Thesis for the Master's degree (MSc) in Molecular Biosciences, Physiology Programme

Environmental enrichment and its effects on telencephalic neurogenesis and behaviour in isolated adult zebrafish, *Danio rerio*

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Acknowledgments

Abstract

Adult neurogenesis has been subject to increasing interest over the past decades. In fish, this ability to create new neurons is impressive compared to most other vertebrate taxa. Several factors are known to influence adult neurogenesis, like environmental enrichment, learning, exercise, stress and aging. In this study, zebrafish were isolated and exposed to two different environments, a barren environment (control group) and an enriched environment (enriched group). The enriched environment was aquaria supplied with gravel and plastic plants, while the barren aquaria were left empty.

Neurogenesis in the zebrafish was determined by proliferating cell nuclear antigen (PCNA) – immunohistochemistry. After development of a suitable protocol, this method gave staining of a quality that allowed quantification of stained nuclei in the telencephalic tissue. Number of PCNA positive cells per telencephalic volume, response time to distributed food, locomotion activity, cortisol levels and growth rate were measured and compared between the groups. Environmental enrichment led to a strong tendency towards higher neurogenesis, which is in concert with several studies performed of mammals. Also, a significantly higher inter-individual variation in number of new telencephalic cells was observed in the enriched group, indicating that heterogenic environments leads to greater heterogeneity in neurogenesis within a population. Response time to the distributed food was not significantly different between the groups, although the enriched group tended to have longer response time. The control group had significantly higher locomotor activity than the enriched group. In both experimental groups, the effect of time was significant and led to a decrease in both response time and locomotor activity. Whole-body cortisol levels were significantly higher in the enriched group, although this was likely an effect of longer capture time in this environment.

This is the first study to examine environmental enrichment and its effects on neurogenesis and behaviour in zebrafish.

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Abbreviations

ACTH: adrenocorticotropic hormone

BrdU: 5-bromo-2'-deoxyuridine

BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

CRH: corticotrophin-releasing hormone

D: area dorsalis

DAB: 3, 3'- diaminobenzidine

DE: diethyl ether

DG: dentate gurys

Dl: lateral pallium

Dm: medial pallium

E: epinephrine

GC: glucocorticoids

HPA: hypothalamic-pituitary-adrenal

HPI: hypothalamic-pituitary-interrenal

HRP: horse-raddish peroxidase

Ig: immunoglobulin

IGF: insulin-like growth factor

NE: norepinephrine

NMDA: N-methyl-D-aspartate

NGF: nerve growth factor

OB: olfactory bulb

PAP: peroxidase anti-peroxidase

PBS: phosphate buffered saline

PBT: PBS with Triton X-100 and BSA

PCNA: proliferating cell nuclear antigen

S.D: standard deviation

S.E.M: standard error of mean

SA: sympathetic-adrenomedullary

SGZ: subgranular zone (of the dentate gyrus)

SVZ: subventricular zone of the lateral ventricle

TelV: telencephalic ventricle

V: area ventralis

VEGF: vascular endothelial growth factor

1.0 Introduction

1.1 Adult neurogenesis

Adult neurogenesis, the formation and survival of new neurons in adult individuals, has for the last few years been the aim of numerous studies. Until recently, scientists believed that adult individuals did not have any neural regeneration, but in the early sixties, Altman (1962) reported the possibility that new neurons were formed in adult rats. Over the next few years, via the use of $[H^3]$-thymidine, a proliferation marker, Altman and co-worker Das reported that there were indeed new neurons formed in a variety of brain structures from adult rat and cat (Altman, 1963; 1969a; 1969b; Altman and Das, 1965; 1966; Das and Altman, 1970; 1971). Their theories were later confirmed by Kaplan and Hinds (1977), but the subject received considerably more attention after Eriksson et al. (1998) showed that adult neurogenesis also occurs in humans. Today it is generally accepted that most animals have adult neurogenesis, with studies from animals as diverse as insects (Cayre et al., 1996; Malaterre et al., 2002; Scotto-Lomassese et al., 2003; Dufour and Gadenne, 2006), crustaceans (Schmidt and Demuth, 1998 ; Harzsch et al., 1999; Hansen and Schmidt, 2004; Sullivan and Beltz, 2005), fish (Meyer, 1978; Raymond and Easter, 1983; Zupanc and Zupanc, 1992; Zupanc and Horschke, 1995), amphibians (Bernocchi et al., 1990; Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993; Wullimann et al., 2005), reptiles (Lopez-Garcia et al., 1988; Garcia-Verdugo et al., 1989; Perez-Sanchez et al., 1989; Perez-Canellas and Garcia-Verdugo, 1996; Font et al., 2001), birds (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Burd and Nottebohm, 1985; Alvarez-Buylla, 1990; Alvarez-Buylla, 1992; Alvarez-Buylla et al., 1992) and mammals (Altman and Das, 1966; 1967; Das and Altman, 1970; 1971; Kaplan and Hinds, 1977; Eriksson et al., 1998; Coe et al., 2003; Guidi et al., 2005). However, neurogenesis does not occur in all brain tissues and structures of all animals. In mammals, the proliferation zones are restricted to the subventricular zone (SVZ) in the walls of the lateral ventricles, from where new neurons migrate to the olfactory bulbs, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Gould et

al., 2000; Brown et al., 2003b; Emsley et al., 2005). In teleost fish, on the other hand, proliferation zones have been described in all subdivisions along the rostocaudal axis (Grandel et al., 2006; Zupanc, 2006).

1.2 Possible functions of adult neurogenesis

It is likely that the function of neurogenesis reflects the function of the specific brain structure in which it occurs. In the teleost fish, the brain grows in size throughout life, with brain size positively correlated to age, body weight and length (Birse et al., 1980; Brandstatter and Kotrschal, 1990; Zupanc and Horschke, 1995). In this case it is plausible that at least some of the adult born neurons are a necessity for growth.

Reduced neurogenesis has been shown to correlate with depression and impaired learning abilities (Jacobs et al., 2000; Shors et al., 2001; Shors et al., 2002; Kempermann and Kronenberg, 2003). Fuchs et al. (2006) suggest that the downregulation of neurons observed during stress can be explained by rearrangements in the brain network in order to cope with changes in the external or internal environment.

There seems to be a common agreement that the hippocampus is involved in processes such as memory and learning (Eichenbaum et al., 1992; Gould et al., 2000; McEwen, 2000a; Kempermann, 2002). There have been several reports suggesting that the function of adult neurogenesis in the hippocampus is indeed related to learning and/or learning processes (Gould et al., 1999b; Kempermann, 2002; Kempermann et al., 2004; Prickaerts et al., 2004; Leuner et al., 2006). There seems to be a dual link between adult hippocampal neurogenesis and learning. Learning increases neurogenesis (Gould et al., 1999a) and neurogenesis enhances the ability to learn (van Praag et al., 1999a). However, this notion is not without some ambiguity. It has been much discussed whether animals need new neurons to learn. Shors et al. (2001) showed that rats depleted of new hippocampal neurons, via the use of a toxin acting against proliferating cells, were impaired in their ability to learn the hippocampal-dependent task of trace eyeblink conditioning. Still, the rats were able to learn the hippocampal-independent task of delay conditioning (Shors et al., 2001). They also showed that, once the population of new

neurons were replaced in the rats, the ability to acquire hippocampal-dependent memories was restored. This suggests that new neurons somehow participate in the formation of these memories. Moreover, depletion of newly generated neurons did not affect subsequent acquisition of special memories in the Morris water maze, nor contextual fear conditioning, although both task are reportedly hippocampus-dependent (Shors et al., 2002). This means that neurogenesis can not be a necessity for all types of learning, with or without hippocampus-dependence. This is further emphasized by the observation that older animals, with reduced neurogenesis, still learn quite well (Kempermann et al., 1998b; Kempermann, 2002).

Depression has been associated with reduced neurogenesis in the hippocampal area (Steckler and Prickaerts, 2004). Some studies have shown hippocampal volume loss in patients with untreated depression (Bremner et al., 2000; MacQueen et al., 2003), while a study by Sheline et al. (2003) showed that this loss was not observed in patients treated with anti-depressants. Low levels of the neurotransmitter serotonin, 5-hydroxytryptamine, are often found in the hindbrain of depressed patients (Coppen and Doogan, 1988). Neurogenesis in the mammalian DG has been shown to increase by activation of serotonergic receptors, and treatment with the serotonin-releasing drug, *d*-fen-fluramine gives similar results (Gould, 1999). In addition, treatment with serotonin reuptake inhibitors increases neurogenesis (Malberg et al., 2000; Malberg and Duman, 2003). However, other brain areas than the hippocampus are thought to be involved in the pathogenesis of depression, so reduced neurogenesis is not likely the whole explanation for this disorder (Steckler and Prickaerts, 2004).

Neurogenesis in different parts of the brain is not necessarily regulated by the same modulators, which further complicates the search for the function of the new cells. A study done by Brown et al. (2003b) on adult mice, showed that voluntary wheel running and environmental enrichment doubled the amount of new hippocampal granular cells, while it had no effect on the number of newly generated cells in the lateral ventricle walls. Rochefort et al. (2002) showed the opposite scenario, where an odour-enriched environment affected the neurogenesis in the olfactory bulb, but had no effect on the neurogenesis of the hippocampus.

Kozorovitskiy and Gould (2003) suggest that neurogenesis might be a mechanism for brain repair. New granule cells that migrate to the olfactory bulb are thought to

replace old or damaged cells. This is supported by the observation that the volume of the olfactory bulbs does not increase in older mice despite neurogenesis, and that apoptosis is observed in the layers where the newly generated cells are incorporated (Petreanu and Alvarez-Buylla, 2002). Studies show that in the DG cell loss or brain lesions are associated with compensatory neurogenesis in mammals (Gould and Tanapat, 1997; Kernie et al., 2001). The new cells exhibit markers of granule cells, but they are not able to restore the granular cell layer to its previous undamaged state. In a study by Scharff et al. (2000), cell death of specific neuron types was induced in the high vocal centre of male zebra finches, which resulted in compensatory replacement by neurons of the same type. However, here the replacement was associated with some degree of functional recovery. The birds that exhibited song deterioration showed subsequent song improvement over the next three months (Scharff et al., 2000). Even more striking, some fish and reptiles have been shown to be able to regenerate whole brain parts after injury, revealing an enormous potential for neuronal regeneration in these animals (Font et al., 2001; Zupanc, 2006).

It seems that during evolution the amount of adult neurogenesis has decreased with increasing brain complexity. Could it be that higher vertebrates are in less need of new neurons than lower vertebrates? And why is it so? There is presently no answer for this, but the fact that many lower vertebrates continue to grow throughout life could provide a demand for neurogenesis. One thing that is known, is that the functional benefits of neurogenesis can not be acute, because it takes days for the new neuron to be functionally integrated (Hastings and Gould, 1999). Thus it is more likely that neurogenesis represents a long-term adjustment, allowing for a strategic increase in brain network complexity (Kempermann, 2002).

1.3 Modulators of adult neurogenesis

Several factors are known to have a positive influence on adult neurogenesis. There have been a number of reports showing the effect of voluntary exercise on brain cell proliferation, growth and survival in adult mammals (van Praag et al., 1999b; Rhodes

et al., 2003; Holmes et al., 2004; van Praag et al. 2005; Kronenberg et al., 2006; Olson et al., 2006; Redila and Christie, 2006; Redila et al., 2006; Stranahan et al., 2006). For instance, in a study done by van Praag et al. (2005), it was shown that in 19 months old mice, with the opportunity for voluntary wheel running, there was a significant increase in neurogenesis compared to the control group without such an opportunity. This study also reported that running had a positive influence on learning, tested in a Morris water maze, which is in agreement with many other studies (Fordyce and Farrar, 1991; van Praag et al., 1999a; Anderson et al., 2000). There are also studies that show no effect on learning from physical exercise (Barnes et al., 1991), although it should be noted that here forced treadmill training was used, while in the other studies the training was voluntary. There are several reports postulating that learning itself, especially hippocampal-dependent learning, is a positive modulator of hippocampal neurogenesis (Gould et al., 1999a; Shors et al., 2001), but the results are not uniform, making it hard to draw any conclusions (Shors et al., 2002; Dobrossy et al., 2003; Rhodes et al., 2003; Prickaerts et al., 2004; Ehninger and Kempermann, 2006; Leuner et al., 2006).

