Early rearing environment impacts cerebellar growth in juvenile salmon

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Summary

The size and structure of an animal's brain is typically assumed to result from either natural or artificial selection pressures over generations. However, because a fish's brain grows continuously throughout life, it may be particularly responsive to the environmental conditions the fish experiences during development. Salmon are an ideal model system for studying these effects because natural habitats differ significantly from the hatchery environments in which these fish are frequently reared. For example, in the wild, salmon alevins (i.e. yolk-sac fry) are buried in the gravel, while hatchery environments lack this structural component. We show that the simple manipulation of adding stones to a standard rearing tank can dramatically alter the growth of specific brain structures in steelhead salmon alevins (*Oncorhynchus mykiss*). We found that alevins reared with stones grew brains with significantly larger cerebella than genetically similar fish reared in conventional tanks. This shift to a larger cerebellar size was, in turn, accompanied by changes in locomotory behaviors – behaviors that correlate strongly to the function of this brain region. We next show that hatchery fish reared in a more naturalistic setting in the wild had significantly larger brains than their lab-reared counterparts. However, relative cerebellar volumes were similar between wild-reared alevins and those reared in the complex treatment in the laboratory. Together our results indicate that, within the first three weeks of life, variation in rearing environment can result in brain differences that are commonly attributed to generations of selection. These results highlight the need to consider enrichment strategies when designing captive rearing facilities for both conservation and laboratory use.

Key words: salmon, *Oncorhynchus mykiss*, fish, brain, enrichment, development, conservation, hatchery.

Introduction

In a recent study, we demonstrated that the brains of wild salmonids (*Oncorhynchus mykiss*) are significantly larger than those of fish reared in captivity (Marchetti and Nevitt, 2003). This study did not investigate to what degree these differences reflected 'nature' or 'nurture', i.e. genetic selection for a type of brain that is adaptive for captive living, or, developmental responses to the hatchery environment within a single generation. Variation in brain size is routinely observed among wild and domesticated strains of animals following generations of artificial selection in captive environments. For example, turkeys, rabbits, pigs, sheep, llamas, ferrets, cats and dogs all show dramatic reductions in brain size in domesticated forms (Ebinger and Rohrs, 1995; for a review, see Kruska, 1988). Because of this link to domestication, large neuroanatomical variation between strains is often attributed to selection processes. In fishes, developmental plasticity has also been cited as a potential contributing factor to neural phenotype, but the degree to which brain growth can be influenced by proximate environmental conditions is largely unknown (e.g. Francis et al., 1993; Huber et al., 1997; Brandstatter and Kotrschal et al., 1998; Hofman and Fernald, 2000; Lema et al.,

2005). In this study, we examine how the early rearing environment influences neural and behavioral development in steelhead trout *O. mykiss*.

A fish's brain grows continuously throughout its lifetime, suggesting that brain growth may be impacted by the environmental conditions a fish experiences. Such environmental feedback on brain development in teleost fishes is likely to influence individual behavior and habitat preferences into adult life (Zaunreiter et al., 1991; Kotrschal and Palzenberger, 1992). Salmon are an ideal model system for studying environmentally induced changes because, in addition to brain size, many phenotypic traits vary among individuals reared in wild and captive environments. Differences include variation in growth rate, timing of sexual maturity, and anti-predator, feeding and sexual behaviors (Olla et al., 1994; Fleming et al., 1997; Gross, 1998; Flagg et al., 2000). These differences may influence the low survival observed in hatchery fish upon release into the wild (Jonsson et al., 2003). However, little is known about how the hatchery environment itself may be playing a role in generating phenotypic differences between strains.

In nature, salmon spend the first year of their life in dynamic

and heterogeneous fresh-water streams. Eggs are laid in gravel nests and hatch into alevins (yolk-sac fry). Over a period of weeks, alevins absorb their yolk sac before emerging from the gravel as free-swimming fry. By contrast, fish reared domestically are nourished by clean, well-aerated water, but they are also packed in rearing tanks, with little environmental variability or enrichment, and no natural substrate. Previous studies suggest that the structural environment experienced by alevins may initiate a trajectory for later juvenile development. For example, alevins reared in standard hatchery tanks enriched with naturalistic substrate are larger as fry than fish reared without structure (Leon, 1975; Hansen and Møller, 1985). Moreover, alevins reared with this type of enrichment (i.e. gravel) may also develop into better swimmers that are more able to avoid predators as fry (Bams, 1967). These results indicate that structure influences alevin development, but whether or not experiencing structure during ontogeny affects brain growth is unknown.

