm-long PVC tube with an internal diameter of 1.27 cm. The test-tank was fitted with an external pump to create a recirculating flow. Alarm cue and control water for the post-stimulus observations were administered through 2 mL syringes through openings on the side of the test-tank just above the water level (see Materials and methods for more detail).



resulting in four treatment groups as follows: (1) pre-exposed fish acutely exposed to alarm cue, (2) pre-exposed fish that received distilled water, (3) non-exposed fish acutely exposed to alarm cue, and (4) non-exposed fish that received distilled water during behavioural trials. Pilot tests using this concentration of alarm cue showed it to be sufficient to elicit behavioural responses in fry compared to control water. Fish movements and interaction were video recorded for 5 min after the addition of the alarm cue or the control water (post-stimulus).

Each 5 min test period (baseline or post-stimulus) was scored for behavioural measures (using Solomon Coder (https://solomon.andraspeter.com), by an experimenter blind to the specific treatment), including total time spent motionless (s), total time spent associated with the shelter (s), and number of aggressive acts—these behavioural metrics have been correlated with anti-predator responses in other studies using juvenile Atlantic salmon (Jackson and Brown 2011; de Mestral and Herbinger 2013). Total time spent motionless is defined here as the total time the salmon spent not moving, wherein movement is defined as a change of

location by at least half a body length (Jackson and Brown 2011). A fish was considered to be associated with the shelter when the head was one body length or less away from the PVC shelter (Salvanes 2017; Clark and Moore 2018). An individual was associated with the shelter when the head of the fish was within one body length of the shelter. Aggressive acts were calculated as number of biting motions or rapid approaches towards other fish. Four of the 60 behavioural video recordings were corrupted and so ultimately 56 trials ($n = 112$ fish) were analyzed ($n = 26$ non-exposed/control, $n = 30$ non-exposed/alarm stimulus, $n = 28$ pre-exposed/control, and $n = 28$ pre-exposed/alarm stimulus).

Statistical analyses

Neuromorphology

We used general linear models to investigate the effect of pre-exposure on the size of the brain and five brain regions. All measures used in these analyses were $\log_{10}$-transformed prior to analyses (Kotrschal et al. 2012). To investigate the role of pre-exposure on total brain volume, we fitted a linear model with total brain volume as the response variable, treatment (pre-exposed and non-exposed) as a fixed factor, and body mass (excluding the mass of the brain) as a covariate (Kotrschal et al. 2017). Preliminary analysis included an interaction term between treatment and body mass but the interaction term was not statistically significant and so was removed from subsequent analyses to preserve degrees of freedom (Beck and Bliwise 2014). To investigate the role of pre-exposure on the relative brain region volumes, we used separate linear models for each brain region with the volume of the brain region of interest as a response variable, treatment (pre-exposed and non-exposed groups) as a fixed factor, and the total brain volume, excluding the volume of the brain region of interest (referred to as "rest of brain"), as a covariate (Fong et al. 2019). Similar to the previous analysis, an interaction term between the covariate and the fixed factor was tested and subsequently removed from analysis. The assumption of homoscedasticity was analysed using Levene's test, and differences were found to be nonsignificant ($P > 0.05$); normality of the data was tested with Shapiro–Wilk test, and no significant deviations from normality were detected.

Behaviour

The three behaviours measured were indexed as combined *Z*-scores to increase sensitivity for analysis and producing a variable of measure representing overall activity score (Labots et al. 2018). We followed the methods used by a previous study measuring Atlantic salmon anti-predator behaviour in the presence of alarm cue administration (Lau et al. 2021). Briefly, a *Z*-score was calculated for each behavioural measure for each observation period; the measures were then combined into an index of activity scores as follows: *Z*-score (log [number of aggressive acts + 1]), *Z*-score (time spent motionless), and *Z*-score (time spent sheltering). The minimum *Z*-score was subtracted from each *Z*-score to produce positive *Z*-scores across measures. General linear mixed models were used to analyze activity scores for baseline (pre-acute exposure to alarm cue) and post-acute-exposure observations. For baseline analysis, activity score was predicted by pre-exposure as a fixed factor, with body mass as a covariate and trial number as a random factor to account for experimental tank effects. A developmentally plastic response to alarm cue on baseline behaviour (Poisson et al. 2017) would be indicated by a significant main effect of pre-exposure (e.g., if developmentally pre-exposed and non-exposed fish differ in activity score prior to acute exposure of alarm cue). For post-stimulus observations, activity score was predicted by developmental pre-exposure and acute alarm exposure as fixed factors as well as an interaction term between these two factors, with body mass and pre-stimulus activity score as covariates and trial number and fish ID as random factors. An innate response to alarm cue would be indicated by a significant main effect of acute alarm exposure. A significant interaction effect between pre-exposure and acute alarm exposure would indicate a differential response to alarm cue between developmentally pre-exposed and non-exposed fish. All statistical analyses were conducted in R Studio version 1.4.1103. Linear mixed-effects models were run using the lme4 package (Bates et al. 2015).