Seasonal changes have been reported to play an important role in neurogenesis in several species, like the shore crab, *Carcinus maenas* (Hansen and Schmidt, 2004), the red-backed salamander, *Plethon cinereus* (Dawley et al., 2000), and in the wild meadow vole, *Microtus pennsylvanicus* (Galea and McEwen, 1999). However, this phenomenon has been most widely described in birds (Nottebohm et al., 1986; Nottebohm et al., 1987; Cynx and Nottebohm, 1992; Barnea and Nottebohm, 1994; Dawson et al., 2001). Barnea and Nottebohm (1994) did a whole-year study on neurogenesis in adult black-capped chickadees, *Parus atricapillus*. These authors determined the rate of newly born neurons by a single injection of $[H^3]$-thymidine, followed by release of the birds and recapture approximately six weeks later. They observed new neurons in the hippocampal area all year, with a distinct peak in the autumn. The autumn is a season of many changes for the chickadees; the landscape changes in appearance, the birds change diet from insects to seeds, and they cease their territorial behaviour and form flocks. The addition of new neurons might reflect an adjustment to these changes (Barnea and Nottebohm, 1994).

Several other factors are known to enhance neurogenesis. These are named in table 1. Finally, environmental enrichment has been shown in numerous reports to have a

positive effect on adult neurogenesis. This phenomenon is discussed in detail in chapter 1.4.

A number of negative influences on adult neurogenesis have also been demonstrated. Aging, for instance, is considered to be one of the biggest threats to plasticity and regeneration of the brain. Hattiangady and Shetty (2006) found that, during aging, it is not altered numbers of neural stem/progenitor cells that causes the decline in neurogenesis, but rather the environment surrounding these cells, and it seems like the cells enter a state of quiescence and lengthening of cell cycle time. When an animal ages the levels of several factors necessary for cell proliferation, like the brain-derived neurotrophic factor (BDNF) (Hattiangady et al., 2005), insulin–like growth factor (IGF) and vascular endothelial growth factor (VEGF) (Shetty et al., 2005) decline. Angiogenesis has also been shown to be delayed in aged animals, and a decrease in number of vessels per given area of tissue has been reported (Sadoun and Reed, 2003; Wang et al., 2004).

In addition to age, stress is known to be one of the most potent inhibitors of adult neurogenesis. Stress can be defined as a state in which there is a perceived threat to an organism's homeostasis, real or imagined. The factor causing this threat is the stressor, and the physiological and behavioural responses attempting to re-establish the homeostasis is the stress-response (McEwen, 2000b; Charmandari et al., 2005). Neurogenesis has been reported to decrease both under acute and chronic stress (Gould et al., 1997; Pham et al., 2003).

The stress response is characterized by an activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenomedullary (SA) system (Habib et al., 2001; Charmandari et al., 2005; Herman et al., 2005). These activations lead to enhanced levels of corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), glucocorticoids (GC) (Habib et al., 2001; Charmandari et al., 2005), epinephrine (E) and norepinephrine (NE) (Stratakis and Chrousos, 1995; Navarro-Oliveira et al., 2000). In fish, the HPA-axis of mammals has a homologous system called the hypothalamic-pituitary-interrenal (HPI)-axis (Bernier and Peter, 2001; Flik et al., 2006).

GCs have been shown to affect neurogenesis. In an initial study by Cameron and Gould (1994), treatment with corticosterone, the main GC in rodents, produced a

significant decrease in the number of new cells, whereas adrenalectomy resulted in a significant increase. Granule cell progenitors in the adult rat DG, however, show very few adrenal steroid receptors, suggesting that adrenal steroids do not act directly upon these progenitor cells (Cameron et al., 1993). Indeed, it has been shown that adrenal steroids and N-methyl-D-aspartate (NMDA) receptors influence granule cell production in the rat DG through a common pathway, and that NMDA receptors operate downstream of corticosterone in this pathway (Cameron et al., 1998).

The direction of some of the effects of GCs is dose-dependent. Basal levels of GCs enhance hippocampus excitability, synaptic plasticity and are thought to play a role in memory formation, while stress levels of GCs have been reported to have the opposite effects in rodents (Diamond et al., 1992; McEwen, 1999). Furthermore, there are reports from mammals that show that despite elevated GC levels, there are no registered effects on cell proliferation (for review, see Mirescu and Gould, 2006).

In fish social interactions, such as dominant/subordinate relationships have been shown to affect neurogenesis. In her master thesis performed at Oslo University in 2005, Sørensen studied pairs of juvenile rainbow trout, *Oncorhynchus mykiss* (Sørensen, 2005). The fish were allowed to interact for four days before they were sacrificed, and during this time dominant/subordinate relationships were formed. By the use of the thymidine analogue 5-bromo-2´-deoxyuridine (BrdU)-labelling it was shown that subordinate animals had a significant reduction in the number of labelled cells compared to the unstressed controls (Sørensen et al., 2006). This is, to date, the only study done on stress and how it affects neurogenesis in fish.

To be unable to control a situation is often a stressor in itself. Malberg and Duman (2003) reported that uncontrollable stress reduced neurogenesis in the hippocampus of adult rats, but that this was reversed by antidepressant treatment. Several reports suggest that controllability over a stressful situation seems to counter some of the negative effects on brain proliferation related to stress (Bland et al., 2006; Shors et al., 2007). Table 1 lists further factors shown to inhibit neurogenesis.

Table 1 Summary of the main modulators of adult neurogenesis

Factors	Stimulating neurogenesis	Inhibiting neurogenesis	Animal group	References
Enriched environment	Yes, see chapter 1.4	No	M	Altman and Das, 1964; Kempermann et al., 1997b; Kempermann et al., 1998a; Mohammed et al., 2002; Brown et al., 2003b
Voluntary exercise	Yes	No	M	Fordyce and Farrar, 1991; van Praag et al., 1999a; van Praag et al., 1999b; Anderson et al., 2000; van Praag et al., 2005
Hippocampal dependent learning	Yes	No	M	Gould et al., 1999a; Shors et al., 2001
Growth factors as IGF and VEGF	If in high levels	No	M	Aberg et al., 2000; Anderson et al., 2002; Jin et al., 2002; Fabel et al., 2003
Neurotrophic factors as NGF and BDNF	Yes, see chapter 1.4	No	M	Scharfman et al., 2005; Frielingsdorf et al., 2007
Estrogen	Transiently	No	B, M	Hidalgo et al., 1995; Tanapat et al., 1999; Tanapat et al., 2005; Suzuki et al., 2007
Testosterone	In some animals	No	B, M	Nottebohm, 1980; Nottebohm, 1981; Absil et al., 2003; Galea et al., 2006
Serotonin	Yes	No	M	Gould, 1999; Malberg et al., 2000; Malberg and Duman, 2003
Wnt proteins	If in high levels	No	M	Lie et al., 2005
Seasonal changes	In some animals	No	B, M	Cynx and Nottebohm, 1992; Barnea and Nottebohm, 1994; Galea and McEwen, 1999

Genetic background	Yes	Yes	M	Kempermann et al., 1997a; Kempermann and Gage, 2002
Thyroid hormones	Yes	No	M	Montero-Pedrazuela et al., 2006
Age	No	Yes	M	Shetty et al., 2005; Hattiangady and Shetty, 2006
Stress	No	Yes	M	Gould et al., 1997; Malberg and Duman, 2003; Pham et al., 2003
NMDA receptor activation	No	Yes	M	Cameron et al., 1995; Cameron et al., 1998
Cortisol/Corticosterone	No, but se chapter 1.3	Yes	M	Diamond et al., 1992; Cameron and Gould, 1994; McEwen, 1999
Intoxicating substances as cocaine, alcohol, amphetamines, morphine and nicotine	No, but some positive effects have been reported from nicotine	Yes	M	Eisch et al., 2000; Teuchert-Noodt et al., 2000; Abrous et al., 2002; Herrera et al., 2003; Dominguez-Escriba et al., 2006; Mudo et al., 2006
Subordinate social position	No	Yes	F, M	Kozorovitskiy and Gould, 2004; Sørensen, 2005
Nitric oxide	No	Yes	M	Packer et al., 2003

The table lists the main modulators of adult neurogenesis of which some are discussed in text. Abbreviations: BDNF = brain-derived neurotrophic factor, IGF = insulin-like growth factor, NMDA = *N*-methyl-D-aspartate, NGF = neurotrophic growth factor, VEGF = vascular endothelial growth factor, B = birds, F = fish, M = mammals

1.4 Environmental enrichment and adult neurogenesis

Environmental enrichment includes complex structures and new items, objects, experiences, odours, etc. in an animal's milieu. Numerous studies have been done on how environmental enrichment influences neurogenesis, brain size and brain structural plasticity (Altman and Das, 1964; Kempermann et al., 1997b; 1998a; 1998b; Nilsson et al., 1999; Brown et al., 2003b; Komitova et al., 2005; Lema et al., 2005; Segovia et al., 2006), for review, see Mohammed et al., 2002. One example is a study by Altman and Das (1964), where the effects of enriched environment on glia cell proliferation was investigated in rats. These authors used a "restricted" environment, represented by small isolation cages, and an "enriched" environment, represented by a large communal cage, with both males and females. In the communal cage food and water sources were shifted from different locations in the cage every second or third day, forcing the animals to follow new routes to forage. After approximately three months, rats from each group were injected with $[H^3]$-thymidine. The animals lived in their respective environments for one week post injection before they were sacrificed. The authors reported a significant increase in the number of labelled glia cells in the neocortex of the rats from the enriched environment, compared to those from a restricted one. In addition, the mean brain weight of the individuals from the former group, were 9.7 % higher than that of the latter (Altman and Das, 1964). There have also been several reports on measurable differences in brain size, especially in the telencephalon, between domesticated and wild animals, for example fish (Marchetti and Nevitt, 2003) rats, pigs, turkeys, wolves and foxes (for review, see Kruska, 2005).

Environmental enrichment has also been shown to influence the expression of several gene products, like neurotrophic factors, in the brain. Neurotrophic factors are endogenous signalling proteins, which promote survival, growth and division, as well as differentiation and morphological plasticity, of neural cells. One well-known neurotrophic factor is the nerve growth factor (NGF). The NGF's mRNA levels are highly expressed in the hippocampus, the cerebral cortex and the olfactory bulb (Korsching et al., 1985). NGF increases the levels of choline acetyltransferase, an essential enzyme in acetylcholine synthesis (Mobley et al., 1986). NGF is also necessary

in the development and maintenance of cholinergic neurons, which together with acetylcholine is crucial for cognitive function (Calamandrei and Alleva, 1995). Cholinergic neurons starved of NGF have been shown to atrophy and lose important phenotype characteristics (Williams et al., 2006). Pham et al. (1999a) showed that enriched environment significantly increased NGF in the hippocampal, visual and entorhinal cortices of rats. Similar results were obtained by Torasdotter et al. (1998), even though the rats in this study had only lived in the enriched/impoverished environment for 30 days. NGF has also been shown to improve the performance of aged rats in spatial and memory tasks (Calamandrei and Alleva, 1995; Chen and Gage, 1995; Chen et al., 1995). The expression of another neurotrophic factor, BDNF, is also found to increase in the hippocampus of rats reared in enriched environments (Falkenberg et al., 1992). Both NGF and BDNF have in behavioural studies been shown to play roles in mediating learning and memory (Henriksson et al., 1992; Mizuno et al., 2000; Woolf et al., 2001). Furthermore, animals from enriched environments perform better in tasks involving memory and learning (Nilsson et al., 1999; Pham et al., 1999b).

　　　Not much is known about how environmental stimuli affect the brain of fish. Lema et al. (2005) did a study on effects of environmental enrichment on telencephalic proliferation rate, but the control and enriched group differed in two aspects, namely spatial variation in water flow velocity (greater variation for the control group) and the presence of physical structures (absent for the control group). In addition, the number of fish in each group was low (n= 3), so any conclusion about the validity of this experiment is at best ambiguous.

1.5 Fish behaviour and stress

In this thesis the zebrafish, *Danio rerio*, was used as the experimental animal. Zebrafish is a shoaling teleost fish, originally from the fresh water lakes in India. It is a widely used model organism due to its short generation time, easy handling and sequenced genome (Barinaga, 1990; Lele and Krone, 1996; Pickart et al., 2006). During this experiment the zebrafish were kept in isolation, and since the zebrafish are shoaling fish, the isolation might be a stressor.

