SOCIAL STRESS REDUCES CELLULAR PROLIFERATION IN THE FOREBRAIN OF ZEBRAFISH (*Danio rerio*)

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Abstract

Many animals, including zebrafish (*Danio rerio*), form social hierarchies as a result of competition for limited resources. Socially subordinate fish experience chronic activation of the hypothalamic-pituitary-interrenal (HPI) axis, leading to prolonged elevation of plasma cortisol, the glucocorticoid end-product of HPI axis activation. Elevated cortisol levels can reduce cellular proliferation and neurogenesis in the brain. Thus, the present study tested the hypothesis that social stress suppresses cellular proliferation in the brain of subordinate zebrafish via a cortisol-mediated mechanism. Cellular proliferation was assessed using the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, as a marker. After 48 and 96 h of social interaction, significantly lower numbers of BrdU-positive cells were present in the forebrain of subordinate male zebrafish compared to dominant or control fish, suggesting a suppression of cellular proliferation in fish experiencing chronic social stress. Treatment of interacting male zebrafish with metyrapone, a cortisol synthesis inhibitor, attenuated the suppression of cellular proliferation in subordinate fish. Subordinate female zebrafish did not experience elevation of plasma cortisol or suppression of cellular proliferation in the forebrain. Collectively, these data provide evidence that cortisol plays a role in regulating cellular proliferation in the forebrain of male zebrafish during social interactions.

Résumé

Plusieurs animaux, y compris le poisson zèbre (*Danio rerio*), forment des hiérarchies sociales en conséquence des ressources limitées. Les poissons subordonnés sociales ont une activation chronique de l'axe hypothalamique-hypophysaire-interrénal (HHI), qui force à faire l'élévation prolongée du cortisol plasmique, le produit final des glucocorticoïdes de l'activation de l'axe HHI. Les niveaux élevés de cortisol peuvent réduire la prolifération cellulaire et la neurogenèse dans le cerveau. Ainsi, cette étude vise à tester l'hypothèse selon laquelle le stress social supprime la prolifération cellulaire dans le cerveau antérieur du poisson zèbre par un mécanisme impliquant le cortisol. La prolifération cellulaire a été évaluée en utilisant BrdU, un analogue de la thymidine et marqueur de la prolifération cellulaire. Après 48 and 96 heures d'interaction sociale, les poissons zèbres subordonnés masculins ont moins cellule BrdU-positif dans le cerveau antérieur par rapport aux poissons dominant. Ceci suggère une suppression de la prolifération cellulaire chez les poissons ayant un stress social chronique. Le traitement de metyrapone, un inhibiteur de la synthèse du cortisol, aux poissons zèbres masculin au cours d'interaction sociale a atténué la suppression de la prolifération cellulaire chez les poissons subordonnés. Les poissons zèbres féminin subordonnés n'ont pas une élévation de cortisol plasmique ni une suppression de prolifération cellulaire dans le cerveau antérieur. Collectivement, ces données démontrent que le cortisol joue un rôle dans la régulation de la prolifération cellulaire au cours des interactions sociales.

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List of abbreviations

5-HT	5-hydroxytryptamine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
$BrdU^+$	5-Bromo-2'-deoxyuridine-positive
BrdU	5-Bromo-2'-deoxyuridine
CRF	Corticotropin releasing factor
D	Dorsal telencephalic area
Dc	Central zone of dorsal telencephalic area
Dd	Dorsal zone of dorsal telencephalic area
DiV	Diencephalic ventricle
Dl	Lateral zone of dorsal telencephalic area
Dm	Medial zone of dorsal telencephalic area
Dom	Dominant
Dp	Posterior zone of dorsal telencephalic area
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPI	Hypothalamic-pituitary-interrenal
HSI	Hepatosomatic index
LX	Vagal lobe
MC2R	Melanocortin-2 receptor
Met	Metyrapone
MR	Mineralocorticoid receptor
NeuN	Neuronal nuclei
NPY	Neuropeptide Y
OB	Olfactory bulb

PCA	Principal components analysis
PGZ	Periventricular grey zone
PPa	Parvocellular preoptic nucleus, anterior part
PPn-C	Prepacemaker nucleus
PVZ	Periventricular zone
PCNA	Proliferating cell nuclear antigen
qPCR	Quantitative polymerase chain reaction
RGC	Radial glial cells
RM	Repeated measure
TeIV	Telencephalic ventricle
Sub	Subordinate
V	Ventral telencephalic area
Vd	Dorsal nucleus of ventral telencephalic area
Vp	Postcommissural nucleus of ventral telencephalic area
Vs	Supracommissural nucleus of ventral telencephalic area
Vv	Ventral nucleus of ventral telencephalic area

Chapter 1: Introduction

The extent to which the structure of the brain in an adult vertebrate can be influenced by the environment is an area of active research. Teleost fish retain the capacity for cellular proliferation and neurogenesis across the entire brain even as adults (Zupanc et al., 2005; Schmidt et al., 2013), whereas in mammals, the capacity to undergo neurogenesis exists in only a few, specific areas of the adult brain (Taupin, 2006;~~(Taupin, 2006;~~ Ming and Song, 2011). Thus, teleost fish provide an excellent opportunity to study structural plasticity in the brain, where plasticity may be defined as the brain's ability to respond to changes in the internal or external environment by adopting new phenotypes or restoring old phenotypes (Dennis et al., 2013). Stress has been linked to decreases in cellular proliferation and neurogenesis in mammalian brains (Gould et al., 1999; Tamashiro et al., 2007; Levone et al., 2015), but little work has been done in teleost fish to date. Thus, the purpose of the present thesis was to test the hypothesis that chronic stress and the associated elevation of circulating cortisol levels impair cellular proliferation and neurogenesis in socially subordinate zebrafish.

1.1 Cellular proliferation and neurogenesis

The idea of neurogenesis in adult mammals initially was met with skepticism, because neurogenesis was thought to be restricted to pre- and post-natal periods (Balu and Lucki, 2009). Evidence of neurogenesis was reported as early as 1912 by Ezra Allen, who observed specific phases of mitosis in albino rat brains (Allen, 1912). The use of [^{3}H]-thymidine as a marker for DNA synthesis provided additional evidence of neurogenesis in the brain of adult rats (Altman and Das, 1965). However, it was not until the development of 5-bromo-3'-deoxyuridine (BrdU), a thymidine analogue that is incorporated into nucleic acids during the synthesis phase of mitosis

(Taupin, 2007) and that can be visualized via immunohistochemistry and therefore can be visualized in conjunction with neuronal or glial markers, that neurogenesis in mammalian adult brains became widely accepted (Ming and Song, 2011).

The extent to which cellular proliferation and neurogenesis occur in the brain of adult vertebrates differs among vertebrate groups (Taupin, 2006; Ming and Song, 2011; Schmidt et al., 2013; Alunni and Bally-Cuif, 2016). A 'neurogenic zone' is a region that expresses immature precursor cells that allow for the growth and development of neurons. In mammals, there are two neurogenic zones that are widely accepted, the hippocampus (Cameron and Gould, 1994; Gould et al., 1998) and the olfactory system (Lois and Alvarez-Buylla, 1993). In contrast, teleost fish possess the capacity for neurogenesis in all parts of the brain (Grandel et al., 2006; Kizil et al., 2012; Schmidt et al., 2013). Neurogenic zones have been characterized in several fish species, including brown ghost knifefish (*Apteronotus leptorhynchus*; Zupanc and Horschke, 1995), medaka (*Oryzias latipes*; Kuroyanagi et al., 2010), several species of the genus *Austrolebias* (Fernández et al., 2011), and zebrafish (*Danio rerio*; Grandel et al., 2006; Lindsey et al., 2012). Neurogenic zones in zebrafish are of particular interest owing to widespread and increasing use of this species for biomedical research in neuroscience (Kalueff et al., 2014; Stewart et al., 2014).