Working in both laboratory and wild settings, we examine whether the structural environment fish experience immediately after hatching influences neural and behavioral development in steelhead trout (*O. mykiss*). In the laboratory, we reared hatchery-origin steelhead from the egg through the alevin life-stage in simple and structurally complex rearing treatments. For the field study, alevins were reared in a more naturalistic setting in artificial nests deployed in the American River (Sacramento County, CA, USA). For both experiments we measured total brain volume in addition to the relative volumes of four clearly defined structures (Fig. 1): the olfactory bulb (OB), the telencephalon (TE), the optic tectum (OT) and the cerebellum (CE).

Materials and methods

Laboratory study

Central Valley steelhead (*Oncorhynchus mykiss* Walbaum) eggs were collected, on day 27 post-spawn, from the Nimbus Salmon/Steelhead hatchery in Rancho Cordova, California. Eggs were transferred, to the Center for Aquatic Biology and Aquaculture at the University of California, Davis, and were distributed haphazardly into three simple and three complex temperature-controlled (~12°C), flow-through (20 l min^{-1}) rearing tanks (1.2 m×0.6 m×0.6 m, density 2000 eggs/tank). These two rearing environments were identical except that the bottom surface of complex tanks was scattered with small stones (~4 cm diameter, 1 stone/10 cm^2). The photoperiod matched ambient conditions. Hatching began on day 35 post-spawn and all fish were hatched by day 37 post-spawn. The alevin life-stage lasted 12 days, at which time the fish began to emerge from the bottom of the tank.

Each day from hatching to emergence, a video recording (Sony Mini-DV video camera DCR-TRV18; Tokyo, Japan) was made of a tank, from above. The recordings were for 2 s at 10 min intervals during daylight hours (08:00 h to 16:30 h) for each treatment (one treatment tank/day). To assess differences in movement between treatments, we counted the number of alevins swimming along the bottom of the tank during each 2 s period (50 periods/tank/day). For both treatments, the number of moving fish per day did not change across the 10-day sampling period (regression, linear fit, $F=0.0395$, d.f.=9, $P=0.8475$). Data were normalized to area (number of moving fish m^{-2}) because the video camera could not record the entire tank at once. On day 47 post-spawn, 30 fish from each tank were collected, over-anesthetized with MS222 (tricaine methane sulfonate: 10 mg kg^{-1} water), weighed and measured. Five fish from each tank were sampled for subsequent neural analysis. These fish were immersed in Bouin's fixative overnight, dehydrated in a graded ethanol series, and embedded in paraffin.

Field study

We next evaluated how brain growth compared between fish reared in the laboratory and in a more typical setting in nature. Eggs were again obtained from the Nimbus Salmon/Steelhead Hatchery on day 27 post-spawn and positioned in two artificially constructed redds (i.e. nests) at sites chosen to approximate natural steelhead nests in the American River, CA, USA (sites A: 38°37.8′N, 121°17.6′W and B: 38°35.4′N, 121°19.8′W). Within each redd we placed six egg incubation tubes containing 25 eggs each and stones (~3 cm diameter) collected from the river. Egg tubes were constructed from PVC pipe (44.5 mm diameter, 300 mm long), drilled with 18× 19 mm holes that were covered with mesh (0.35 mm). For each redd, a 22 cm deep depression was made in the gravel and tubes were buried in an upstream progression. The temperature and flow rate of the river varied throughout the course of the rearing period (0.76–1.17 m s^{-1}), but both were consistently higher than the laboratory conditions. Alevins were reared in the river for 13 days until yolk sac absorption. All fish were then removed and sacrificed (MS222: 10 mg kg^{-1} water) on site. Of these fish, 36 individuals were randomly selected for neural analysis. These fish were immersed in Bouin's fixative overnight, dehydrated in a graded ethanol series, and embedded in paraffin.