In addition to CRH, ACTH and GC levels, the turnover of the neurotransmitter serotonin is reported to increase during the stress-response in fish (Chaouloff, 1993; Winberg et al., 1997). In most vertebrates serotonin has been shown to affect different behavioural aspects associated with the stress response, such as decreased food-intake and appetite, and increased locomotor activity (Wedderburn and Sillar, 1994; De Pedro et al., 1998; Leibowitz and Alexander, 1998; Brocco et al., 2002; Lowry and Moore, 2006), although contrary results on locomotor activity have also been reported (Winberg and Nilsson, 1993; Winberg et al., 1997) Like serotonin, CRH suppresses feeding behaviour (for review, see Bernier and Peter, 2001). De Pedro et al. (1993) tested the effects of intraperitoneal and intracerebroventricular CRH administration on food-intake in goldfish, *Carassius auratus*. The intraperitoneal injections of CRH did not show any effect on food-intake. In contrast, the intracerebroventricular injections lead to an inhibition of food-intake for several hours after the injection. This indicates that CRH acts centrally to regulate feeding. Conversely, with higher doses of CRH, the food-intake was enhanced, implying a dose-dependent effect of CRH on appetite (De Pedro et al., 1993). The same scenario has also been shown for GCs. With basal levels of GCs, appetite and feeding are stimulated, but during stress, with enhanced levels of GCs, appetite is suppressed. GCs induce insulin secretion from the pancreas, and high levels of insulin suppress appetite (Dallman et al., 1993).

Both CRH and cortisol have been reported to regulate locomotor activity in fish. Clements et al. (2002) did a study on juvenile spring chinook salmon, *Oncorhynchus tshawytscha*. The fish were injected intracerebroventricularly with CRH, which resulted in an increased locomotor activity. Simultaneous injection of a CRH-antagonist prevented this activity increase, which strengthens the role of CRH as an enhancer of locomotor

activity in fish (Clements et al., 2002). In a study by Øverli et al. (2002a), cortisol-treated food was fed to isolated juvenile rainbow trout, and locomotor activity upon subsequent encounter with a conspecific intruder was measured. One group (short-term) was fed once with this food, while another group (long-term) was fed three times over a 48 hour period. The fish displayed no significant change in locomotor activity when left in isolation, regardless of the duration of the cortisol treatment. However, upon intruder encounter, the fish with short-term cortisol treatment showed enhanced locomotor activity, while the long-term treated fish showed an inhibition of locomotor activity (Øverli et al., 2002a). In addition to show that cortisol may affect locomotor activity, this study also shows that the action of cortisol may be time- and context- dependent.

Since the brains of the experimental fish in the current study were used for analyses of cell proliferation, the fish were closely monitored for behavioural signs of stress. The behavioural analyses were locomotor activity and response time to presented feed. In addition, whole-body cortisol levels were measured.

1.6 The teleost telencephalon – morphology and function

In this section the names of different structures follow Wulliman et al. (1996).

The telencephalon of actinopterygian fish undergoes a process of eversion of the hemispheres during development. This is in contrast to other vertebrates, whose hemispheres during development undergo an evagination process (Nieuwenhuys, 1962; Northcutt and Davis, 1983; Yamane et al., 1996; Butler, 2000; Rodriguez et al., 2002a). As a result, the hemispheres of actinopterygian fish are separated by a single ventricle, whereas other vertebrates have two internal ventricles. The pallial region of the actinoptergyian telencephalon is assumed to be homologous with pallial areas from other vertebrates, such as amniotes (Rodriguez et al., 2002a; Broglio et al., 2005). See figure 1.1 for a schematic representation of the differences in telencephalic development between these two groups.
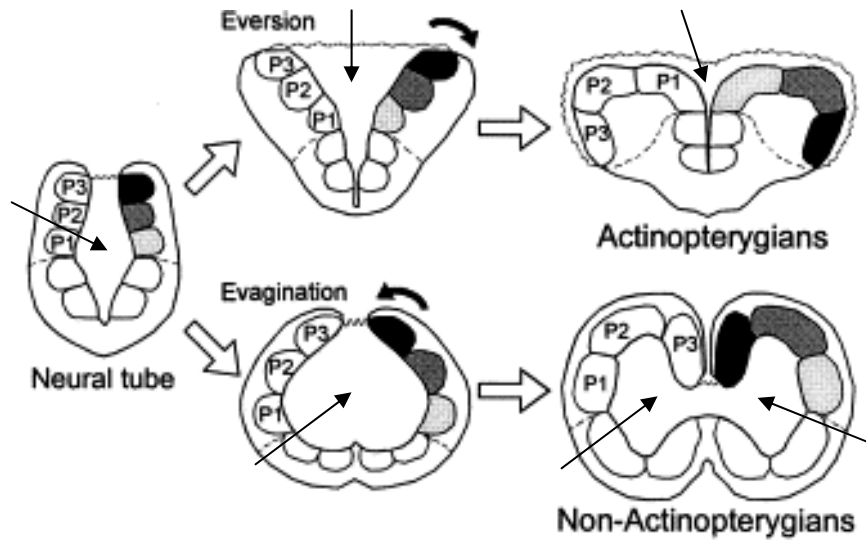
Figure 1.1 A schematic representation of the eversion and evagination processes occurring in actinopterygians and non-actinopterygians, respectively. The ventricles are indicated with arrows. P1, P2, P3 are the three main subdivisions of the pallium. The figure is adapted from Rodriguez et al. (2002a).

As in amniotes, the telencephalon of actinopterygians consist of a dorsally located pallial mantle, the area dorsalis (D), and supallial areas, the area ventralis (V) (Northcutt and Davis, 1983; Wullimann et al., 1996). There is much experimental evidence showing that roles assigned to the hippocampus in mammals, birds and reptiles have their counterpart in the teleost telencephalon (Lopez et al., 2000; Portavella et al., 2002; Rodriguez et al., 2002a; Rodriguez et al., 2002b; Salas et al., 2003; Vargas et al., 2006).

More precisely, it is the lateral pallium (Dl) that is considered to be a homologue to the amniote hippocampus, while the medial pallium (Dm) is assumed to be homologous to the amniote amygdala (Northcutt and Davis, 1983; Broglio et al., 2005).

1.6.1 Proliferation zones in the zebrafish brain

As mentioned previously, proliferation zones have been reported all along the rostocaudal axis of the teleost brain (Zupanc and Horschke, 1995; Grandel et al., 2006; Zupanc, 2006). By the aid of BrdU-labelling, Zupanc et al. (2005) mapped the proliferation zones in the zebrafish. They found labelled cells in the olfactory bulb, telencephalon, preoptic area, hypothalamus, optic tectum, torus longitudinalis, vagal lobe, medulla oblongata and in the cerebellum. Interestingly, a similar study was performed by Grandel et al. (2006), and some of their observations diverged from the findings of Zupanc et al. (2005). One example is that Zupanc et al. found abundant proliferation in the mantle zone of the mesencephalon (optic tectum and torus longitudinalis), whereas Grandel et al. only observed proliferation in the ventricular zone of the mesencephalon (Zupanc et al., 2005; Grandel et al., 2006).

In the telencephalon, proliferation zones have been described both in D and V, along the ventricular surfaces (Zupanc et al., 2005; Grandel et al., 2006), see figure 1.2. Due to the eversion process, some of the ventricular zone of D lies along the dorsal and lateral telencephalic surface, as shown in figure 1.1.
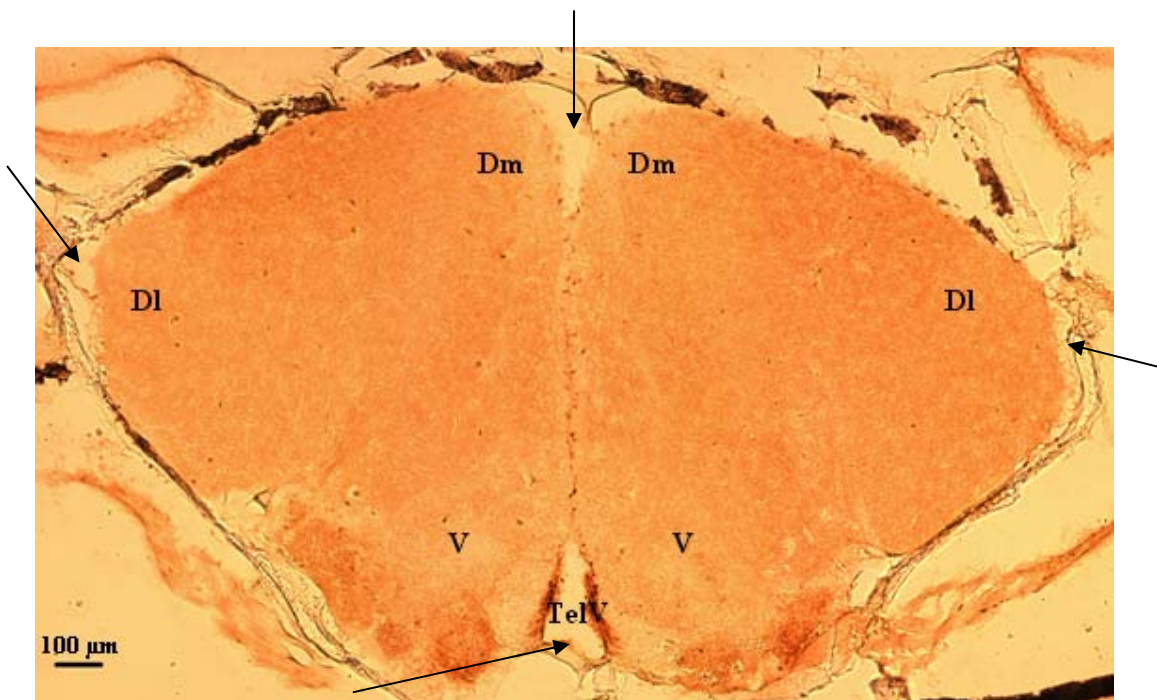
Figure 1.2 A picture of a telencephalic section. The proliferation zones of D (Dm and Dl) and V are marked with arrows (TelV = Telenchephalic Ventricle).

1.7 Proliferating cell nuclear antigen (PCNA)

PCNA is a ring-shaped, 29 kDa protein involved in various parts of DNA metabolism, such as replication, recombination, repair and even apoptosis (Kelman, 1997; Maga and Hubscher, 2003). It has also been shown to play roles in cell cycle control and check point processes (Kelman, 1997). Because of its functional diversity, it interacts with many different proteins and is present in most parts of the cell cycle (see figure 1.3), but it is seldom expressed in G_0 (Bolton et al., 1994). Thus, the proliferating cells are characterized by the presence of PCNA. The level of PCNA expression is quite low in G_1, but it rises rapidly in the S phase, with a two to three fold increase from G_1. The level continues to rise through late S phase and early G_2, though at a lower rate, before it starts to decline at late G_2/M phase (Bolton et al., 1992; Bolton et al., 1994).

Through immunohistochemistry, using a specific antibody against PCNA, it is possible to detect the presence of this protein in the cell nucleus, and thereby obtain an indication of whether the cell in question is in a proliferating stage or not.
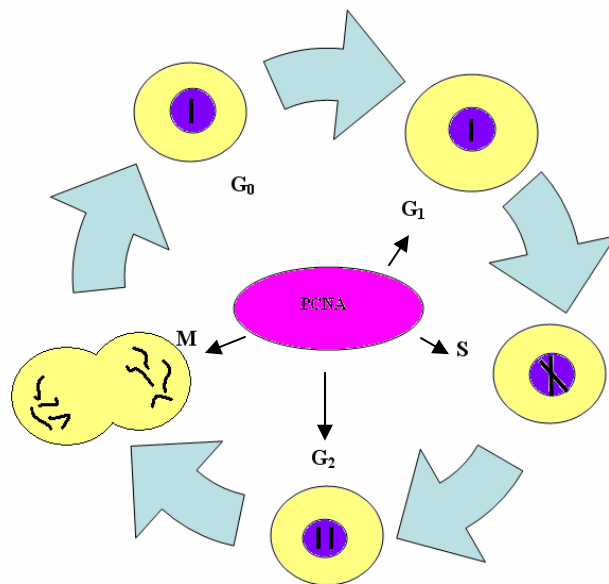
Figure 1.3 A schematic representation of the cell cycle. In G_1-phase the cell grows and organelles are duplicated, in S-phase the DNA replicates, in G_2-phase there is protein synthesis and in M-phase the cell divides. In G_0-phase, the cell cycle is finished. The cell then carries out its specialized functions or re-enters the cell cycle. PCNA is present from G_1 to M, with its highest levels in S. Chromosomes are represented by bold lines.