With the use of BrdU, 16 neurogenic zones have been characterized in the brain of adult zebrafish, most of which are located in the forebrain or telencephalon (Grandel et al., 2006). These niches have been characterized and have been mapped in the brain (Fig. 1.1; Lindsey & Tropepe, 2012) The telencephalon develops by eversion (outward folding; Wullimann and Mueller, 2004; Folgueira et al., 2012), meaning that the periventricular zones (PVZ) are located on the outer portion of the telencephalon. The PVZ contain many proliferating cells in the brain

of adult zebrafish, with some zones having higher rates of cellular proliferation than others (Lindsey et al., 2012; Schmidt et al., 2013). The dorsal region of the telencephalon (D) as well as the ventral nucleus of the ventral telencephalon (Vv) exhibit the highest rates of proliferation (Grandel et al., 2006; Schmidt et al., 2013), with little proliferation being apparent in the posterior or central zones of the dorsal telencephalon (respectively, Dp and Dc), or in the supracommissural or postcommissural nuclei of the ventral telencephalon (respectively, Vs and Vp; Lindsey et al., 2012). The density of $BrdU^+$ cells can also be used to distinguish the boundaries of some zones. For example, dense populations of cells are usually found in the ventral (Vv) and dorsal (Vd) nuclei of the ventral telencephalon, whereas the D and medial zone of the dorsal telencephalon (Dm) exhibit equal distributions of proliferating cells (Lindsey et al., 2012). These proliferating cells can migrate and may differentiate into neurons (Grandel et al., 2006). The number of newborn neurons peaks about two weeks after cell birth, with some variation among brain regions in the rate at which proliferating cells differentiate into neurons (Lindsey et al., 2012).

Radial glial cells (RGC) are cell types that arise from the ectoderm and are comprised of actively mitotic cells in the brain (Barry et al., 2014). These cells are key progenitors for the formation of new neurons (Schmidt et al., 2013) and are able to self-renew as well as generate new neurons (Götz and Barde, 2005; Mori et al., 2005; Rothenaigner et al., 2011). The ability to retain RGCs is an embryonic feature; RGCs are found in the telencephalon of developing mammals but disappear after birth with the exception of the few areas of the brain that can undergo neurogenesis (Alvarez-Buylla and García-Verdugo, 2002; Doetsch, 2003). However, the brain of adult zebrafish appears to retain this embryonic feature (Schmidt et al., 2013) and RGCs in adult zebrafish exhibit expression of genes that is similar to that of embryonic RGCs,

including glial acidic fibrillar protein (GFAP; Chen et al., 2009) and the brain lipid-binding protein (Pellegrini et al., 2007). Most cells in the proliferation zones of zebrafish have been found to differentiate into neurons that express the marker Hu C/D (Grandel et al., 2006). The protein Hu is expressed exclusively in neurons and because it does not discriminate between neuron subtypes, it is an attractive marker for neurons (e.g. known as 'pan-neuronal' marker; Desmet et al., 2014). Progenitor cells in the brain of adult zebrafish have the ability to generate multiple types of neurons, with neurons expressing histamine, tyrosine hydroxylase, or serotonin (5-hydroxytryptamine, 5-HT) having been detected (Kaslin and Panula, 2001). This situation contrasts with that in mammals, where only a few subtypes of neurons are produced in neurogenic zones of the adult brain (Grandel et al., 2006).

1.2 Social stress and its influence on the brain

The capacity for neurogenesis in the brain of adult teleost fish raises questions about the factors that may influence rates of cellular proliferation and neurogenesis, and the mechanisms through which such factors are linked to changes in cellular proliferation and neurogenesis. To date, several studies have investigated environmental impacts on cellular proliferation and neurogenesis in the brain of adult teleost fish. Adult male electric fish (*Brachyhypopomus gauderio*) exhibited higher levels of cellular proliferation during the breeding season (Dunlap et al., 2011a). Additionally, during the mating season, electric fish held in groups exhibited higher cellular proliferation than fish held in isolation within the brain region responsible for electrocommunication (Dunlap et al., 2011a). These data suggest an impact of the social environment experienced by the fish on cellular proliferation and neurogenesis. This possibility is supported by data for another species of weakly electric fish, brown ghost knifefish. When

knifefish were held with a conspecific for 7 days, they exhibited higher levels of cellular proliferation than isolated fish in the PVZ adjacent to the diencephalic prepacemaker nucleus (PPn-C), the area in the brain responsible for electrocommunication (Dunlap et al., 2006). Similarly, zebrafish that were held in isolation for two weeks exhibited lower levels of cellular proliferation in the periventricular grey zone (PGZ) compared to fish housed in a group (Lindsey and Tropepe, 2014). Interestingly, two weeks exposure to social novelty, where fish were introduced to an unfamiliar group of male and female zebrafish, also led to a trend of decreased cellular proliferation in the PGZ, vagal lobe (LX) and the olfactory bulb (OB; Lindsey and Tropepe, 2014). These data suggest that while social interactions with a familiar group of conspecifics promote cellular proliferation and neurogenesis, social interactions that feature an element of stress, e.g. social novelty, may inhibit cellular proliferation and neurogenesis.

Many species of fish establish social hierarchies as a result of competition for limited resources. For example, juvenile salmonids form social hierarchies through competition for feeding territories (Gilmour et al., 2005), and adult males of the cichlid *Astatotilapia burtoni* form social hierarchies as a result of competition for mating opportunities (Fox et al., 1997). These social hierarchies often form through agonistic interactions, with winners and losers, or socially dominant versus socially subordinate individuals exhibiting behavioural and physiological differences (Johnsson et al., 2006; Sorensen et al., 2014). Dominant fish monopolize limited resources, typically space and food in experimental settings, whereas subordinate fish are less active, retreat from aggression and may be excluded from feeding opportunities (Abbott and Dunbrack, 1985; Noakes and Leatherland, 1977; Sloman and Armstrong, 2002). In *A. burtoni*, dominant or territorial males are able to monopolize food and territory as well as reproductive opportunities with females (Fernald and Maruska, 2012).

Interestingly, these dominant males can supress reproduction of subordinate or non-territorial males, but the social status of an individual male can quickly change (Burmeister et al., 2005; Maruska, 2014). When a non-territorial male is given the opportunity to occupy a new territory it transforms physiologically to become capable of reproduction (Maruska, 2014).

Zebrafish, like many other fish species, form social hierarchies under certain conditions (Saverino and Gerlai, 2008; Dahlbom et al., 2011a; Roy and Bhat, 2015; Suriyampola et al., 2015). When confined in a tank with a conspecific, zebrafish display a range of aggressive social behaviours that escalate in intensity until one fish wins the encounter, becoming dominant over the losing fish (Paull et al., 2010; Oliveira et al., 2011). Social interactions begin with the fish erecting their fins at the sight of a conspecific. Strikes consisting of chases and bites are given by both fish until one fish retreats and no longer directs aggressive acts towards the other, which becomes the dominant fish (Oliveira et al., 2011). Even after hierarchy establishment, dominant fish continue to engage in behaviours such as aggressive acts, patrolling the tank and monopolizing food, whereas subordinate zebrafish retreat from the dominant fish, stay immobile at the bottom of the tank, and feed last (Oliveira et al., 2011). Males appear to be more aggressive than females, with dominant and subordinate males differing in physiological condition as well as behaviour (Filby et al., 2010b). For example, dominant males had a positive change in body weight during social interactions, and a higher hepatosomatic index (HSI), a measure of liver energy reserves, than subordinate fish after 5 days of interaction (Filby et al., 2010b).

In addition, low social status frequently is associated with activation of the hypothalamic-pituitary-interrenal (HPI) or stress axis and resultant elevation of plasma concentrations of the glucocorticoid stress hormone cortisol, suggesting that low social status serves as a chronic

stressor (Øverli et al., 1999; Sloman et al., 2001). For example, subordinate salmonids exhibit prolonged elevation of circulating cortisol levels, which in turn is thought to contribute to negative consequences associated with chronic social stress such as low growth rates (Sloman et al., 2001; Gilmour et al., 2005; Jeffrey et al., 2014; Culbert and Gilmour, 2016). In zebrafish, social interactions resulted in elevated plasma cortisol concentrations in subordinate compared to dominant fish (Filby et al., 2010b), and zebrafish engaging in social interactions exhibited elevated trunk cortisol levels in comparison to fish held in isolation, where 'trunk cortisol' refers to tissue cortisol levels for the trunk of the zebrafish after severance of the head (Pavlidis et al., 2011). The effects of social interactions on cortisol levels appear to be more subtle in zebrafish than in salmonid fish. For example, Filby et al., (2010b) reported differences of ~40 ng mL^{-1} between plasma cortisol concentrations in dominant and subordinate fish, whereas circulating cortisol levels in subordinate salmonids typically are ~100 ng mL^{-1} as compared to values of <10 ng mL^{-1} (the unstressed value; reviewed by Gamperl et al., 1994) in dominant fish (Jeffrey et al., 2014; Culbert and Gilmour, 2016).