Results

Neuromorphology

We found no significant difference in body mass between the two treatment groups (pre-exposed and non-exposed) ($t_{[54]} = 0.006, P = 0.093$). Pre-exposed fish had a mean body mass of 0.17 ± 0.022 g, and non-exposed fish had a mean body mass of 0.18 ± 0.23 g.

We found no significant effect of pre-exposure on total brain volume (beta = 0.031, $t_{[54]} = 1.071$, $P = 0.29$). For the five brain regions measured, we found a significant effect of pre-exposure on OB volume (beta = 0.18, $t_{[54]} = 2.06$, $P = 0.045$; Table 1 and Fig. 2). Pre-exposed fish had significantly smaller OB volume (0.017 ± 0.005 mm^3) compared to non-exposed fish (0.02 ± 0.007 mm^3). We found no significant effect of pre-exposure on the volume of the remaining four brain regions (see Table 1 and Fig. 2).

Behaviour

A linear mixed-effects model found no significant effect of pre-exposure on baseline measures of activity score in the pre-stimulus observations (beta = −0.34, $t_{[107]} = -0.92$, $P = 0.36$). A linear mixed-effects model for activity score for the post-stimulus observations found no significant main effect of pre-exposure on activity score (beta = 0.52, $t_{[104]} = 1.02$, $P = 0.31$), no significant main effect of acute exposure (beta = 0.082, $t_{[104]} = 0.15$, $P = 0.88$), and no interaction between pre-exposure and acute exposure to alarm cue (beta = −0.45, $t_{[104]} = -0.60$, $P = 0.55$).

Discussion

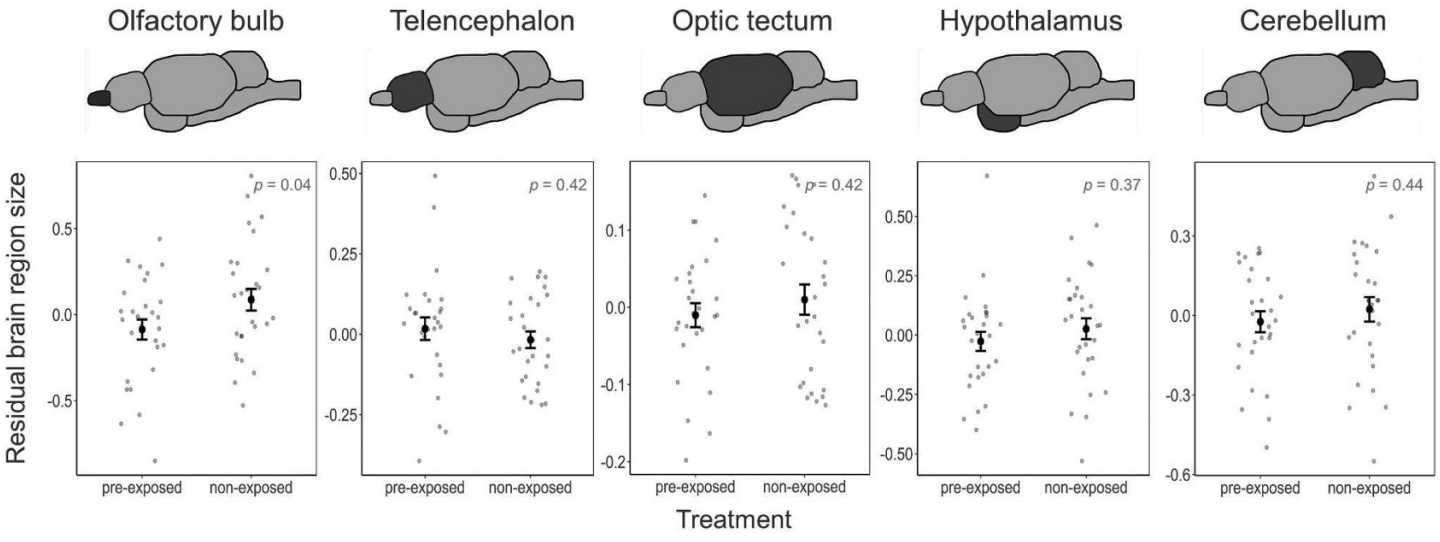
This study was designed to test whether early developmental exposure to conspecific alarm cue (pre-exposure) leads to

Table 1. Results from the general linear models showing the effect of pre-exposure on the total brain volume and regional volume for each of the five brain regions studied in juvenile Atlantic salmon (*Salmo salar*).