1.8 Aim of the study

Today, many animals are kept in captivity in zoos, hatcheries, research institutions, fur farms, etc. Hatchery rearing has been shown to affect the growth and development of brain structures in fish (Marchetti and Nevitt, 2003), and also the ability to forage and avoid predators (Brown et al., 2003a). In addition, not to live in its natural habitat or with its kin might be a stressful experience for an animal and reduce its welfare. In this study, the aim was to detect whether environmental enrichment had an effect on response time to presented food, locomotor activity, cortisol levels and neurogenesis of the zebrafish. These parameters were chosen because cortisol levels and behaviour are often used as indicators on an animal's welfare, and neurogenesis has been reported in many animals to be modulated by environmental enrichment.

In the present study zebrafish were held in isolation, either in an enriched or barren environment, and four different hypotheses were tested. Directionality of hypotheses is based on the background literature presented in previous sections.

Hypotheses:
1. After transfer to their new environment, zebrafish in an enriched environment will initiate feeding faster than those in a barren environment
2. Zebrafish in a barren environment will have increased locomotor activity compared to those in an enriched environment
3. Zebrafish in an enriched environment will have increased neurogenesis compared to those in a barren environment
4. Zebrafish in an enriched environment will have a lower level of whole-body cortisol than those in a barren environment

2.0 Methods and materials

2.1 Animals

Approximately 100 zebrafish were purchased from a local zoo store. After transfer to the university, they were kept in a 40 litre tank, at 26ºC, for two weeks before the start of the experiment. The tank water was partly replaced with fresh dechlorinated tap water twice a week. The water was well aerated and the light/dark cycle was 14:10 hours, with light from 0700 to 2100 hours, during both holding and experiments. The fish were fed daily with Hikari tropical micro pellet food (Kyorin co.).

2.2. The experimental setup

To avoid effects of social interactions, such as aggression, fish were kept isolated during the experimental period. The two groups each consisted of 12 fish. The enriched group was kept in an enriched environment, which consisted of stones and plastic plants, while the control group was kept in a barren environment, with empty aquaria. Individuals from both groups were kept in their respective aquaria for seven days.

Water temperature and the light/dark cycle during the experiment were identical to those of the holding tank. Twelve 15 litre aquaria with dividing walls of opaque PVC plastic were used, giving 24 separate compartments. Each aquarium was covered in grey plastic on all sides, except the top and the front side to allow feeding, water exchange, and aeration from the top, and filming from the front. To keep the zebrafish from jumping out, all the aquaria were covered with a transparent lid, with small holes to allow feeding. The enriched aquaria, i.e. twelve compartments in six aquaria, were filled with English sea stones, size 4-9 mm, to a height of 3 cm. These compartments were also equipped with two, 20 cm tall, *Acorus spp*. plastic imitations. The remaining twelve compartments (six aquaria) were left empty. The experimental setup is shown in figure 2.1.

Each aquarium was supplied with water from a common tank at a rate of 24 ml/min. Excess water was drained through small tubes in the aquaria walls at desired water height. The common tank was equipped with English sea stone and 11 *Acorus spp.* plastic imitation, so that any chemicals released from these items were distributed to all aquaria.

a)   b)
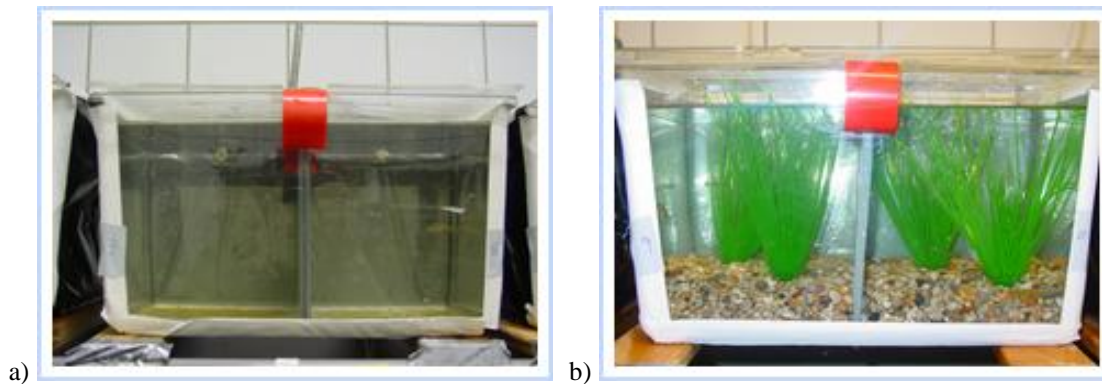
Figure 2.1 Aquaria used in the experiment. a) shows a barren aquarium, while b) shows an enriched aquarium.

2.3 The experiment

Materials and protocols for making the solutions mentioned in the following text can be found in Appendix 1 and 2, respectively. The fish were randomly selected from the common aquaria. They were gently netted and anesthetized in a 5 % benzocaine solution. The fish were then individually weighed and measured (Figure 2.2), and subsequently placed in a small container filled with water, where they were allowed to recover before they were placed in their respective aquaria, where they were kept for seven days. The zebrafish were filmed for ten minutes at day 1 and 4, using a digital video camera. They were hand-fed each day between 3 and 4 p.m. except on day 7. The latency from when the food was distributed until the fish started eating, denoted as the response time from now on, was recorded each day.

On day 7 the fish were quickly netted out of the aquaria, anesthetized, weighed and measured before they were sacrificed. The heads were separated from the bodies immediately behind the operculi and placed in 4 % paraformaldehyde, while the bodies were quickly frozen in liquid nitrogen and stored at -80°C.
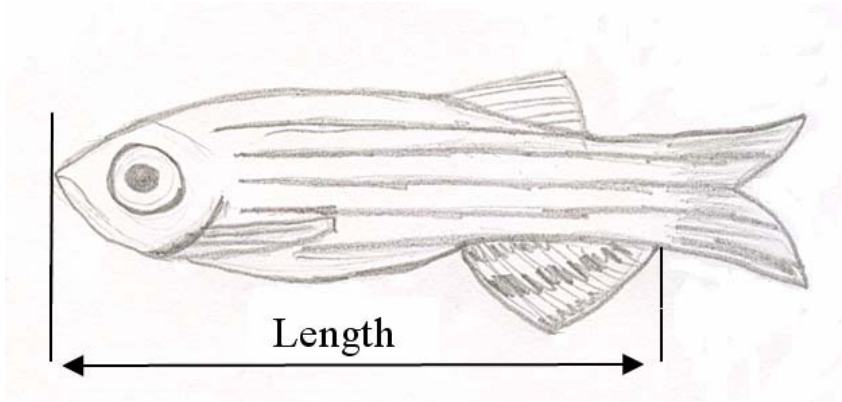
Figure 2.2 A schematic representation of how the length of the zebrafish was measured, from the tip of the mouth to where the tail fin begins.

2.4 Analysis of behavioural parameters

The zebrafish have an intermittent swimming activity and spend much of their locomotor periods coasting passively (McHenry and Lauder, 2005). Thus, the zebrafish are essentially in some kind of movement almost constantly. Hence measuring the time spent moving as an indicator of behaviour, is meaningless. Instead, locomotor activity was measured as the number of turns per unit time. The time unit was three minutes, and one turn was defined as any direction change exceeding 90° (see figure 2.3). Using a counter, the data from each fish was registered. The analysis was carried out for eight fish from each group, sixteen fish in all. The registration included films from both day 1 and day 4.
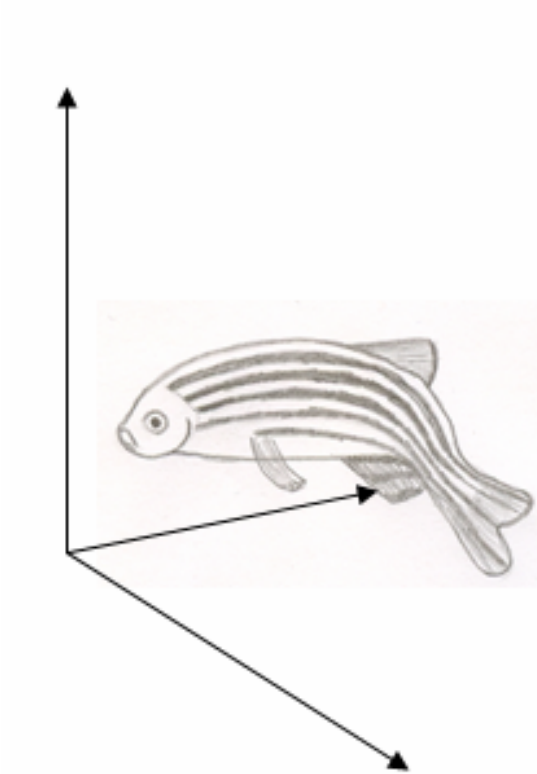
Figure 2.3 A schematic representation of a zebrafish inside a coordinate system. In the behavioural analysis, movements of the zebrafish were imagined to be inside such a system. Any movement exceeding 90°, in any direction, was recorded.

2.5 Processing of the brains

To fix the heads, they were kept in the paraformaldehyde solution at room temperature over night. They were then transferred to a 30% sucrose solution, where they were kept until the next day. The heads were then placed in small rubber moulds filled with Tissue – Tek (optimal cutting temperature medium) and frozen at -80°C

After fixation, 25 µm transverse sections were made by the use of a cryostat (Cryo-star HM560 M). Four sections were mounted per slide (Superfrost®plus slides). The sections were allowed to dry for two days at room temperature, before storage at -80°C.

2.5.2 Immunohistochemistry

Immunohistochemistry is a method used to detect specific antigens in cells or tissues, founded in the antigen-antibody binding. For this experiment, a specific polyclonal antibody against PCNA and a horseradish peroxidase (HRP) polymer conjugated to secondary antibodies were used. Subsequently, the chromogen 3, 3'-diaminobenzidine (DAB) was added. DAB polymerizes to a dark brown residue upon encounter with peroxidase, thereby marking the spot of the antigen-antibody interaction, see figure 2.4. This spot can easily be observed trough the microscope.
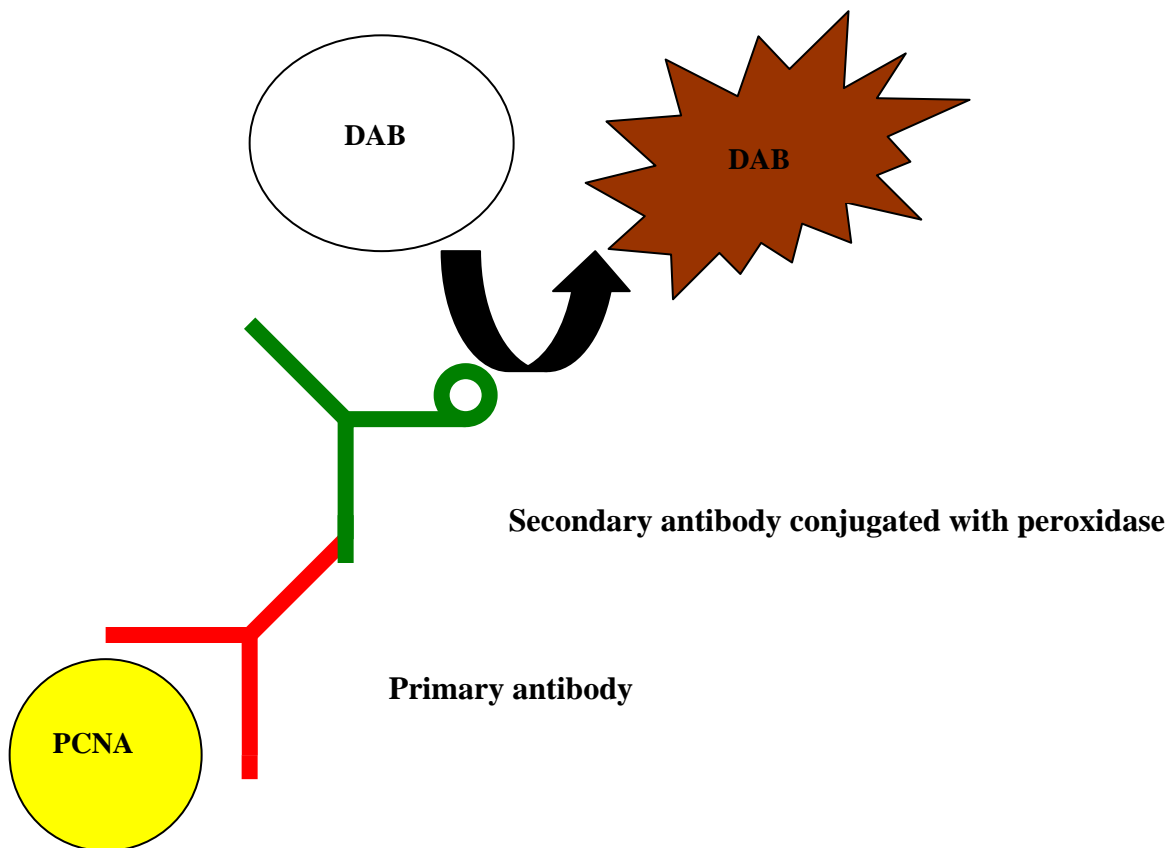
Figure 2.4 A simple representation of the immunohistochemistry method.