Social stress also influenced brain monoaminergic activity in zebrafish (Dahlbom et al., 2011). Specifically, subordinates exhibited higher serotonergic activity (5HIAA:5-HT, where 5HIAA is 5-hydroxyindoleacetic acid, the metabolite of serotonin) than dominant fish in the hindbrain. Similar effects have been observed in dominance hierarchies in other fish species. For example, in the cichlid *A. burtoni*, subordinates exhibited higher serotonergic activity than dominants in several brain regions after 5 weeks of interaction (Loveland et al., 2014). Elevated serotonergic activity also is characteristic of subordinate salmonids including Arctic charr *Salvelinus alpinus* (Winberg and Nilsson, 1993) and rainbow trout *Oncorhynchus mykiss* (Øverli et al., 1999). In addition to changes in monoaminergic activity in the brains of fish that have

engaged in social interactions, changes in gene expression have been reported. Filby et al., (2010a) reported altered transcript abundance for genes linked to aggression in the brains of male zebrafish after one day of social interaction. Interestingly, several of these genes are implicated in cellular proliferation and neurogenesis in mammals, including *npy* (Decressac et al., 2011; Geloso et al., 2015), *crf* (Koutmani et al., 2013) and oxytocin-like (oxytocin in mammals; Leuner et al., 2012), suggesting possible mechanisms through which cellular proliferation and neurogenesis may be influenced by chronic social stress.

Chronic elevation of cortisol also has been associated with reduced cellular proliferation in the brains of mammals, and more recently, fish. In mammals, mice subjected to a social defeat paradigm (repeated aggressive attacks by a conspecific over several days) exhibited reduced cellular proliferation in the dentate gyrus (Yap et al., 2006). Similarly, rats exposed to social defeat for 18 days experienced reduced cellular proliferation and neurogenesis, together with significant elevation of circulating corticosterone, the main glucocorticoid stress hormone in rodents (Czéh et al., 2002), suggesting a specific role for cortisol in inhibiting cellular proliferation during social defeat. Correspondingly, treatment of rats with corticosterone reduced cellular proliferation (Cameron and Gould, 1994) in the dentate gyrus and also reduced the survival of neurons produced by cellular proliferation (Wong and Herbert, 2004). Removal of corticosterone by adrenalectomy increased neuronal birth (Cameron and Gould, 1994), but also led to low survival of neurons, indicating that glucocorticoids are needed for neuron survival (Wong and Herbert, 2004). Similarly, in fish, socially subordinate rainbow trout exhibited reduced cellular proliferation in the telencephalon after 4 days (Sørensen et al., 2012), and the same trend was seen when trout were treated with cortisol (Sørensen et al., 2011). In the cichlid *A. burtoni*, socially supressed males exhibited reduced cellular proliferation compared to

dominant fish and males that were given the opportunity to ascend after the dominant fish was removed (Maruska et al., 2012). Socially suppressed males also experience elevated cortisol levels (Maruska et al., 2013). By contrast, exposure of the weakly electric fish, *B. gauderio* and *A. leptorhynchus* to a simulated predatory stimulus reduced cellular proliferation; however cortisol was not elevated (Dunlap et al., 2017). These data indicate that the link between glucocorticoids and cellular proliferation/neurogenesis is not simple. Similarly, exercise in mice activated the hypothalamic-pituitary-adrenal (HPA) axis while at the same time increasing cellular proliferation and neurogenesis (van Praag et al., 1999). The mechanisms linking cellular proliferation and neurogenesis with HPA axis activity remain under investigation in mammals (reviewed by Levone et al., 2015; Srikumar et al., 2011).

1.3 Hypothesis

With this background in mind, the present study tested the hypothesis that social interactions in zebrafish inhibit cellular proliferation and neurogenesis in the forebrain of subordinate zebrafish through a cortisol-mediated mechanism. Thus, subordinate zebrafish would be predicted to exhibit chronically elevated circulating cortisol concentrations, and lower rates of cellular proliferation and neurogenesis compared to dominant fish. Cellular proliferation was visualized and quantified using the BrdU approach. To investigate the role of cortisol specifically in social stress-induced changes in cell proliferation, the cortisol synthesis inhibitor metyrapone was used. Effects of chronic social stress on cellular proliferation would be predicted to be eliminated in zebrafish treated with metyrapone to prevent elevation of circulating cortisol levels during social interactions.

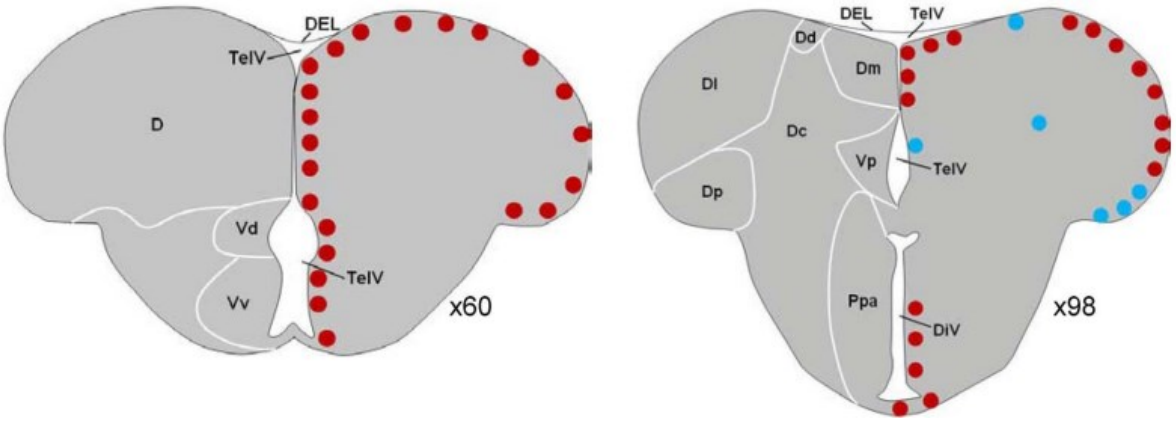


Figure 1.1 Schematic indicating proliferating zones in the forebrain of zebrafish (modified from Lindsey et al., 2012). These areas are characterized as niches where cellular proliferation occurs. The red dots indicate areas of higher density of BrdU-positive cells compared to regions with blue dots. *D*, Dorsal telencephalic area, *Vd*, dorsal nucleus of ventral telencephalic area, *Vv*, ventral nucleus of ventral telencephalic area, *Ppa*, parvocellular preoptic nucleus, anterior part, *Vp*, postcommissural nucleus of ventral telencephalic area, *Dm*, medial zone of D, *Dd*, dorsal zone of D, *Dl*, lateral zone of D, *Dp*, posterior zone of D, *Dc*, central zone of D, *DiV*, diencephalic ventricle, *TeIV*, telencephalic ventricle

Chapter 2: Materials and methods

2.1 Experimental animals

Male and female zebrafish (*Danio rerio*) 6-12 months in age were obtained from an in-house breeding program in the aquatic animals facility of the University of Ottawa. Fish were housed in 5 or 10 L polycarbonate tanks with a constant supply of aerated, dechloraminated city of Ottawa tap water at 28.5°C. A photoperiod of 14L:10D was used. Zebrafish were fed three times per day with a mixture of equal parts of three foods; Zeigler zebrafish diet, Brine Shrimp Direct Golden pearls (300-500 μm) diet, and Zeigler AP 100 5 diet (250-400 μM), with an additional serving of brine shrimp daily. Zebrafish were acclimated to these holding conditions for at least one week before any experimentation was performed. All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care (CCAC) for the use of animals in research and teaching, and after approval of the University of Ottawa Animal Care Committee (protocol BL-2118).