Neural analysis

Sections were cut transversely at 10 μm, mounted on charged slides, Nissl stained and mounted with Permount® under a coverslip. Cross-sectional area of the total brain and identified structures [the olfactory bulb (OB), telencephalon (TE), optic tectum (OT) and cerebellum (CE)] were measured serially and analyzed at regular 40 μm intervals using Zeiss AxioVision® Software (Fig. 1). Volumes (including total brain volume) were calculated by multiplying the area of each section by the section thickness and summing the results. Measurements of total brain volume began on the section where the first cells of the olfactory bulb were observed in the brain case, and ended at the caudal pole of the corpus cerebella. Total brain volume measurements included the medulla through to the termination of the cerebellum. Subdivision demarcations followed published descriptions (Northcutt and Davis, 1983; Wullimann et al., 1996). All
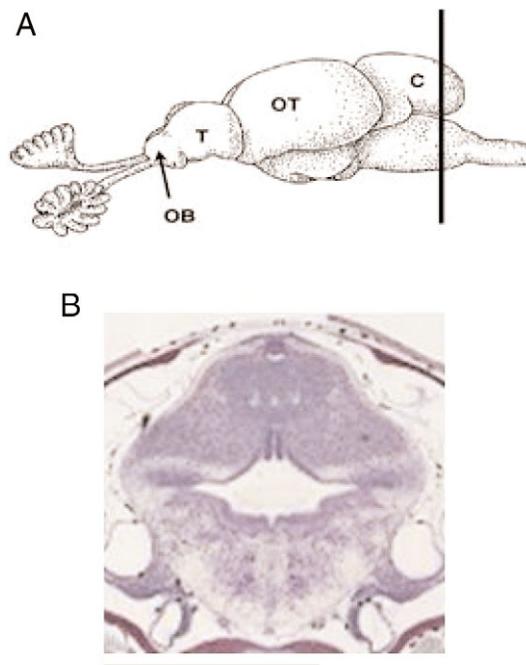
Fig. 1. Anatomy of the salmonid brain. (A) The four subdivisions measured: olfactory bulb (OB), telencephalon (TE), optic tectum (OT) and cerebellum (CE). (B) A representative histological thin section cut through the cerebellum at the vertical line indicated in A. Scale bar, 1 mm.

measurements were done blind to the treatment groups being examined.

Statistical analysis

Differences in body weight and length between laboratory rearing treatments were analyzed using a two-level nested analysis of variance (ANOVA), where tanks were nested within treatments. Movement behavior was analyzed using a Z-test. Variation among treatments in relative total brain volume was analyzed using a Student's t-test. The relative volume of each structure (olfactory bulb, telencephalon, optic tectum and cerebellum) was analyzed using either a Student's t-test or a Wilcoxon Signed Ranks test if the data did not conform to the assumptions of a parametric test. Body and brain size comparisons among field sites were analyzed using a Student's t-test. Comparisons of body size and the relative volume of each brain structure between laboratory and wild-reared fish were analyzed using a one-way ANOVA. If the model was significant, then this analysis was followed by a Tukey–Kramer HSD to determine which groups differed from each other. The relative total brain volume between laboratory and wild-reared fish was analyzed with a Wilcoxon Signed Ranks test.

Results
Laboratory study

We found that adding natural substrate produced significant neural and behavioral differences among treatments. Fish
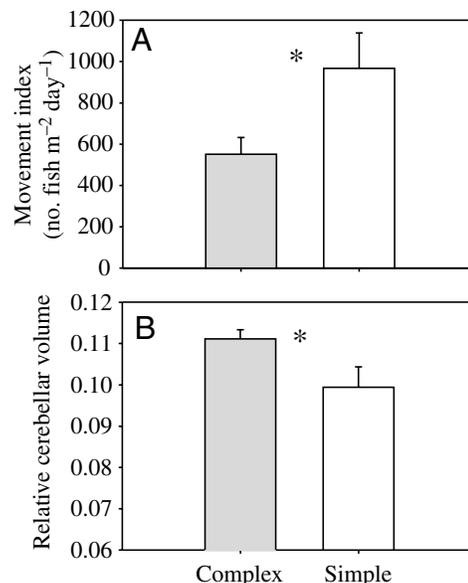