The protocol used for PCNA staining is attached in Appendix 3. However, several options were examined before deciding on this protocol, including BrdU-staining. In all, 21 different variations of the immunohistochemistry method were tested before the staining was successful. They are listed in Appendix 5.

Before commencement of the staining procedure, the sections were thawed and allowed to dry. The area around each section was then outlined with a PAP – pen to keep liquid droplets in place. The slides were washed in phosphate buffered saline (PBS) for five minutes, repeated twice. Further, for epitope retrieval, the slides were placed in a 10mM citric acid buffer, pH 6.0, and incubated for one hour at 70 °C. The citric acid solution was allowed to cool to room temperature before the slides were removed and washed three times in PBS. To prevent unspecific binding of the antibody, approximately 50 µl of blocking solution containing 6 % milk powder, was added to each section. The slides were placed in a humidified chamber and incubated for one hour at room temperature. The blocking solution was removed and replaced with a PCNA primary antibody (Rabbit polyclonal IgG), diluted 1:50 in PBT. The slides were then incubated over night at room temperature, in a humidified chamber.

On day two the slides were washed three times in PBS, five minutes per wash. To block endogenous peroxidase activity, the slides were placed for 15 minutes in a 3 % hydrogen peroxide solution, before they were washed three times in PBS. Approximately 50 µl of secondary antibody (Labelled polymer HRP, Anti-rabbit) was added to each section before the slides were incubated in a humidified chamber for 30 minutes at room temperature, and then washed three times in PBS. 50 µl of active DAB solution was added to each section. The effect of this chromogen was observed through the microscope. When the staining was complete, normally after 10 – 15 minutes, the slides were washed twice for five minutes with dH_20. They were then allowed to dry before being mounted with permanent mounting medium and coverslips.

2.5.3 Quantification of PCNA-positive nuclei

The sections were investigated using a Zeiss Axioplan 2 Imaging microscope and photographed using an Axiocam HR camera (2600 x 2060 pixels resolution) and the computer program Axiovision 3.1. The pictures were taken at 200 x magnification. To ensure the best resolution of the final image, each section was photographed in smaller parts and then joined to create a complete image. The joining of the images was done with the photo stitching software called PTGui.

The images were analyzed in Adobe Photoshop CS2. Four sections per 400 µm, giving a mean of twelve sections per telencephalon, were investigated. Since the size of the fish brain increases with body size (Zupanc and Horschke, 1995) it would be of little interest to detect the absolute number of new cells in the telencephalon. Therefore, the number of stained cell nuclei per investigated brain volume was calculated. The investigated volume of each telencephalon was calculated by multiplying the area of each section, as determined using Photoshop CS2, by its thickness, which was 25 µm. The nuclei were counted in the proliferation zones described in chapter 1.6.1. Nuclei were occasionally observed outside these zones (which might have been migrating cells), but these were not counted. Sometimes a cell displayed two nuclei, i.e. the cell was in the telophase of mitosis, in which case each nucleus was counted. As this experiment focus on how environmental enrichment affects the proliferation rate in the telencephalon, and not how the specific areas within the telencephalon are affected, the proliferation zones were not counted separately. Figure 2.5 shows a typical section of the telencephalon after PCNA-immunohistochemistry.
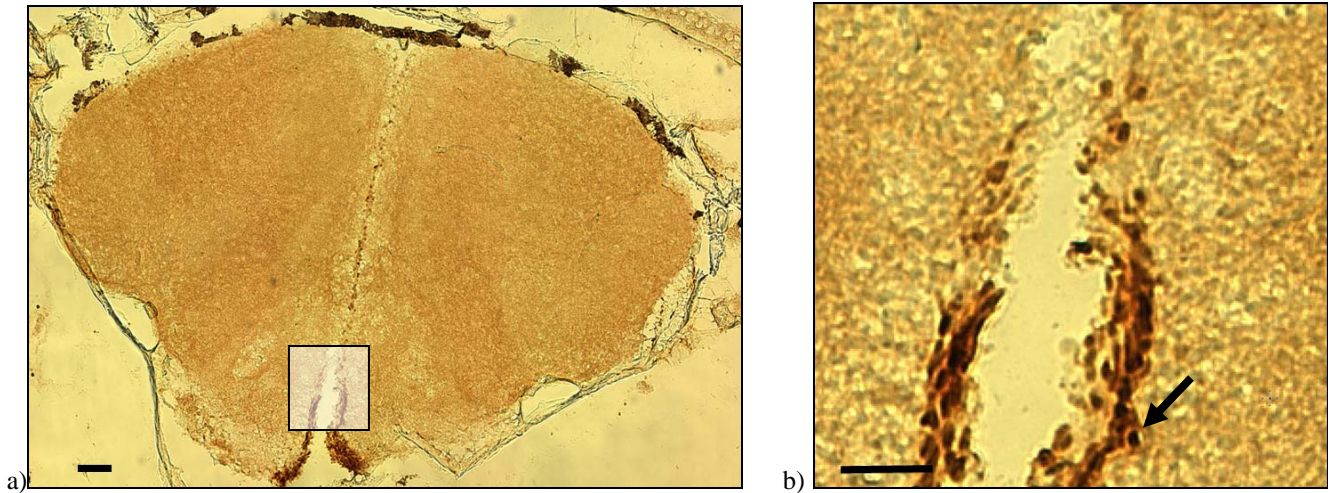
Figure 2.5 a) One telencephalic section stained by immunohistochemistry. Scale bar is 100 µm. b) An enlargement of the frame from a) Here the scale bar is 50 µm. The arrow points to a typical nucleus.

2.6 Cortisol assay

Since the zebrafish are small (average 0.3g and 2.5 cm), plasma samples are very hard to obtain. A whole-body extraction was therefore carried out. (In this case, "whole-body" refers to the body minus the head). The fish bodies were weighted and homogenized using a T25 homogenizer (IKA labor technik), in 1:3 (weight : volume) PBS. The samples were then stored at -80°C.

Lipophilic components of the specimen were extracted with diethyl ether (DE). 1 ml of DE was added to each sample, followed by approximately 30 sec of vortexing. The samples were then centrifuged, at 4°C and 5000 rpm, for 10 min. After centrifugation, the samples were frozen at -80°C, whereupon the ether layer was decanted off and collected. The pellets were allowed to thaw on ice, before extraction was repeated twice. The supernatant from all three extractions were combined in one eppendorf tube, and the DE evaporated at room temperature in a fume hood over night. Subsequently, 250 µl of EIA Kit assay buffer and 250 µl of carbon tetrachloride (CCl_4) were added to each tube. The remaining lipids dissolved in the CCl_4. A pre-test, with known amounts of cortisol added

to samples (n = 5), was performed to determine the amount of cortisol lost in the extraction procedure. This test showed that an average of 77.6 % of the cortisol was extracted and this was corrected for in all samples. The assay buffer/ CCl_4 mixture was vortexed well, and then centrifuged for 10 min at 5000 rpm at 4°C. The aqueous phases were subsequently transferred to new eppendorf tubes. Cortisol concentrations of the extracts were determined using a Cortisol Correlate-EIA[TM] Kit (Assay Designs/USA).

2.7 Statistical analysis

Statistical analyses were performed using STATISTICA software (StatSoft, Inc., Tulsa, Oklahoma) for ANOVA procedures and Student's t-tests. Graph Pad (Software Inc., San Diego, California) was used for descriptive statistics, Kolmogorov Smirnov test for normality, Levene's test for variance homogeneity and Grubb's test for outlier identification.

3.0 Results

Of the 24 fish originally included in the experiment, three fish were lost before completion of the behavioural studies. Total number of fish was therefore 21, where 10 fish were from the enriched group and 11 from the control group. The analyses were based on these numbers unless otherwise stated.

3.1 Growth

Both length and weight of all the tested fish were recorded at day 1 and day 7 in the experiment. Growth rates were compared between the groups and the results are presented in figure 3.1 as percent increase in body weight and length per day. There were no significant difference in either weight or length increase (Student's t-test: $t_{(19)}= 0.36$, p=0.72 and $t_{(19)}=1.22$, p= 0.24, for weight and length respectively).
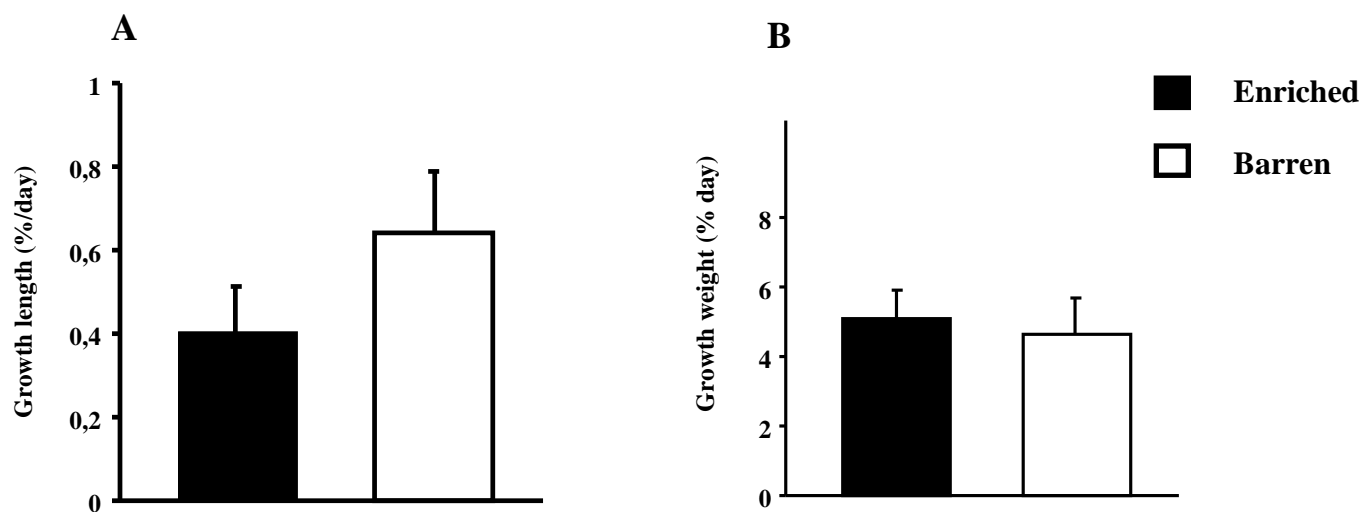
Figure 3.1 Growth presented as percentage length (A) and weight (B) gain per day, respectively. There was no significance in length or weight between the groups, as tested by Student's t-test ($t_{(19)}=1.22$, p= 0.24 and $t_{(19)}= 0.36$, p=0.72, respectively).

3.2 Behaviour

3.2.1 Response time

Each fish was fed approximately 5 % of the body weight each day. It was not feasible to quantify the exact amount of food consumed by each individual, but the response time from when food was presented until the first food item was consumed was registered for each individual on each day, except on day 7. These data are graphed in figure 3.2. One individual in the barren environment showed response times (average 57.6 s) that were over ten times longer than the mean group value (average 2.16 s). This fish was identified as an outlier (Grubb's test $p<0.05$) and removed from further analysis.