Experiments were carried out on dominant and subordinate zebrafish that were obtained by housing fish in pairs to establish a social hierarchy. Fish were lightly anaesthetised by immersion in a buffered solution of MS-222 (0.24 mg mL^{-1} 3-aminobenzoic acid ethyl ester; 21 mM Tris, pH 7; Sigma-Aldrich; Westerfield, 2000) and mass and fork length were recorded. Each fish was given a unique mark using alcian blue (Sigma-Aldrich) for identification (Jeffrey and Gilmour, 2016). The fish within a pair were of the same sex, size-matched for length (dominant mean length 3.2 ± 0.05 cm, subordinate 3.2 ± 0.05 cm; mean difference in length within a pair 0.004 ± 0.005) and mass (dominant mean mass 0.2973 ±0.0160 g, subordinate 0.2932 ± 0.0176 g, mean difference in mass within a pair 0.02 ± 0.008 g) and obtained from different holding tanks to avoid prior exposure to each other. The members of a pair were placed in an experimental

chamber (4.5 L) separated by an opaque divider for a 24 h recovery and isolation period. A 24 h isolation period was selected based on pilot trials (see below) to ensure strong interactions within a pair (Larson et al., 2006; Oliveira et al., 2016). Following the 24 h recovery and isolation period, the divider was removed and the fish were allowed to interact, typically for 48 or 96 h although other interaction periods were used in some trials. The time required for hierarchy formation was recorded, i.e. the time between removal of the divider and establishment of a hierarchy based on one fish ceasing to initiate aggressive behaviour and instead repeatedly retreating from the other fish. Pairs were observed twice a day for 2 min each time; observation periods occurred at 1 pm and 5 pm. A scoring system similar to that used in other studies (e.g. Filby et al., 2010; Paull et al., 2010; Dahlbom et al., 2011) was used to assess the strength of the hierarchy and assign social status. Dominant behaviours such as biting, chasing, patrolling and feeding were given high scores, whereas submissive behaviours such as retreats, multiple bouts of freezing and absence of feeding were given low scores (Table 2.1). Acts of aggression and retreats were counted over the 2 min observation period, and position in the tank was assessed at the end of the observation period. Finally, a pellet of food was placed in the tank to determine which fish fed first. Fish were pinch-fed after every observation period. Scores for specific behaviours were weighted according to their reliability in indicating social status [e.g. dominant fish always patrol (10 points) and are aggressive (5 points) whereas subordinates exhibit little movement; however, subordinates occasionally will take food first, so this behaviour is weighted at only 1 point]. Scores for specific behaviours were averaged over the observation periods for each fish and the mean was used in a principle components analysis (PCA) to generate behaviour scores. The fish with the higher score within a pair was assigned dominant status, and the fish with the lower score was assigned subordinate status. Pairs that did not exhibit divergent

behaviour scores were removed from subsequent analyses (18 of 127 pairs). Studies of social interactions frequently use sham-treated fish as a control group, i.e. fish that are handled in the same way as fish paired with a conspecific, but held in isolation (Jeffrey and Gilmour, 2016). However, holding fish in isolation was found to be a stressor for zebrafish (Pagnussat et al., 2013) and also resulted in reduced cellular proliferation (Lindsey and Tropepe, 2014). Therefore, fish housed in a stable group were chosen as the control for the present study (Lindsey and Tropepe, 2014).

2.2 Experimental protocols

Four series of experiments were carried out. First, social hierarchies in pairs of zebrafish were characterized in terms of their behaviour and selected physiological variables, including circulating cortisol concentrations. A second experiment investigated the consequences of social hierarchy formation for cellular proliferation, using the BrdU approach. To determine whether neurogenesis was affected by social interaction, a third experiment was carried out in which fish that had been held in pairs for 48 h of interaction were returned to group-holding conditions for two weeks to allow newly formed cells to differentiate. Finally, pairs of zebrafish were exposed to metyrapone to establish a causal link between cortisol and effects of social interaction on cellular proliferation. Male and female zebrafish (in separate experiments) were used for series 1 and 2, whereas only male fish were used in series 3 and 4. Experiments on female zebrafish were carried out in conjunction with S. McDonald and are reported in part in McDonald (2017).

2.2.1 Series 1: Characterization of social stress

An initial set of experiments was used to characterize the behaviour and cortisol levels of zebrafish during social interactions to establish an appropriate protocol for subsequent experiments. Using the general approach described above, male zebrafish were held in pairs for 1 (N = 7 pairs), 2 (N = 7), 4 (N = 8), 8 (N = 8), 24 (N = 11), 48 (N = 12), 96 (N = 8) or 120 h (N = 8). Observations were carried out at the times indicated in Table 2.2. Following the interaction period, fish were euthanized with MS-222 (as described above, but using a concentration of 0.72 mg mL^{-1}), and the trunk was flash frozen in liquid nitrogen and stored at -80°C for later measurement of trunk cortisol concentrations. In addition, two feeding regimes were tested. Pairs were allowed to interact for 80 h and were fed either by adding food to the tank until both fish were satiated (N = 11) or by adding the food in small pinches until the dominant fish stopped feeding (N = 10).

To measure trunk cortisol concentrations, the trunk was ground to powder under liquid nitrogen with a mortar and pestle, and cortisol was extracted using the method of Folch et al., (1957) as modified by Al-Habsi et al., (2016). Briefly, powdered tissue was homogenized in 15 mL of 2:1 chloroform:methanol and incubated at room temperature for 15 min. Next, 5 mL of a solution of 2 M KCl and 5 mM EDTA was added, and the samples were vortexed and incubated at room temperature for 20 min. The bottom layer was extracted into a glass tube and exposed to a nitrogen stream at 50°C until the liquid had evaporated. The resulting lipids were re-suspended in 0.2 mL of ethylene glycol monomethyl ether (EGME; Sigma-Aldrich) and stored at -80°C until analysis of cortisol concentrations using a commercially available radioimmunoassay (RIA) kit (MP Biomedical). Addition of [^{3}H]-hydrocortisone to homogenized samples yielded an

extraction efficiency of 87% (Marilyn Vera Chang, personal communication). The intra- and inter-assay coefficients of variation were 9.7% and 16%, respectively.

Owing to variability in trunk cortisol concentrations (see Results), subsequent experiments focused on measurement of plasma cortisol concentrations. Blood and extracellular fluid were collected using the method of Babei et al., (2013). In brief, zebrafish were euthanized as described above. The caudal fin was severed using a scalpel dipped in saline containing 500 IU heparin (Sigma Aldrich), and the fish was placed in a small 0.5 mL microcentrifuge tube, the bottom of which had been perforated using a needle. This tube was placed in a 1.5 mL microcentrifuge tube containing 10 µL of heparinized (500 IU) saline and centrifuged at 40 *g* for 5 minutes at 11°C. The fish was removed, the trunk was sectioned immediately rostral to the previous incision, and the fish was replaced in the collection tube and centrifuged one more time. The blood collected in this fashion was centrifuged at 13,800 *g* for 15 min at 4°C. The plasma was extracted and stored at -80°C until analyzed for cortisol concentration with a commercially-available enzyme-linked immunoassay (EIA; Neogen). The intra assay coefficient of variability was 3.37% and all samples within a given experiment were analyzed in a single assay.

To confirm that a stress-induced elevation of plasma cortisol concentrations could be detected with this approach to blood collection, fish were randomly allocated into two tanks and allowed to recover overnight. Fish in one tank were euthanized and plasma was collected for the measurement of baseline cortisol concentrations. Fish in the second group were subjected to an acute air-exposure stressor consisting of 3 min in a net held in air, 3 min of recovery in the holding tank, and 3 min in a net held in air (Ramsay et al., 2009). After 5 min of recovery in the holding tank, fish were euthanized and plasma was collected. Analysis of these plasma samples revealed baseline cortisol concentrations of 16.4 ± 2.0 ng mL^{-1}, increasing significantly

(Student's *t*-test, $P = 0.005$) to 130.6 ± 27.5 ng mL^{-1} in fish exposed to the stressor. Following this successful validation trial, plasma cortisol concentrations were assessed in pairs of male zebrafish that had been allowed to interact for 48 ($N = 7$) or 96 h ($N = 8$), in pairs of female zebrafish that had interacted for 96 h ($N = 9$), and in control fish sampled from a holding tank ($N = 7$ male and 6 female fish).