Fig. 2. Effects of rearing environment on locomotory behavior and relative cerebellar size. (A) Locomotory behavior. 'Movement index' indicates the number of moving fish m²/experimental day. (B) Relative cerebellar volume (cerebellar volume/total brain volume) between treatments. Asterisks indicate statistical significance between treatments $P<0.01$.

reared with stones were less active than fish reared in simple tanks ($Z=-16.77$, $P<0.01$), suggesting that they held onto the positions they established in tanks (Fig. 2A). These same fish had larger relative cerebellar volumes than fish reared in simple tanks without substrate (Fig. 2B, $Z_{1,18}=2.60795$, $P=0.009$). We found no differences among rearing treatments in relative total brain, OB, TE or OT volumes (volume/body mass: complex 20.64 ± 1.06 mm³ mg⁻¹, simple 22.16 ± 0.93 mm³ mg⁻¹, $t_{1,18}=1.086$, $P=0.292$; volume/total brain volume: OB: complex 0.012 ± 0.00023, simple 0.012 ± 0.00034, $t_{1,18}=0.021$, $P=0.984$; TE: complex 0.078 ± 0.001, simple 0.080 ± 0.0012, $Z_{1,18}=-0.64254$, $P=0.520$; OT: complex 0.35 ± 0.0037, simple 0.33 ± 0.018, $Z_{1,18}=0.41576$, $P=0.678$). Lengths and masses were also similar among treatments (lengths: complex 23.93 ± 0.13 mm, simple 22.95 ± 0.18 mm, $F_{1,4}=4.4154$, $P=0.106$; masses: complex 11.87 ± 0.15 mg, simple 10.44 ± 0.17 mg, $F_{1,4}=4.3093$, $P=0.118$).

Field study

Seventy-three percent ($N=110/150$) and 69% ($N=104/150$) of river-reared fish hatched and survived to collection in nest 1 and 2, respectively. There were no body or brain size differences between the two nest sites (length: site A 26.46 ± 0.18 mm, site B 26.49 ± 0.18 mm, $t_{1,10}=-0.122$, $P=0.905$; body mass: site A 15.29 ± 0.31 mg, site B 14.95 ± 0.31 mg, $t_{1,10}=0.764$, $P=0.463$; relative total brain volume: site A 23.11 ± 0.42 mm³ g⁻¹, site B 24.27 ± 0.34 mm³ g⁻¹, $t_{1,8}=-2.152$, $P=0.0636$), even though the temperatures varied between sites by as much as 1.2°C (site A 13–13.8°C and site B 13.62–15.0°C).

We found significant phenotypic variation in river-reared fish compared to their lab-reared counterparts. River-reared fish were larger than fish reared in both laboratory treatments (length: river 26.48 ± 0.09 mm, $F_{2,15}=83.3585$, $P<0.0001$; body mass: river 15.12 ± 0.17 mg, $F_{2,15}=55.2434$, $P<0.0001$). With respect to brain growth, fish reared in the river had larger total brain volumes than those reared in the laboratory (river: 23.8050 ± 0.84 mm^3 g^{-1}, laboratory: 21.40 ± 0.59 mm^3 g^{-1}, $Z_{2,28}=2.39168$, $P=0.016$). However, river-reared fish had similar relative cerebellar volumes to fish reared with stones and both of these groups had larger relative cerebella than fish reared in simple tanks (Fig. 3; $F_{2,28}=7.7342$, $P=0.002$). River-reared fish had larger relative telencephalon volumes than fish reared with stones, but they were not larger than those of fish reared in simple tanks (TE: river 0.083 ± 0.001, $F_{2,28}=4.4309$, $P=0.022$). We did not find differences among treatments in relative OB or OT volumes (OB: river 0.012 ± 0.0003, $F_{2,28}=0.2605$, $P=0.773$, OT: river 0.32 ± 0.016, $F_{2,28}=1.1802$, $P=0.323$).