The data for response time were tested with repeated measures ANOVA. In both groups, the response time decreased significantly with time ($p<0.001$). The enriched group tended to have a higher mean response time than the control group each day, but the difference between the groups was not significant ($p=0.12$).
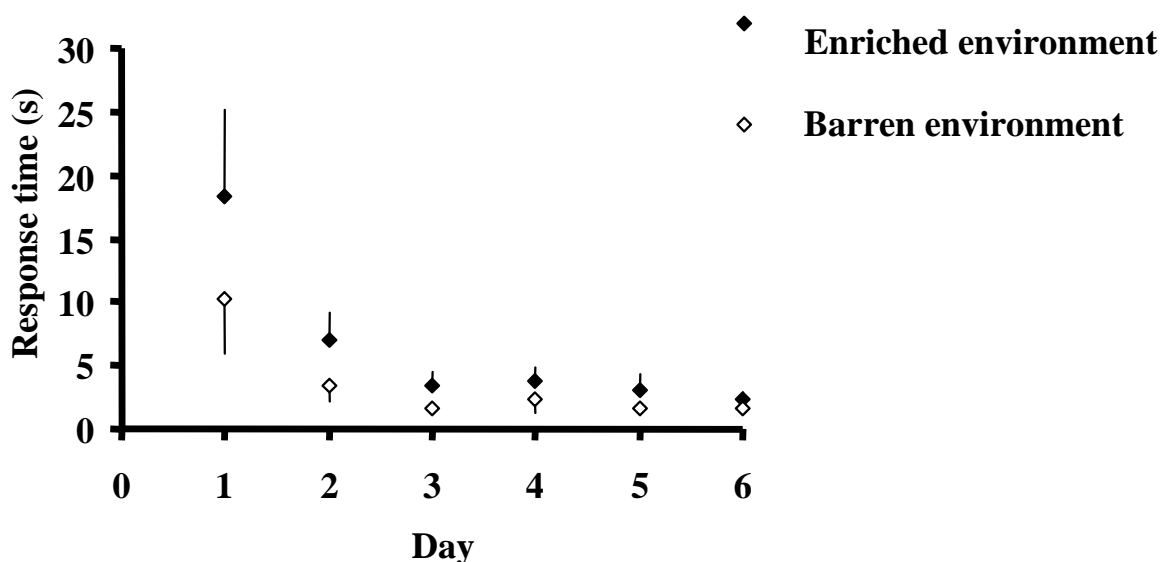
Figure 3.2 Response time to food in zebrafish kept in enriched and barren environments. The results are presented as mean ± S.E.M. Response times decreased significantly with time ($p<0.001$), while the difference between groups did not reach statistical significance ($p=0.12$) (repeated measures ANOVA). For detailed ANOVA results, see appendix 4.

3.2.2 Locomotor activity

The locomotor activity, in terms of numbers of turns per time unit (three minutes), was examined in eight fish from each group. This was measured from films obtained at day 1 and 4. The data obtained were analysed in a two-way ANOVA, with day of observation and environment as independent variables. The results are presented in figure 3.3. Both time and treatment significantly affected locomotor activity ($p<0.001$ and $p= 0.005$, respectively). There was however no interaction effect ($p = 0.78$). In general, zebrafish from the barren environment showed higher locomotor activity on both days.
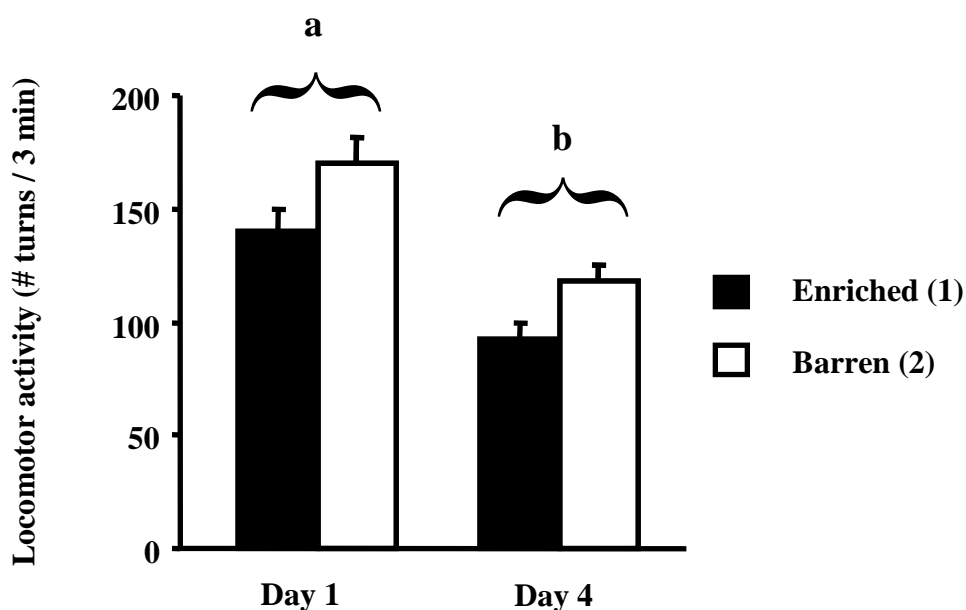
Figure 3.3 Locomotor activity in zebrafish kept in an enriched or barren environment, as indicated by the frequency of turns observed at day 1 and 4 after transfer from group rearing to rearing in isolation in experimental environments. Different letters (a, b) and numbers (1, 2) indicate statistically significant effects of the factors day ($p<0.001$) and environmental complexity ($p=0.005$). For detailed ANOVA statistics see appendix 4.

3.3 Brain cell proliferation

Brain cell proliferation was visualised by PCNA immunohistochemistry in eight fish from each group. After the immunohistochemical procedure, six brains were found to be deviant in appearance due to freeze-damage, and were therefore excluded from the subsequent analysis. The total number of brains examined was thus 10, five from each group. Nuclei stained positively for PCNA were found in the proliferation zones described in chapter 1.6.1 (see figure 3.5).

The number of stained nuclei per telencephalic volume is shown in figure 3.4. Levene's test showed that the variance of the data was significantly larger in the enriched group than the barren (original data mean ± S.D.: 3239 ± 940 vs. 2892 ± 332, lack of homogeneity, p=0.03). Due to the lack of variance homogeneity, the data were log transformed before they were tested in a Student's t-test. After log-transformation, data met the criteria for parametric statistics. There was no significant difference in stained nuclei between the group mean cell counts ($t_{(8)}$=1.62, p=0.14).
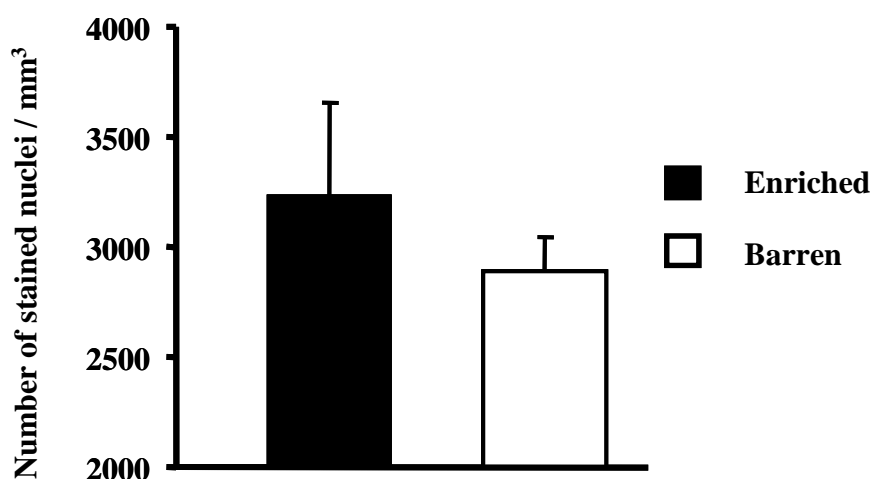
Figure 3.4 Number of PCNA positive nuclei per telencephalic volume in zebrafish reared in enriched and barren environments. Data are presented as mean ± S.D. No significant difference was detected between the groups (Student's t-test on log-transformed data, p= 0.14).

Figure 3.5 Proliferation zones as described in chapter 1.5.1. All sections are stained by PCNA immunohistochemistry. **A** is a rostal section from the zebrafish telencephalon, **B** is an enlargement of the marked area in **A**. **C** is a more caudal section of the telencephalon, and **D** and **E** are the marked areas from **C**. **F** is a typical freeze-damaged section. Examples of stained nuclei are marked with black arrows. Scale bars are 100µm for **A**, **C** and **F**, and 50 µm for **B**, **D** and **E**. Section **A**, **C** and **F** were taken 75µm, 600µm and 800µm from the rostal tip of the telencephalon, respectively. Abbreviations: D = area dorsalis, Dm = medial pallium, Dl = lateral pallium, OB = olfactory bulb, TelV = telencephalic ventricle, V = area ventralis

3.4 Whole-body cortisol

Cortisol were extracted from whole-body (minus head) samples, and concentrations calculated in relation to body weight. The data were analysed by Student's t-test. Fish from the enriched group had significantly higher whole-body levels of cortisol than fish from the control group ($t_{(19)}$=3.41, p=0.003). The results are presented in figure 3.6.
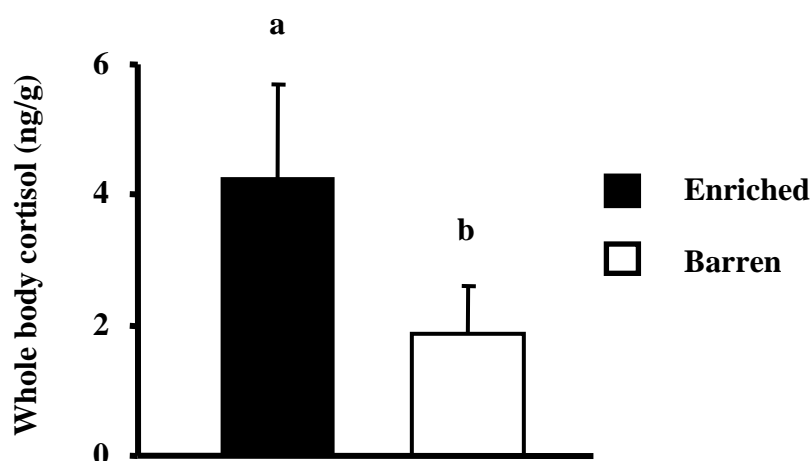
Figure 3.6 Whole-body cortisol levels, presented as ng cortisol per g body weight (mean ± S.E.M), in zebrafish reared in enriched and barren environments. Testing by a Student's t-test revealed that the enriched group had significantly higher levels of whole-body cortisol than the control group (p=0.003), indicated by different letters (a, b).

4.0 Discussion

4.1 Neurogenesis in isolated zebrafish in enriched and barren environments

In order to estimate neurogenesis in zebrafish a protocol for immunohistochemical PCNA staining was developed. Staining of a quality that allowed quantification of stained nuclei in the telencephalic tissue was obtained after a number of attempts (listed in Appendix 5). The results revealed no significant effect of environmental enrichment on average levels of neurogenesis in the zebrafish. There was however a strong tendency towards higher neurogenesis in the enriched group, which may have failed to reach statistical significance due to the small sample size. Furthermore, the inter-individual variability was significantly higher in the enriched group than in the control group. This latter observation is in agreement with several other studies (Mering et al., 2001; Tsai et al., 2002). Tsai et al. (2002) found that, compared to control group, mice reared in an enriched environment had higher coefficients of variance (defined as S.D/mean value) in most parameters tested, which were body weight, organ (heart, kidney, liver, adrenal, uterus and spleen) weight and haematology (red and white blood cells, haemoglobin and haematocrit). This indicates that a heterogeneous environment leads to greater heterogeneity in several physiological parameters within a population. An experimental consequence of this is that more animals are needed in order to reach statistical significance. However, high inter-individual variance is not always reported in animals from enriched environments and seems to be dependent on certain circumstances, such as animals strain, type of enrichment, duration of the experiment and type of statistical analysis (Tsai et al., 2002; Augustsson et al., 2003).

Although neurogenesis was not significantly higher in the enriched zebrafish it is a possibility that, if the proliferation zones were compared separately, there would have been a significant difference between the groups. This was not investigated here due to time constraints. Nevertheless, since it has been reported that the proliferation zones in

mammals are affected differently by different stimuli (Rochefort et al., 2002; Brown et al., 2003b), it would be interesting to investigate this option further in the future.