2.2.2 Series 2: Characterization of cellular proliferation during social stress

Cellular proliferation in the forebrain was assessed using a single pulse of BrdU in pairs of male zebrafish that had been allowed to interact for 48 ($N = 4$) or 96 h ($N = 4$), and in pairs of female zebrafish that had interacted for 96 h ($N = 4$). Control experiments utilized fish sampled from a holding tank ($N = 4$ male and 4 female fish). BrdU was administered 24 h prior to the end of the interaction period using the procedure described by Lindsey et al., (2012). In brief, the fish in each pair were lightly anaesthetised as described above and given a 10 mM bolus of BrdU (Sigma Aldrich) via intraperitoneal injection at a volume of 50 μL g^{-1}. Pairs were returned to their tank and allowed to recover for an hour with the divider in place before removing the divider for the remainder of the interaction period. At the end of the 48 or 96 h interaction period, pairs were euthanized as described above. Control fish were euthanized from their holding tanks. The brains were removed and placed in 4% paraformaldehyde (PFA) overnight at 4°C; all subsequent processing also was carried out at 4°C. The fixed brains were washed with 1x phosphate-buffered saline (PBS), immersed in 30% sucrose overnight and transferred to optimal cutting temperature (OCT; Thermo Fisher) compound for 45 min. The brains were then mounted in a mould in OCT and cryosectioned (Leica CM3050s) at 20 μm intervals. Sections

were collected onto slides (Thermo Fisher), dried overnight in an incubator at 4°C and stored at -20°C until immunohistochemistry was performed.

For immunohistochemistry, slides were allowed to thaw at room temperature and sections were rehydrated by immersion in (autoclaved) 1x PBS twice for 5 min each time. For antigen retrieval, the slides were then immersed in 10 mM sodium citrate containing 0.05% Tween (pH 6) at 65°C for 30 min. The slides were allowed to cool to room temperature and were then exposed to 2N HCl at 37°C for 15 min. This reaction was quenched twice with 0.1 M borate buffer (pH 8.5; Fisher Scientific) for 5 min each time. Permeabilization was achieved with three treatments of 1x PBS containing 0.05% Triton X-100 (Sigma Aldrich) for 5 min each time. To block non-specific binding, sections were exposed to 1% skim milk powder in 1x PBS containing 0.05% Triton X-100 for 45 min at room temperature. Sections were incubated in a 1:100 dilution of primary mouse anti-BrdU antibody (Developmental Studies Hybridoma Bank) in 1% skim milk powder in 1x PBS overnight at 4°C. This antibody was developed by S.J. Kaufman at the University of Illinois and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa (Department of Biology, Iowa City, IA 52242). Following incubation with the primary antibody, sections were washed with 1x PBS three times for 10 min each time on a shaker, and then incubated with a 1:300 dilution of donkey anti-mouse IgG coupled to Alexa fluor 488 (Invitrogen) for 3 h at room temperature. Sections were washed 3 times for 5 min each time with 1x PBS and mounted using an antifade medium containing the nuclear marker 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Vectashield Laboratories).

Imaging of brain sections was achieved using a Nikon A1RsiMP confocal microscope. Every third section through the rostrocaudal axis of the forebrain was examined, with areas of

the forebrain being distinguished by comparing sections stained with cresyl violet (Sigma) to a neuroanatomical atlas of the zebrafish brain (Wullimann et al., 1996). Within each brain region, all cells exhibiting BrdU immunofluorescence ($BrdU^+$ cells) were counted from a z-stack image created from 20 μm optical sections at 20x magnification. Cells were identified by the co-localization of DAPI staining. The number of $BrdU^+$ cells was normalized to the total number of cells based on DAPI staining. A circle of 4,000 μm^2 was positioned in a representative area of each brain region to count DAPI-stained cells using the optical dissector principle (West, 1999) and was kept in the same place for every section. The ratio of $BrdU^+$ cells to total cells within the counting box was calculated for each forebrain region in each fish. Cell counts were carried out by an observer who was blind to the treatment group to which the fish belonged using the NIS Elements Advanced Research Imaging Software, Version 4.20.

2.2.3 Series 3: Characterization of neuron differentiation during social stress

To determine the fate of progenitor cells labelled with BrdU during social interactions, a separate experiment was carried out on male zebrafish that were confined in pairs for 48 h (N = 4). Establishment of pairs and administration of BrdU was carried out as described above, but after the 48 h social interaction period, fish were returned to their original holding tanks for two weeks. This holding period was chosen because Lindsey et al. (2012) reported that neuronal differentiation peaked two weeks after BrdU treatment. Pairs were euthanized with a lethal dose of MS-222 two weeks after being returned to their holding tanks and brains were collected and processed as described above. In addition to immunofluorescent detection of BrdU, sections were processed for immunofluorescent detection of the neuronal nuclear protein NeuN as a

marker of neuronal differentiation (Gusel'nikova and Korzhevskiy, 2015); NeuN has been used as a marker for mature neurons in zebrafish (Arslan-Ergul et al., 2016). Sections were incubated with mouse anti-BrdU as described above and a 1:1000 dilution of rabbit anti-NeuN (Abcam). Donkey anti-rabbit IgG coupled to Alexa fluor 633 (1:700 dilution, Invitrogen) was used to label rabbit anti-NeuN. Sections were examined as described above, and $BrdU^+$ cells as well as cells that exhibited co-localization of BrdU and NeuN were counted. The percentage of $BrdU^+$ cells that also expressed NeuN was used as an index of neurogenesis.

2.2.4 Series 4: Metyrapone treatment

To examine the role of cortisol in forebrain cell proliferation during social interactions, administration of metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) was used to inhibit cortisol synthesis. Metyrapone inhibits 11ß-hydroxylase, the enzyme that catalyzes the conversion of 11-deoxycortisol to cortisol in the final step in cortisol synthesis, and has been used to block cortisol synthesis in several fish species (see Mommsen et al., 1999). An initial set of experiments focused on developing a non-invasive protocol for metyrapone administration in zebrafish. Metyrapone was administered orally using the approach of Bennett and Rhodes (1986). Metyrapone (VWR/Enzo life science) dissolved in ethanol was sprayed over the food (same type of food as above) and ethanol was allowed to evaporate for 3 days. Control food was sprayed with ethanol alone. Male fish ($N = 6$ in each group) were randomly allocated into 'baseline' and 'stressed' tanks and fed food treated with 0 (control), 0.01, 0.03, 0.050, 0.1, 0.500 or 1 mg g^{-1} metyrapone (Bennett et al., 1986). Fish were fed 5% of their body weight 3 times a day for 7 days; to accustom the fish to this feeding regime, all tanks were fed regular (untreated)

zebrafish food for 6 days prior to commencing treated food. After the 7-day exposure period, fish in 'baseline' tanks were euthanized for the measurement of baseline plasma cortisol concentrations as described above. Fish in 'stressed' tanks were exposed to the acute netting/air-exposure stressor developed by Ramsay et al., (2009), as described above. Fish were euthanized and blood was collected for the measurement of plasma cortisol concentrations as described above.

Because effective blockade of the cortisol response to an acute netting stressor was not achieved with oral administration of even the highest dose of metyrapone, which was 20 times higher than the effective dose in rainbow trout (Bennett et al., 1986), a waterborne exposure treatment developed by Marilyn Vera Chang (personal communication) was tested. Male fish (*N* = 6) were randomly allocated into 'baseline' or 'stress-induced' metyrapone exposure groups which were held in static tanks. Water changes (100%) were carried out every 24h and all tanks were covered to reduce degradation of metyrapone by light exposure. After an overnight acclimation period, water in treatment tanks was replaced with 325.4 μM metyrapone (Marilyn Vera Chang, personal communication) in system water. Fish were exposed to these conditions for 6 days, after which all fish in the baseline group were immediately euthanized and the stress-induced group were exposed to the acute stressor of Ramsay et al., (2009) and then euthanized. Blood was collected for the measurement of plasma cortisol concentrations as described above. To assess the minimum length of metyrapone exposure required to achieve effective blockade, 12 male fish were randomly allocated into either a baseline or stress-induced group with metyrapone-exposure (325.4 μM). Two fish from each of the baseline and stress-induced groups were sampled at two, four and six days of exposure. Sampled fish were exposed to the acute

stressor of Ramsey et al. (2009) and then euthanized for collection of blood and measurement of plasma cortisol concentrations as described above.