	Estimate	SE	df	*t* value	*P*
Total brain					
Body mass	0.43	0.10	53	4.10	<0.001
Pre-exposure	0.031	0.029	53	1.07	0.29
Olfactory bulb					
Rest of brain	0.67	0.37	53	1.83	0.073
Pre-exposure	0.18	0.089	53	2.06	0.045
Telencephalon					
Rest of brain	1.21	0.19	53	6.36	<0.001
Pre-exposure	−0.036	0.045	53	−0.81	0.42
Optic tectum					
Rest of brain	0.49	0.082	53	5.99	<0.001
Pre-exposure	0.021	0.026	53	0.822	0.415
Cerebellum					
Rest of brain	1.02	0.27	53	3.83	<0.001
Pre-exposure	0.049	0.063	53	0.78	0.44
Hypothalamus					
Rest of brain	0.15	0.25	53	0.60	0.55
Pre-exposure	0.056	0.062	53	0.90	0.37

Note: The model for the total brain volume included body mass (excluding the mass of the brain) as a covariate and pre-exposure as a fixed effect. The models for each brain region (volumes in mm^3) included the total brain volume, excluding the volume of the brain region of interest (referred to as "rest of brain"), as a covariate and pre-exposure as a fixed effect. All variables were log_{10}-transformed prior to analysis (see Materials and methods for details).

Fig. 2. Panels depict the size of each brain region in the pre-exposed and non-exposed treatments of Atlantic salmon (*Salmo salar*) juveniles (see Materials and methods for details). Pre-exposed fish are those that received early developmental exposure to alarm cue, and non-exposed fish are those that received distilled water during early development. Light grey points represent relative brain region sizes of individuals from each treatment group. Dark grey points represent means, and error bars represent 95% confidence interval.



developmentally plastic changes in anti-predator-related behaviour (time spent motionless, sheltering, and aggressive acts) and corresponding neuromorphological (regional brain volume) investment in Atlantic salmon destined for stocking to the wild for reintroduction efforts. We found no evidence to suggest that pre-exposure had an effect on baseline anti-predator behaviour or the behavioural response to acute alarm cue exposure (alarm response) for yearling hatchery-

reared fish. We did, however, find differences in neural investment to select regional volumes. Of the five brain regions measured—telencephalon, optic tectum, OB, hypothalamus, and cerebellum—we found a significant and negative effect of pre-exposure on the relative volume of the OB of pre-exposed fish, with no significant effect on the remaining brain regions. Interestingly, the difference in OB volume did not translate to differences in behaviour for the behavioural metrics we observed.

Perceived predation risk is known to elicit differential neural investment in fishes, including changes in overall size and size of various brain structures (reviewed in Gonda et al. 2013). Studies of predator-mediated brain variation have primarily focused on brain size differences as metrics of comparison, but the results across studies often conflict (see Walsh et al. 2016; Reddon et al. 2018). Under natural settings, population-level comparisons in environmentally induced structural changes to the brain are often dependent on species (Joyce and Brown 2020), sex (Reddon et al. 2018), and life history (Gonda et al. 2012). For example, male guppies (*Poecilia reticulata*) that are experimentally exposed to cues of predation risk develop larger brains for their body size than non-exposed males (Reddon et al. 2018). By contrast, wild guppies (*Poecilia reticulata*) that were translocated from high- to low-predation sites evolved relatively larger brains compared to those from low- to high-predation sites (Mitchell et al. 2020) Similarly, three-spine stickleback (*Gasterosteus aculeatus*) experimentally exposed to increased predation risk develop smaller rather than larger brains (Samuk et al. 2018). These contrasting effects of predation risk on brain size may represent a trade-off between neural tissue investment and other fitness-related traits (Dunbar and Shultz 2013) or the employment of different anti-predator responses (Samuk et al. 2018).Samuk et al. (2018) suggest that fish that employ a change in habitat as an anti-predator response will experience a different suite of cognitive challenges compared to fish that employ increased vigilance, leading to differential investment to neural tissue (for cognitive and sensory tasks) and other tissues (such as swimming muscles).

In the context of our results, perceived predation threat during early development (pre-exposure) did not affect overall size of the brain but led to a developmentally plastic response in neural investment (i.e., pre-exposed fish developed smaller OBs as fry), with no corresponding change in behavioural activity level when exposed to alarm cue as yearling. Variation in OB size has been demonstrated under experimental manipulation of perceived predatory risk in ninespined sticklebacks (Gonda et al. 2012). In that study, fish whose parents originated from pond environments (characterized by low levels of predation) developed larger OBs in the presence of perceived predation, but fish whose parents originated from marine environments (characterized by high levels of predation) showed no plastic response in OB size under perceived predation risk manipulation. Similarly, northern redbelly dace (*Phoxinus eos*) exposed to perceived predation (in the form of alarm cue) developed larger OBs compared to non-exposed fish (Joyce and Brown 2020). In contrast, an observational study found a significant negative relationship between predator biomass and OB volume in guppies (*Poecilia reticulata*) (Kotrschal et al. 2017). In that study, variation in OB size was associated with biomass of only one of the four predator species measured, suggesting that variation in OB size is at least indirectly dependent on predator–prey dynamics. It is difficult to draw conclusions about OB size variation given the scarce literature pertaining to the topic. And, given our experimental design—brain measurements were collected at the fry stage, while behavioural measures were collected at the parr stage—we were unable to directly link variation in predation-related behavioural measures to neural correlates.