Not much work has been published on how environmental enrichment affects the brain of fish. However, Kihslinger and Nevitt (2006) showed that, by simply adding stones to the standard rearing tank, salmon alevins grew significantly larger cerebella than fish reared in conventional tanks. It has also been shown that the brain volume of wild fish is larger than that of fish reared in hatcheries (Kihslinger et al., 2006). In rodents, on the other hand, a large number of studies confirm that environmental enrichment increases neurogenesis (Altman and Das, 1964; Kempermann et al., 1997b; Kempermann et al., 1998a; Nilsson et al., 1999; Kempermann et al., 2002; Brown et al., 2003b; Komitova et al., 2005; Olson et al., 2006; Segovia et al., 2006).

New neurons might be a necessity in fish, that grow in size throughout life, in order to provide central neurons to the increasing amount of peripheral sensory and motor elements (Zupanc, 2001). Consequently, an increase of neurogenesis might reflect an increase in growth. However, in this experiment, growth rates did not differ between the groups, and are thus not likely an explanation to why the enriched animals tended towards higher neurogenesis.

There are at least three possible reasons why significant differences in neurogenesis were not detected in the present study: First, the fish were kept in their respective environments for only seven days, which might be too short a period to create detectible differences. Second, the individual variation was so large that more fish were needed in order to reach statistical significance. Third, environmental enrichment of this kind may simply not be sufficient to induce increased neurogenesis in the zebrafish telencephalon.

4.2 Behaviour of isolated zebrafish in enriched and barren environments

This is the first study to demonstrate an effect of environmental enrichment on behavioural variables in zebrafish. Two such variables were measured; response time to food presentation and locomotor activity.

4.2.1 Response time to food presentation

In both groups the response time to presented food fell significantly over time, but there was no significant difference between the groups. In this study, only the response time was measured, and not the amount of food consumed. Previously it has been reported that transfer to a new environment may cause reduction or cessation of food-intake in fish (Øverli et al., 2002b; Schjolden et al., 2005), but this was not seen in this experiment. All the fish observed showed food-intake each day. At day 1 the fish received food approximately three hours after their transfer to their new environment. It was essentially at this day that the fish showed long response time. Since no cortisol samples were taken at day 1, it is difficult to say whether the fish were stressed by the handling and the anaesthesia performed at that day. In a previous study by Lepage et al. (2000), it was found that transfer to a novel environment led to a significant increase in plasma cortisol levels in anadromous brown trout, *Salmo trutta*. High cortisol and CRH levels have been showed to decrease appetite in fish (Bernier and Peter, 2001; Volkoff et al., 2005; Bernier, 2006). If the zebrafish indeed were stressed at day 1, this might explain the slow response to food. The decrease in response time observed over the next few days was probably caused by a habituation to the new environment.

It should be noted that great individual variations in response time were observed. Some fish started to eat immediately, even at day 1. Although not significant, the enriched group had an overall tendency towards a higher response time in the whole experimental period. It might be that the plants that were present in the enriched aquaria served as a visual obstruction for the fish, leading to a longer response time.

In conclusion there was no significant difference in response time to food between the zebrafish from different experimental groups, and all fish seemed to acclimate rapidly to their new environment. For future experiments cortisol samples after handling and exposure to novelty at day 1 should be collected, to determine the stress level of the animals after these actions.

4.2.2 Locomotor activity

In the two- way ANOVA, the control group had significantly higher locomotor activity than the enriched group, while both groups of fish showed a significant decrease in locomotor activity with time. Hence, as with response time to food, habituation may have affected locomotion. A study on ratsnakes, *Elaphe obsoleta*, revealed that snakes reared in an enriched environment habituated more quickly to a novel environment than ratsnakes reared in standard conditions (Almli and Burghardt, 2006). Provided that the increased locomotor activity in the barren group can be taken as a sign of anxiety or stress (see below), this also seemed to be the case with the enriched zebrafish.

Transfer to a novel environment is often associated with a high GC response (Hennessy, 1991). As with food-intake, locomotor activity can be affected by GCs and CRH (Lowry and Moore, 2006). Clements et al. (2002) showed that high CRH levels led to increased locomotor activity in spring chinook salmon. If the zebrafish were stressed at day 1 after the handling and transfer to a novel environment, the high locomotor activity might reflect this state. Stress has also been shown to elevate the serotonergic activity in fish (Winberg and Nilsson, 1993). High levels of serotonin may lead to a decrease in locomotor activity, prominently in subordinate animals, and lowered aggression (Winberg and Nilsson, 1993). Since all fish lived in isolation during the experiment, this scenario might not be transferable to the present study. Increased locomotion as a result of high serotonin levels has also been reported (Green and Grahame-Smith, 1974; Wedderburn and Sillar, 1994; O'Neill and Sanger, 1999), making it hard to draw any conclusions about this monoamine's effect on locomotion. However, in future

investigations of the present kind it would be interesting to measure brain serotonergic activity.

Animals reared in enriched environments have previously been shown to be less affected by stress and show reduced fearfulness in encounters with novelty, in addition to faster habituation and decreased locomotor activity (Larsson et al., 2002; Benaroya-Milshtein et al., 2004; Hattori, 2007). Yet, different results in behavioural tasks from animals reared in enriched environments have been reported (Hattori, 2007). This is likely due to the period of time the animals have spent in their respective environments. It would be interesting to see if the behavioural differences between the groups in the present study would have been more pronounced if the experimental period was prolonged. It has previously been shown in mammals that environmental enrichment enhances memory and learning, and that animals reared under such conditions perform better at tasks such as the Morris water maze (Falkenberg et al., 1992; Nilsson et al., 1999). For the future, it would be interesting to investigate if zebrafish reared in enriched environments show enhanced performance skills in tasks of memory and learning, such as active avoidance training (Laudien et al., 1986).

4.3 Whole-body cortisol levels

Whole-body cortisol levels were measured to determine whether the fish were stressed by the isolation and if the experimental environments led to different stress levels. The cortisol levels turned out to be significantly higher in the enriched zebrafish than in the control in this experiment. However, basal levels of GCs are often moderately higher in animals reared in enriched environments, compared to controls (Haemisch et al., 1994; Klont et al., 2001; Marashi et al., 2003; Moncek et al., 2004), although other studies report no increase in GC levels from environmental enrichment (Pham et al., 1999a; Schrijver et al., 2002). Interestingly, studies have shown that animals reared in enriched environments have lower GC levels compared to controls during stress, which might indicate that these animals are less affected by stress (Larsson et al., 2002; Benaroya-Milshtein et al., 2004). Additionally, the moderately elevated basal levels of

GCs in animals from enriched environments do not seem to be accompanied by the negative health consequences such as decreased neurogenesis (Kempermann et al., 1997b; Mohammed et al., 2002; Moncek et al., 2004) and lowered immune functions (de Groot et al., 2000) associated with high GC levels during stress.

Whole-body cortisol levels vary between different fish species (Sakakura et al., 1998; Pottinger et al., 2002). In a time-study on cortisol levels performed in our lab, the zebrafish whole-body cortisol levels was found to lie in between 0.07-3.95 ng/g tissue (unstressed fish, n=5) and 17.66- 48.83 ng/g tissue (30 minutes of stress, n=4) (Sørensen, Øverli and Nilsson: unpublished results). It was also found that the cortisol levels increased significantly to 1.03-12.49 ng/g tissue (n= 5) after only one minute of stress exposure.

In the present study, cortisol levels in the zebrafish ranged from 0.34 to12.48 ng/g tissue (mean ± S.E.M; enriched: 7.49±1.75, barren: 1.95±0.59). These data are comparable with the levels found at 0 and 1 minute of stress in the study by Sørensen et al. (unpublished) as well as other studies (Ramsay et al., 2006), and do not indicate high levels of stress for neither experimental group of fish.

There is some reason to suspect that the higher cortisol levels observed in the enriched zebrafish were at least partly caused by handling. Due to the plastic plants in the enriched aquaria, more time (approximately 30 sec) was spent to net the enriched fish than the control fish (approximately 5 sec). This time difference might have been sufficient to initiate activation of the HPI-axis, which would lead to increased cortisol levels in the enriched group. . There was also no correlation between plasma cortisol or any of the behavioural patterns analysed (data not shown). In view of the known effects of cortisol on behaviour in teleost fish (Øverli et al., 2002, and references therein), it is therefore reason to suspect that cortisol levels were at least in part an effect of sampling stress, and not a chronic feature of the different environments.

Isolation has been shown to be a stressor in many social species, such as pigs and rats (Brown and Grunberg, 1995; Ruis et al., 2001). Not much is known about how isolation affects fish, but Martins et al. (2006) performed a study on this topic on the social African catfish, *Clarias gariepinus*. Isolated catfish showed lowered feed-intake and growth rate than the non-isolated fish, but the cortisol levels did not differ. This indicates that isolation for a short period (15 days) was not a stressor for the catfish

(Martins et al., 2006). No such experiment has been performed on zebrafish, but the low cortisol values found in the control group and the relatively low values from the enriched group in this experiment do not indicate that isolation, at least not for seven days, was a stressor.

4.4 Conclusions

In the current study, environmental enrichment led to lowered locomotor activity and a tendency towards increased neurogenesis. Further, the enriched group showed significantly higher inter-individual variation in neurogenesis than the control group. Isolation was not a stressor to the zebrafish, as indicated by relatively low whole-body cortisol levels and short response time to distributed food.

The tendency towards higher neurogenesis and the activity pattern observed in the enriched group, indicate that environmental enrichment has the potential to increase the welfare of zebrafish. However, further studies must be performed to examine this possibility.

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Appendix 1 – Materials

Material:	Provider:	Art. Nr:	Lot. Nr:
5-Bromo-2' deoxyuridine (BrdU)	Sigma -Aldrich	B-5002	023K1260
Albumin from Bovine serum	Sigma -Aldrich	A2153	073K0654
Benzocaine	Sigma	E-1501	58H0619
BrdU Mouse Monoclonal IgG1	Santa Cruz Biotechonoly	sc-32323	HO305
Carbon tetrachloride	Fluka	87031	1229937
Cortisol Correlate -EIATM Kit	Assay Designs	900-071	06020626
DAB Chromogen	DakoCytomation	S3000	085070
Deconex	Borer Chemic	123375	20569
Diethyl ether	Merck	1.00921.1000	K25841221
EnVision+® System Labelled Polymer - HRP Anti-Mouse	DakoCytomation	K4000	105273
EnVision+® System Labelled Polymer - HRP Anti-Rabbit	DakoCytomation	K4002	115079
Hydrogen peroxide, 30 wt. %	Sigma -Aldrich	H1009	104K1434
KCl	Merck	1.04936.1000	TA104736
KH_2PO_4	Merck	1.04873.1000	A118873
Molico Instant Milkpowder	Nestle	04115-01	1792-2
Na_2HPO_4	Merck	1.06580.1000	K22363880
NaCl	AnalaR®	102415K	K36100833
PAP, Mouse, Monoclonal	DakoCytomation	P0850	00017453
PAP-pen	Daido Sangyo		
Paraformaldehyde	Sigma	P-6148	50K0916
PCNA Mouse Monoclonal IgG_{2a}	Santa Cruz Biotechonoly	sc-56	D0605
PCNA Rabbit Polyclonal IgG	Santa Cruz Biotechonoly	sc-7907	LO105
Permanent Mounting Media	DakoCytomation	S3026	044311
Sucrose	AnalaR®	102745C	K28157286
Superfrost® Plus slides	Menzel-Glaser	J1800AMNZ	11796
Tissue Tek	Sakura	4583	0000530-01
Triton X	Sigma	T-9284	092K0171

Appendix 2 - Solutions

10 X Phosphate buffered saline (PBS)

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

0.8 l dH_2O

Adjust pH to 6.8, and then adjust volume to 1litre with additional dH_2O. Sterilize by autoclaving. The solution can be stored at room temperature. Dilute the solution 1:10 with dH_2O before use. Adjust pH if necessary, pH should be 7.4 for 1 X PBS.

6 % Milk-Powder solution

5 ml PBS

1.5 µl Triton X-100

300 mg Milk powder

Vortex until all the milk powder is dissolved. Solution should be freshly made before use.

0.6 % PBS with Triton X-100 and BSA (PBT)

10 ml 1 X PBS

3 µl Triton X-100

60 mg Bovine serum albumin (BSA)

Vortex until all the BSA is dissolved. Solution can be stored at 4 ºC for one week.

3 % H$_2$O$_2$

10 ml H$_2$O$_2$
90 ml autoclaved dH$_2$O

This volume is adequate for an eight-slide cuvette. The solution should be made freshly before use.