Based on these initial experiments, waterborne exposure to metyrapone was selected as an effective administration protocol. Two further trials were carried out using this approach. For both trials, male zebrafish were randomly allocated to two control and two metyrapone-exposure groups and were exposed to system water or 325.4 μM metyrapone as described above. After 4 days, pairs of length- and mass-matched fish were transferred into experimental chambers (1.5 L) containing control water or water containing 325.4 μM metyrapone, as appropriate, and with the members of the pair being separated by a divider for 24 h. The divider was then removed and the fish were allowed to interact for 48 h, with behavioural observations being carried out as described above. In one trial ($N = 7$ control and $N = 2$ metyrapone-exposed pairs), fish were euthanized after 48 h of interaction for the collection of blood samples and the measurement of plasma cortisol concentrations. In the second trial ($N = 4$ control and $N = 3$ metyrapone-exposed pairs), BrdU was administered after 24 h of interaction and fish were euthanized after 48 h of interaction for the collection of brain tissue and the assessment of forebrain cell proliferation in subordinate fish.

2.3 Statistical analyses

Statistical analyses were carried out using R (version R 3.1.1; R Core Team, 2013) with the exception of principle components analyses, which were conducted using SigmaPlot (v13.0, Systat). Behavioural analyses were carried out using paired Student's *t*-tests or one-way analysis of variance (ANOVA) with repeated measures (RM), as appropriate. Where significant

differences were detected by ANOVA, post hoc multiple comparisons testing was carried out using the Holm-Sidak method. Specific growth rate (SGR) was calculated for interaction periods of 24 h or more as SGR = $[\ln(m_{Final}) - \ln(m_{Initial})] \times 100/D$, where m is the mass of the fish in grams and D is the number of days that elapsed between measurements of mass. Effects of social status and length of the interaction period on specific growth rate and trunk cortisol concentrations were assessed using a two-way RM ANOVA. Plasma cortisol concentrations between dominant and subordinate fish were compared using a paired Student's *t*-test, and comparisons with control fish were carried out using ANOVA. Analyses of cell counts were performed using two-way ANOVA with repeated measures, with the factors being social status and brain region. Student's *t*-tests were used to compare baseline and stress-induced cortisol concentrations, as well as cell counts, in fish treated with metyrapone. In general, comparisons between dominant and subordinate fish were carried out using a paired approach to take into account the likelihood that variables measured for the opponents in an antagonistic interaction are not independent of one another (Briffa and Elwood, 2010). Data are reported as means ± SEM and the fiducial limit of significance for all tests was 0.05. Prior to statistical analysis, data were tested for normality using the Shapiro Wilks test and equal variance using the studentized Breusch-Pagan test. Where data did not meet the assumptions of normality or equal variance, they were transformed prior to analysis.

Table 2.1 Scoring system used to assign points to interacting zebrafish (*Danio rerio*).

Behaviour	Activity/number of acts	Scoring
Position	Patrolling	10 points
	Little movement	5 points
	No movement	0 point
Aggression	0	0 point
	1-5	1 point
	6-10	2 points
	11-15	3 points
	15-20	4 points
	>20	5 points
Retreats	1-4	2 points
	4-10	1 point
	>10	0 point
Feeding	Fed first	1 point
	Fed second	0 point

Table 2.2: Times at which behavioural observations were carried out for zebrafish (*Danio rerio*) held in pairs for interaction periods of varying length. All pairs were observed from removal of the divider to establishment of a hierarchy.

Interaction period	Timing of behavioural observations
1 h	30 min, 1 h
2 h	30 min, 1 h, 1.5h, 2h
4 h	1 h, 2 h, 3 h, 4 h
8 h	2 h, 4 h, 6 h, 8 h
24 h	6 h, 24 h
48 h	6 h, 24 h, 30 h, 48 h
96 h	6 h, 24 h, 30 h, 48 h, 54 h, 72 h, 78 h, 96 h
120 h	6 h, 24 h, 30 h, 48 h, 54 h, 72 h, 78 h, 96 h, 102 h, 120 h

Chapter 3: Results

3.1.1 Series 1: The formation of social hierarchies in pairs of male or female zebrafish

Male or female zebrafish confined with a same-sex conspecific in a behaviour chamber engaged in agonistic interactions that resulted in one fish becoming dominant over its tank mate, as indicated by one fish reliably retreating from the aggressive attacks of the other. Using as a criterion of hierarchy formation the regular appearance of retreats, pairs of male fish required significantly more time to establish a hierarchy than did pairs of female fish (Fig. 1A; Student's *t*-test, $P = 0.0254$). The time that fish were isolated prior to removal of the divider between a pair of fish affected aggressiveness, at least in male fish (Fig. 1B). Dominant male fish were significantly more aggressive following 24 h of isolation compared to 15 h of isolation (Student's *t*-test; $P = 0.0337$). Consequently, subsequent experiments employed a 24 h isolation period prior to removal of the divider between a pair of fish. Aggressive behaviour during feeding was also favoured by a pinch feeding strategy to the point of satiation of the dominant fish, instead of feeding both fish ad lib. To quantify the strength of the interaction within a pair, fish were scored for four behaviours that commonly diverge between dominant and subordinate fish, position in the tank, aggressive behaviours, retreats, and willingness to take a piece of food. Scores for these behaviours were significantly divergent within the members of a pair in male fish after 48 (Fig. 1A-D) or 96 h of interaction (Fig. 1E-H), and in females after 96 h of interaction (Fig. 3). In addition, behaviours such as patrolling, aggressive acts and feeding were robustly maintained by the dominant fish throughout the interaction period (Fig. 4). For example, dominant females displayed no statistically significant differences in any behaviour (one-way RM ANOVA, position $P = 0.109$, aggression $P = 0.728$, retreat $P = 0.120$ and feeding $P = 0.596$) during the 96 h interaction period. In dominant male fish, acts of aggression were significantly higher in the initial observation period (one-way RM ANOVA, $P < 0.001$) and some variation in

feeding scores was detected ($P = 0.031$) although post hoc tests could not detect the origin of this variation, whereas position ($P = 0.433$) and retreats ($P = 0.433$) did not differ across a 96 h interaction period. Therefore, subsequent experiments used behaviour scores derived from a principal components analysis of mean scores over the interaction period for position in the tank, aggressive acts, retreats and feeding behaviour to identify dominant and subordinate fish and characterize the strength of the hierarchy between them.

To determine whether behavioural interactions had physiological consequences for dominant versus subordinate fish, trunk cortisol concentrations and specific growth rates were determined for male zebrafish confined in pairs for times ranging from 1 to 120 h (Fig. 5). Overall, there was no significant effect of social status (two-way RM ANOVA, $P = 0.214$, status x time of interaction $P = 0.209$), however there was an effect of the length of interaction ($P < 0.001$). There is also an effect of status when comparing trunk cortisol concentration of dominant and subordinates to a control group ($P = <0.001$). Owing to concerns about generating consistent trunk samples and inconsistencies between trials in whether dominant and subordinate fish differed in trunk cortisol concentrations, in subsequent experiments, blood was collected for the measurement of plasma cortisol concentrations. With this approach, cortisol concentrations were significantly different between dominant and subordinate male zebrafish at 48 h and 96 h of interaction (Fig. 6; paired Student's *t*-tests, $P = 0.0379$ for 48h and $P = 0.0461$ for 96 h), but no differences were detected between dominant and subordinate females after 96 h of interaction (paired Student's *t*-test, $P = 0.377$), despite the existence of behavioural differences (Fig. 6A). Comparing cortisol concentrations in fish that had experienced social interactions with those of control fish sampled directly from the holding tank, a statistically significant difference was detected at 96 h (one-way ANOVA, $P = <0.001$), where cortisol levels in male subordinates

differed from control values. Dominant and subordinate male zebrafish also differed in specific growth rate at 48 h, 80 h, and 96 h but not 120 h of interaction (Fig. 5B; two-way RM ANOVA; length of interaction period $P = 0.512$, social status $P < 0.001$, length of interaction x status $P < 0.001$). No significant difference was detected between the specific growth rates of dominant and subordinate female zebrafish (Wilcoxon signed rank test, $P = 0.2402$).