Given that we did not find a significant main effect of acute alarm cue exposure on behaviour—the pre-exposed and non-exposed fish did not exhibit an innate alarm reaction—it is possible that the alarm cue concentration used in this experiment ($0.032\ cm^2 \cdot L^{-1}$) was below the behavioural-response threshold for alarm response in Atlantic salmon. However, hatchery-reared Atlantic salmon have been shown to exhibit innate behavioural responses to alarm cue at similar concentrations to the ones used in the current experiment (Lau et al. 2021). Additionally, juvenile rainbow trout (*Oncorhynchus mykiss*) were found to consistently exhibit overt fright reactions to concentrations of alarm cue at $1\ cm^2$ of skin in 134 255 L of water—far below the concentrations used in the current study (Mirza and Chivers 2003). Interestingly, rainbow trout that had been pre-exposed to alarm cue but showed no overt behavioural response to subsequent acute exposure to alarm cue still exhibited an increase in survival during live predator encounters (Mirza and Chivers 2003). Moreover, glowlight tetras (*Hemigrammus* erythrozonus) exposed to subthreshold concentrations of alarm cue only exhibited overt anti-predator responses in the presence of secondary visual predator cues (Brown et al. 2004). It is possible that the behavioural measures we observed were not sensitive to alarm cue exposure at the concentrations we provided and that studies that include secondary cues would aid in our understanding of how alarm cue exposure during early development affects behavioural responses at later developmental stages (Brown et al. 2004).

We found no effect of early developmental pre-exposure to alarm cue on behavioural plasticity in our study. In other words, pre-exposure to alarm cue did not affect the baseline or post-stimulus activity of hatchery-reared salmon. These results are in contrast with an earlier alarm cue study that suggests embryonic exposure to alarm induces behavioural plasticity in rainbow trout (Poisson et al. 2017). That study, however, found the effect of pre-exposure on activity level significantly interacted with time throughout the behavioural trial. It is possible that effects of pre-exposure on activity level in our current experiment were not captured in the two 5-minute recording periods and that behaviour should be compared across a wider range of time. In addition, rainbow trout showed immediate differential responses in activity between exposed and non-exposed fish when a secondary cue (a novel object) was present (Poisson et al. 2017). As noted above, secondary cues may be necessary or may aid to elicit certain alarm responses in fishes (Brown et al. 2004).

Taken altogether, the results from our study are inconclusive as to whether early developmental exposure to alarm

cue leads to a developmentally plastic response in brain morphology or behaviour. A developmentally plastic response would be one that produces long-term plastic changes in developmental trajectory. In fact, previous studies have demonstrated phenotypically flexible (i.e., reversible, rather than developmentally plastic) responses to external stimuli (Näslund et al. 2012; Donaldson and Brown 2022). Atlantic salmon reared during early development in structurally enriched tanks developed differences in brain size as alevin compared to non-enriched counterparts; however, those effects disappeared over time when the enrichment was removed at the fry and parr stages (Näslund et al. 2012). The results from that study suggest no critical early developmental period for enrichment in determining brain growth trajectory. Similarly, juvenile convict cichlids exposed to alarm cues for only 14 days showed significant changes in brain size compared to non-exposed counterparts, but those effects were diminished in the absence of alarm cue after only 11 days (Donaldson and Brown 2022). The results from that study suggest a flexible neuroplastic response to alarm cue exposure; however, it is important to note that the initial alarm cue exposure in that study was administered at the juvenile stage, outside of the potential critical window for olfactory development (Hara and Zielinski 1989; Knudsen 2004). Experiments comparing the effects of early developmental alarm cue exposure on neuromorphology across time and over developmental periods are necessary to establish whether a critical period exists for alarm cue exposure to lead to developmentally plastic responses.

It remains unclear whether pre-exposure would provide an advantage to hatchery-reared animals in the wild. Experiments comparing the alarm response and survivability of pre-exposed and wild fish during live predator exposure would be informative in this regard. Our results highlight the importance of and ability to exploit plastic responses to generate differences in brain structures and the potential role of those changes to behaviour in later developmental stages.

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Data availability

Data are available upon request from the corresponding author (AIM).

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