Diaminobenzidine (DAB) stock-solution

10 ml autoclaved dH$_2$O
1 DAB tablet

Vortex until the tablet is dissolved (will not always dissolve completely). The solution can be stored at 4 ºC for one week. DAB is very poisonous, but can be inactivated with commercial Klorin (hypoclorite).

Activated DAB- solution

4 ml DAB stock- solution
30 µl 3 % H$_2$O$_2$

Mix immediately before use. The solution can be inactivated with commercial Klorin.

Rabbit anti- PCNA [1:50]

4 ml 0.6% PBT
80 µl anti-PCNA

Mix gently by turning the eppendorff tube up-side-down for a few times. This solution should be made freshly before use, and is an adequate amount for 16 slides with 4 sections each.

Benzocaine solution

5 % benzocaine in 96 % ethanol

To anesthetize zebrafish, 300 µl of 5 % benzocaine solution per 100 ml dH_2O is adequate.

30 % Sucrose solution

30 g sucrose
70 ml autoclaved dH_2O

Stir until all sucrose is dissolved. The solution can be stored at room temperature.

4 % paraformaldehyde solution

96 ml 1 X PBS
4 g paraformaldehyde

All handling of paraformaldehyde should be done inside a fume hood. Place the mixed solution on a hotplate-stirrer, and allow the solution to warm. When the solution turns clear it is done. Turn off the hotplate, but allow the solution to stir until it is cool. The solution can be stored at 4°C for one week.

Methacarn

60 % methanol

30 % chloroform

10 % glacial acetic acid

Make fresh before the fixation

10 mM Citric acid buffer

2.1 g citric acid monohydrate

0.9 l autoclaved dH_2O

Adjust to pH 6.0 with NaOH, refill will dH_2O until 1 litre of total volume. The solution can be stored for three months at room temperature.

Appendix 3 – PCNA protocol

Day 1

1. Thaw the frozen slides in room temperature until they are dry
2. Draw around the sections with a PAP pen (in fume hood), allow to dry
3. Wash slides in PBS, 3 x 5 min
4. Epitope retrieval in 10 mM citrate buffer (pH 6.0) at 70 °C, incubate for 1 hour
5. Allow solution, with slides, to cool to room temperature
6. Wash slides in PBS, 3 x 5 min
7. Block with 6 % milk powder for 1 hour at room temperature. The incubation should be done in a humidified chamber
8. Add 50 µl anti – PCNA (1:50) in 0.6 % PBT and incubate over night at room temperature. The incubation should be done in a humidified chamber

Day 2

1. Wash slides in PBS, 3 x 5 min
2. Block peroxidase activity with 3 % H_2O_2 for 15 min
3. Wash slides in PBS, 3 x 5 min
4. Add approximately 50 µl Dako EnVision+ to sections. Incubate at room temperature in a humidified chamber for 30 min
5. Wash slides in PBS, 3 x 5 min
6. Add 50 µl activated DAB solution to sections and incubate for 10-15 min. Observe through a microscope during the incubation time to determine when the reaction is completed
7. Wash slides in H_2O, 2 x 5 min

To conserve the samples, allow sections to dry, add DAKO permanent mounting medium and cover the sections with glass cover slips.

Appendix 4 – Statistical analyses

Table 1 Response time to food distribution

STAT. GENERAL MANOVA	Summary of all Effects; design: (new.sta) 1-VAR1, 2-VAR1					
Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1	238.0083	18	89.84167	2.649198	.120979
2	5*	452.9950*	90*	60.89944*	7.438409*	.000007*
12	5	37.8483	90	60.89944	.621489	.683732

Var1 = Environment, Var2 = Day of observation

Table 2 Locomotor activity

STAT. GENERAL MANOVA	Summary of all Effects; design: (new.sta) 1-VAR1, 2-VAR2					
Effect	df Effect	MS Effect	df Error	MS Error	F	p-level
1	1*	5940.50*	28*	635.2946*	9.35078*	.004865*
2	1*	19900.13*	28*	635.2946*	31.32425*	.000005*
12	1	50.00	28	635.2946	.07870	.781124

Var1 = Environment, Var2 = Day of observation

Appendix 5 – Method development

The following tables show the tests tried for PCNA and BrdU, respectively. The changes made from one test to another are marked with green colour. All sections were 25 µm thick transverse cuttings. For each concentration of antibody tested, there were at least two duplicates per test.

PCNA:

Primary antibody:	Primary antibody concentration:	Epitope retrieval method:	Blocking:	Secondary antibody:	Tertiary antibody:	Sections:	Results and commentary:
Anti – PCNA (Sigma)	a. 1:100 b. 0 incubated over night at 4°C	10 % SDS for 30 min	6% PBT for 60 min	Rabbit anti mouse (1:50) in 0.6 % PBT for 60 min	PAP (1:100) in 0.6% PBT for 45 min	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 24 hours.	A lot of background staining, but no nuclear staining. Several sections loosened from the slides.
Anti – PCNA (Santa Cruz)	a. 1:100 b. 0 incubated over night at 4°C	10 % SDS for 30 min	6% PBT for 60 min	Rabbit anti mouse (1:50) in 0.6 % PBT for 60 min	PAP (1:100) in 0.6% PBT for 45 min	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 24 hours.	No nuclear staining, but less background staining, so will use the Santa Cruz primary antibody from now on. Several sections loosened from the slides.

Anti – PCNA (Santa Cruz)	a. 1:100 b. 1:50 c. 0 incubated over night at 4°C	1 % SDS for 15 min	6% PBT for 60 min	Rabbit anti mouse (1:50) in 0.6 % PBT for 60 min	PAP (1:100) in 0.6% PBT for 45 min	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 24 hours.	The SDS concentration was lowered to see whether it had an effect on the loosening of the sections. Here, no whole sections, but parts, loosened. No staining in the brain tissue.
Anti – PCNA (Santa Cruz)	a. 1:25 b. 1:10 c. 0 incubated over night at 4°C	1 % SDS for 15 min	6% PBT for 60 min	Rabbit anti mouse (1:50) in 0.6 % PBT for 60 min	PAP (1:100) in 0.6% PBT for 45 min	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	The drying period were prolonged, to see whether this had an effect on the sections loosening from the slides. There were fewer sections that loosened, so the sections will be allowed to dry for 48 hours from now on. No staining in the brain tissue.
Anti – PCNA (Santa Cruz)	a. 1:50 b. 1:25 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 20 min at 70 °C.	6% PBT for 60 min	Rabbit anti mouse (1:50) in 0.6 % PBT for 60 min 0.6% PBT for 60 min	PAP (1:100) in 0.6% PBT for 45 min	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Another epitope retrieval method was tested to see whether this could enhance the staining. The results gave only background staining. No sections loosened.

Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:300 c. 1:100 d. 1:50 e. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 20 min at 70 °C.	x. 1% PBT for 60 min y. 3 % PBT for 60 min z. 6% PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Changed secondary antibody to Dako En Vision ++, which is a HRP labelled polymer conjugated to a secondary antibody, excluding the need for the tertiary antibody used until now. All combinations of a-e + x-z were tested. No nuclear staining, only background.
Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:50 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	x. 1 % PBT for 60 min y. 3 % PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	All combinations of a-c + x-y were tested. No nuclear staining, only background.
Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:50 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) boiled in microwave oven for 4 x 5 min. Refilled, if the fluid evaporated, with dH_2O.	x. 1 % PBT for 60 min y. 3 % PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	All combinations of a-c + x-y were tested. No nuclear staining, only background.

Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:50 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	x. 1 % PBT for 60 min y. 3 % PBT for 60 min	Dako EnVision++ for 30 min	----	The brain was not fixated. After cutting, the sections were dried for 48 hours.	All combinations of a-c + x-y were tested. Most sections were completely destroyed, since they where not fixated. A few cells were stained at 1:50 concentration of primary antibody and 1 % BSA, but the results are unsure since the sections were so destroyed.
Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:50 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	1 % PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 30 minutes, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	No nuclear staining, only background.
Anti – PCNA (Santa Cruz)	1:50 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	1 % PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 60 minutes, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	No nuclear staining, only background. Because of troubles with the cryostat, there were so few good sections that only the 1:50 primary antibody concentration was tested.

Anti – PCNA (Santa Cruz)	a. 1:500 b. 1:50 c. 0 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	1 % PBT for 60 min	Dako EnVision++ for 30 min	----	Fixated in 4 % paraformaldehyde for 120 minutes, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	No nuclear staining, only background and a few stained cells in the tissue surrounding the brain.
Anti – PCNA (Santa Cruz)	a. 0 b. 1:50 c. 1:100 incubated over night at 4°C	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	1 % PBT for 60 min	Dako EnVision++ for 30 min	-----	48 hours in 4% paraformaldehyde, then 48 hours in sucrose solution. Dried for 12 hours.	The method was tested on rainbow trout, to see if the species varied in results. The test gave a lot of background staining, and some nuclear staining, but very weak, so it was difficult to quantify.
Anti – PCNA (Santa Cruz)	a. 0 b. 1:50 c. 1:100 d. 1:200 incubated over night at room temperature	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	1 % Milk powder for 60 min	Dako EnVision++ for 30 min	-----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Some background staining. There was also some weak nuclear staining, but it was difficult to quantify.

Anti – PCNA (Santa Cruz)	a. 0 b. 1:10 c. 1:25 d. 1:50 incubated over night at room temperature	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	x. 3 % Milk powder for 60 min y. 6 % Milk powder for 60 min	Dako EnVision++ for 30 min	-----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	All combinations of a-c + x-y were tested. Some background, but nice staining in several nuclei in the brain. The best results were from the sections with 1:50 concentration of primary antibody and 6 % milk powder, and these parameters were set to be retested.
Anti – PCNA (Santa Cruz)	1:50 incubated over night at room temperature	Citric acid buffer (10 mM, pH 6.0) for 60 min at 70 °C.	6 % Milk powder for 60 min	Dako EnVision++ for 30 min	-----	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	This method gave good results, with nice nuclear staining and low background staining. It was tested two additional times, with equally good results. This is the method used in the rest of this study.

BrdU:

These methods were tested alongside the PCNA-testing, and even though PCNA was the antigen finally used in this study, the results for the few BrdU-tests are enclosed for information.

Primary antibody:	Primary antibody concentration:	Epitope retrieval method:	Blocking:	Secondary antibody:	Hours incubated in BrdU-solution (30 mg/l) :	Sections:	Results and commentary:
Anti - BrdU	a. 1:100 b. 1:500 incubated over night at 4°C	2M HCl for 60 min at 37 °C.	6% PBT for 60 min	Dako EnVision++ for 30 min	a. 1 b. 2 c. 4	Fixated in 4 % paraformaldehyde for 4 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	A lot of background staining. A few nuclei stained, but difficult to quantify because of the background staining.
Anti – BrdU	a. 0 b. 1:50 c. 1:100 incubated over night at 4°C	2M HCl for 60 min at 37 °C.	6% PBT for 60 min	Dako EnVision++ for 30 min	BrdU injected 24 hours before sacrifice	48 hours in 4% paraformaldehyde, then 48 hours in sucrose solution. Dried for 12 hours.	The method was tested on rainbow trout to see if the species varied in results. The test gave a lot of background staining, even at the 0 control group. Some nuclear staining, but very weak compared to the background, so it was difficult to quantify.

Anti – BrdU	a. 0 b. 1:250 c. 1:500 incubated over night at 4°C	2M HCl for 60 min at 37 °C.	6% PBT for 60 min	Dako EnVision++ for 30 min	24	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Not much background staining, but also very little nuclear staining.
Anti – BrdU	a. 0 b. 1:200 c. 1:400 d. 1:600 incubated over night at room temperature	2M HCl for 60 min at 37 °C.	6% PBT for 60 min	Dako EnVision++ for 30 min	24	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Quite a lot of background staining in the sections with the 1:200 primary antibody concentrations, somewhat less at the other concentrations. Nuclear staining was weak, and difficult to quantify.
Anti – BrdU	a. 0 b. 1:200 c. 1:400 d. 1:600 incubated over night at room temperature	2M HCl for 60 min at 37 °C.	6% Milk powder for 60 min	Dako EnVision++ for 30 min	24	Fixated in 4 % paraformaldehyde for 24 hours, then over night in sucrose solution. After cutting, the sections were dried for 48 hours.	Some staining in nuclei and some background staining. Still not good enough for quantification.

Fixation with methacarn was also tested, but the sections were completely destroyed during cutting, so no immunohistochemistry was attempted.