Discussion

Our results suggest that adding natural substrate to an otherwise simple early rearing environment can shape the expression of behavioral and neural phenotype in juvenile salmon. The main brain area affected in our fish was the cerebellum. In this study, tissue was embedded in paraffin for histological examination because a high degree of morphological detail was required to accurately and consistently discriminate brain subdivisions. Despite this advantage, this embedding technique could potentially lead to heterogeneous shrinkage of brain tissue (Kotrschal and Palzenberger, 1992; Quester and Schröder, 1997), which might, in turn, obscure differences in other brain regions such as the OB and the TE (Marchetti and Nevitt, 2003). Because we used the same fixation techniques for both treatments, the differences we observe in the cerebellum appear to be robust.

In other groups of fishes, cerebellar size among different species correlates strongly with habitat type (pelagic or benthic), prey maneuverability, as well as swimming ability (Huber et al., 1997). However, this is the first study in fish to show that variation in captive rearing environments can result in brain differences that are on the same scale as those commonly attributed to selection. Our results thus suggest that proximate mechanisms can shape brain structures in fishes, and also initiate a developmental trajectory that may facilitate survival in their local environment (Fig. 4).

In the laboratory, alevins reared with cobble held more stable positions in the tank and had larger relative cerebellar volumes than those reared in simple environments. This correlation is logical given that the cerebellum is involved in controlling movement, body position and orientation in fishes (Kotrschal et al., 1998; Broglio et al., 2003). In our experiment, alevins moved less if stones were present, suggesting that the act of negotiating a more complex habitat influences the development of the cerebellum. It follows that, if alevins can establish a position in the tank, they may also utilize yolk reserves more efficiently because they are less active. More efficient energy consumption is likely to enhance growth, including brain growth, and allow fish reared with cobble to get a 'head start' when they emerge from the gravel to become free-swimming fry. This idea is attractive since results from several other studies suggest that alevins reared with structure experience enhanced growth well into juvenile life (Leon, 1975; Hansen and Møller, 1985). In our study, structure, at least in the laboratory, did not seem to promote enhanced growth since fish grew at similar rates regardless of how they were reared. In addition, the presence or absence of structure did not affect total brain volume, which suggests that the effect of structure on the size of the cerebellum may not be solely the result of changes in energy consumption.
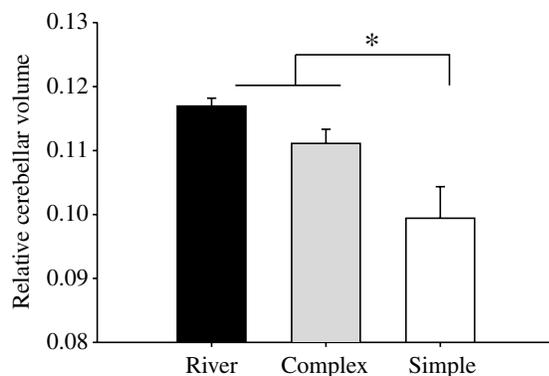

Fig. 3. Effects of river and laboratory (complex and simple) rearing environments on the relative cerebellar volume of steelhead alevins. Relative cerebellar volume is expressed as cerebellar volume/total brain volume. Asterisk indicates statistically significant difference between the simple and river and simple and complex treatments $P<0.01$.
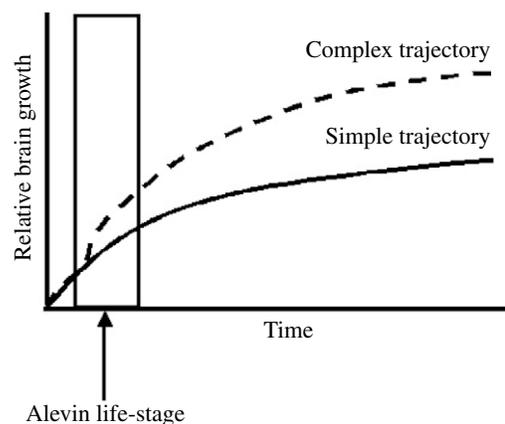

Fig. 4. Variation in the early rearing environment may determine the trajectory for brain growth. The dashed curve describes brain growth in structurally complex captive rearing environments. The solid curve describes brain growth in simple captive rearing environments. Our data suggest that different rearing conditions during the alevin life-stage produce different developmental trajectories with respect to relative brain growth.