3.1.2 Series 2: The impact of social status on cellular proliferation

Immunohistochemical localization of BrdU-positive ($BrdU^+$) cells was used to investigate the effects of chronic social stress on cellular proliferation in the forebrain of male or female zebrafish confined in pairs for 48 h (male only) or 96 h of social interaction (behaviour scores for fish used in this experiment are presented in Table 3.1). Figures 7 and 8 present representative images of brain sections showing immunofluorescence for BrdU, which was eliminated when the primary antibody was omitted from the preparation (Figs. 7C). Proliferative regions were identified using sections stained with cresyl violet (Fig. 7A). Comparison of the number of $BrdU^+$ cells between dominant and subordinate males after 48 h and 96 h of social interaction (Fig. 8A, B) revealed a significant effect of social status that did not depend on the length of the interaction period (Fig. 9A; two-way ANOVA, social status $P = 0.016$, length of the interaction period, $P = 0.727$, status x length $P = 0.585$). That is, regardless of interaction time, subordinate male zebrafish exhibited significantly less cell proliferation in the forebrain than dominant fish (Fig. 9A). In addition, lower levels of cellular proliferation were detected in the brains of male fish that had engaged in social interaction than in control fish sampled from a holding tank (one-way ANOVA, $P = <0.001$). By contrast, cellular proliferation in the forebrain

of female zebrafish was not significantly affected by social status after 96 h of interaction, nor did cellular proliferation in the forebrain of interacting female fish differ from that in control fish (Fig. 9B; one-way ANOVA, $P = 0.921$).

To further explore the effects of chronic social stress on cellular proliferation in the forebrain of male zebrafish, numbers of $BrdU^+$ cells were examined by forebrain region (Fig. 10). Significant effects of both forebrain region and social status were detected for fish that had interacted for both 48 (two-way RM ANOVA, social status $P = 0.133$, brain region $P < 0.001$, social status x brain region $P = 0.001$) and 96 h (two-way RM ANOVA, social status $P = 0.031$, brain region $P < 0.001$, social status x brain region $P = 0.014$). Dominant fish exhibited significantly higher $BrdU^+$ cell counts than subordinates in the D and Dm after both 48 and 96 h of interaction, and in the Vv after 96 h of interaction. Cell proliferation was significantly higher in the POA of subordinates after 48 h of interaction, but this effect had disappeared by 96 h of interaction. The highest number of $BrdU^+$ cells typically was observed in the D in both dominant and subordinate fish, after 48 or 96 h of interaction. In subordinate fish after 48 h of interaction, high $BrdU^+$ cell counts were also detected in the POA, with most other regions being lower and the Dp, in particular, exhibiting low numbers of $BrdU^+$ cells. Low numbers of $BrdU^+$ cells in the Dp were also typical of dominant fish after 48 h of interaction, and of subordinate fish after 96 h of interaction. Cell proliferation was also assessed in the forebrain of control fish that were sampled directly from the holding tank, to provide insight into possible effects of social interaction rather than social status (Fig. 10). Numbers of $BrdU^+$ cells generally were similar between control and dominant fish. The Vv was a notable exception in this regard, with substantially higher cellular proliferation in control fish than in dominant or subordinate fish after either 48 or 96 h of social interaction. Statistically the D (one-way ANOVA, $P = 0.001$), Vv

($P < 0.001$) and Vd ($P = 0.024$) in both the dominant and subordinate were significantly different from the control group after 48h, while only the D in the subordinate ($P = 0.02$) and Vv in the dominant and subordinate ($P < 0.001$) were significantly different from the control group at 96h.

3.1.3 Series 3: The impact of social status on neurogenesis

To assess the proportion of $BrdU^+$ cells that differentiated into a neuronal phenotype, brain sections were probed for both BrdU and the neuronal marker NeuN (Fig. 8C, D). These experiments were carried out in pairs of male zebrafish that interacted for 48 h and were then returned to their holding tanks for 2 weeks (see Table 3.1 for behaviour scores for these fish). In dominant fish, 49.6 ± 5.5% of $BrdU^+$ cells exhibited immunofluorescence for NeuN, whereas a significantly lower (paired Student's *t*-test, $P = 0.042$) percentage of $BrdU^+$ cells were also NeuN-positive in subordinate fish (Fig. 11). Statistical analysis of the percentage of $BrdU^+$ cells that expressed NeuN by brain region revealed a significant effect of social status (two-way RM ANOVA; $P = 0.008$), but not brain region ($P = 0.220$, status x brain region $P = 0.205$).

3.1.4 Series 4: The role of cortisol in social stress-induced changes in cell proliferation

A role for cortisol in eliciting social stress-induced changes in cell proliferation in the forebrain of male zebrafish was suggested by the observation of divergent cortisol levels and forebrain cell proliferation in pairs of interacting male but not female zebrafish. To explore this possibility, male zebrafish were treated with the cortisol synthesis inhibitor metyrapone prior to engaging in social interactions. Pilot trials revealed that the plasma cortisol response to an air-

exposure stressor was not inhibited in zebrafish fed food laced with metyrapone, regardless of the dose of metyrapone added to the food (Fig. 12; Student's *t*-tests). However, addition of metyrapone to the tank water at a concentration of 325.4 μM was effective in inhibiting the plasma cortisol response to an air-exposure stressor (Fig. 12; Student's *t*-test, $p = 0.978$). Pilot trials revealed that an exposure period of at least 4 days was necessary to achieve full blockade of the cortisol response to a stressor.

Exposure of fish to waterborne metyrapone prior to and during social interactions did not affect their social behaviour; these fish formed social hierarchies in which the behaviour scores of dominant and subordinate fish did not differ from those of sham-treated fish of the same status that were not exposed to metyrapone (Fig. 13A, one-way ANOVA, $P = 0.254$ for dominants, $P = 0.445$ for subordinates). However, in metyrapone-exposed pairs, plasma cortisol concentrations did not differ between dominant and subordinate fish (Fig. 14B), whereas sham-treated pairs exhibited divergent cortisol concentrations (paired Student's *t*-test; $P = 0.0305$). The number of $BrdU^+$ cells in the D of subordinate fish that were exposed to metyrapone was significantly higher than that of sham-treated subordinate fish (Student's *t*-test, $P = 0.00362$). Indeed, whereas cellular proliferation in sham-treated subordinates was comparable to that of the subordinate fish described above, cellular proliferation in metyrapone-treated subordinates was on par with that of control fish that had not engaged in social interactions.

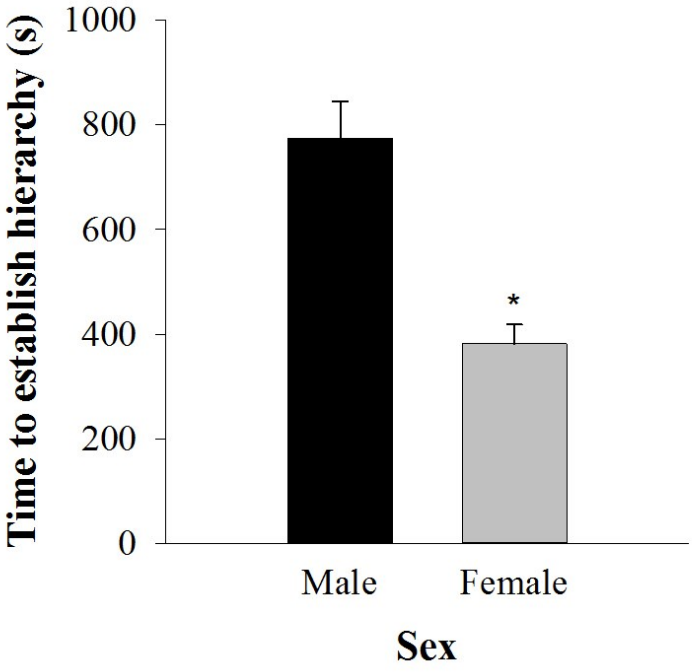
Table 3.1: Behaviour scores derived from a principal component analysis of behavioural observations for pairs of zebrafish (*Danio rerio*) used in the experiments of series 2 and 3

Experiment	**Time of interaction**	**Social status**	
		Dominant	Subordinate
Series 2 (BrdU)	48 h	1.58 ± 0.16	-1.53 ± 0.11
	96 h	1.82 ±0.08	-1.82 ± 0.10
Series 3 ($BrdU^+$ and NeuN)	48 h	1.67 ± 0.19	-0.17 ± 0.22

Values are means ± SE, $N = 4$ pairs in all cases.