We also found significant variation in brain growth between river and laboratory rearing environments. Fish reared in the river were larger and had larger total brain volumes than laboratory-reared fish, perhaps because of slightly warmer and more variable temperatures in the river. However, relative cerebellar volumes were similar between river-reared fish and those reared in the complex treatment. River-reared fish also had larger telencephalon volumes than fish reared with stones, but their telencephalon volumes were similar to fish reared in simple tanks. Thus, while offering fish a physically complex environment early on appears to alter cerebellar growth, our laboratory rearing environments did not produce brain growth comparable to that in the wild.

It is probable that a suite of factors contributed to the brain differences we observed in the laboratory-reared fish. For example, in other fish species, it has been shown that rearing density impacts on dendritic growth and arborization. Jewel fish reared under crowded conditions develop fewer dendritic spines on pyriform interneurons in the optic tectum (Burgess and Coss, 1981). However, in our study, rearing density was similar in both laboratory and field experiments. Social status and other environmental factors (e.g. temperature) also have been shown to influence the size or number of neuroendocrine cells in the forebrain (Miranda et al., 2003; Semsar and Godwin, 2003), suggesting that these factors also contribute to shaping the wild brain phenotype. For example, in cichlid fish, changes in social status alter the size of gonadotropin-releasing and somatostatin-containing neurons in the preoptic area of the hypothalamus. These neurons are presumed to be involved in growth and reproduction (Francis et al., 1993; Hofman and Fernald, 2000).

While the mechanisms are unclear, in other taxa that have been more rigorously studied, environmental enrichment is known to affect brain growth and morphology (Rosenweig and Bennett, 1996; Kempermann et al., 1997). In mice, for example, environmental enrichment has been shown to influence neurotrophin protein and mRNA levels in the brain. Neurotrophins, in turn, impact on neuronal cell proliferation and survival, as well as structural changes, including synaptic connectivity (Ickes et al., 2000; Branchi et al., 2004; Sale et al., 2004). Alternatively, maternal deprivation and other forms of environmental stress during juvenile development have been linked to reduction in the size of specific brain structures as well as inhibition of cell proliferation as animals mature (Coe et al., 2003; Buchanan et al., 2004; Mirescu et al., 2004). For instance, male song birds that experience poor nutrition during early rearing have smaller song control centers in the brain as adult birds, and also have poorer song quality compared to birds that were reared with proper nutrition (Buchanan et al., 2003; Buchanan et al., 2004).

We have not yet determined how morphological variation in cerebellar size correlates with behavior as the fish age, but previous experiments indicate that some types of hatchery enrichment strategies can influence the behavior and survival of salmon. For example, steelhead reared in environments that include underwater feeding, in-stream structure and overhead cover, were socially dominant to fish reared in conventional environments (Berejikian et al., 2000). Further, in some studies, hatchery programs that include enrichment produce fish that survive better during downstream migration than fish reared in conventional hatchery environments, but results are inconsistent (Maynard et al., 2003). In all of these enrichment studies, fish were reared in standard hatchery conditions until the first few months of life, thus enrichment protocols bypassed the alevin life-stage altogether. In the current study, we noted that alevins reared in tanks with structure were able to establish a position in the stones, which allowed them to interact with neighbors in a more predictable way than fish in tanks without stones. This observation suggests that natural substrate may promote social learning in alevins, and may help to explain some of the differences in behavior observed between hatchery and wild fish (Metcalfe et al., 2003; for North Sea cod, see Braithwaite and Salvanes, 2005).

Potential neural correlates to these behavioral differences clearly need to be explored. Because habitat manipulations are easy to implement, fishes may serve as effective model systems for studying underlying mechanisms contributing to these processes. Just as importantly, captive rearing is used to propagate a variety of threatened and endangered fish species for release into the wild. Until recently, little attention has been paid to the proximate effects of the hatchery environment on the phenotype development and survival ability of fish reared in captivity (Braithwaite and Salvanes, 2005). Here, we have shown that rearing conditions dramatically impact upon both behavior and brain growth, and that these effects begin very early in life. These results point to new avenues for conservation researchers to explore.

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