Figure 1: (A) The time required for hierarchy formation following removal of the divider between pairs of male ($N = 58$) and female ($N = 5$) zebrafish (*Danio rerio*). Hierarchy formation was deemed to have occurred when one fish initiated consistent retreating behaviour from the other fish. (B) The mean number of acts of aggression initiated by the dominant fish over a 48 h interaction period as a function of the length of isolation period (15 h, $N = 13$; 24 h, $N = 7$) prior to removal of the divider for pairs of male zebrafish. Values are presented as means ± SE. An asterisk indicates a significant difference (Student's *t*-test; see text for details).

A)


Time to establish hierarchy (s)
1000
800
600
400
200
0
*
Male
Female
Sex

B)

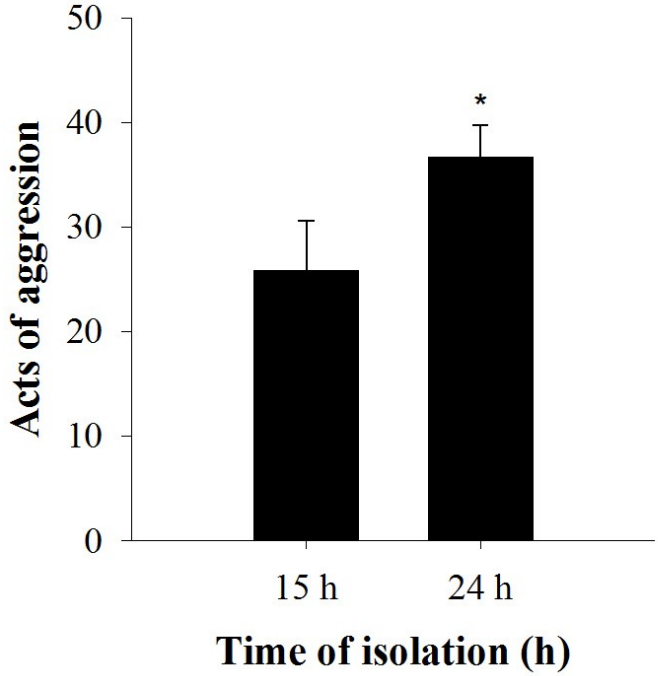

Acts of aggression
50
40
30
20
10
0
*
15 h
24 h
Time of isolation (h)

Figure 2: Behavioural scores for pairs of male zebrafish (*Danio rerio)* confined together for 48 h (A-D; $N = 15$) or 96 h (E-H, $N = 12$) of interaction. Values are mean scores over the interaction period for position in the tank (A, E), aggression (B, F), retreats (C, G) and feeding (D, H). Values are presented as means ± SE. An asterisk indicates a significant difference between the members of a pair (paired Student's *t*-tests or Wilcoxon signed rank test, $P < 0.001$ for all panels except D, where $P = 0.021$).

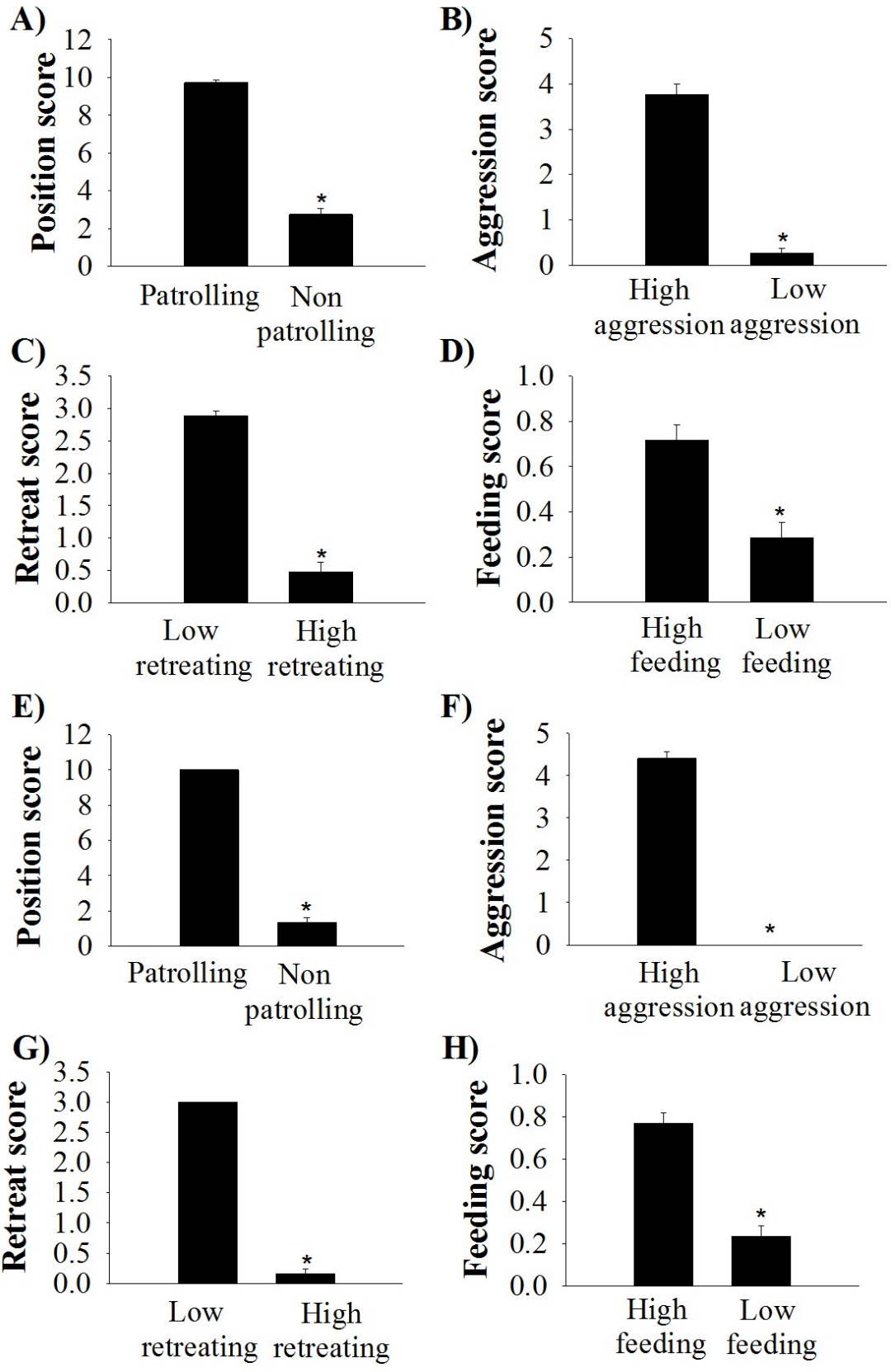

A)
Position score
12
10
8
6
4
2
0
*
Patrolling
Non patrolling
B)
Aggression score
5
4
3
2
1
0
*
High aggression
Low aggression
C)
Retreat score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Low retreating
High retreating
D)
Feeding score
1.0
0.8
0.6
0.4
0.2
0.0
*
High feeding
Low feeding
E)
Position score
12
10
8
6
4
2
0
*
Patrolling
Non patrolling
F)
Aggression score
5
4
3
2
1
0
*
High aggression
Low aggression
G)
Retreat score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Low retreating
High retreating
H)
Feeding score
1.0
0.8
0.6
0.4
0.2
0.0
*
High feeding
Low feeding

Figure 3: Behavioural scores of paired female zebrafish (*Danio rerio)* confined together for 96h ($N = 5$) of interaction. Values are mean scores over the interaction period for position in the tank (A), aggression (B), retreats (C) and feeding (D). Values are presented as means ± SE. An asterisk indicates a significant difference between the members of a pair (paired Student's *t*-tests or Wilcoxon signed rank test, $P = 0.0002$ for panel A, $P = 0.0111$ for panel B, $P = 0.001$ for panel C, and $P = 0.00103$ for panel D).

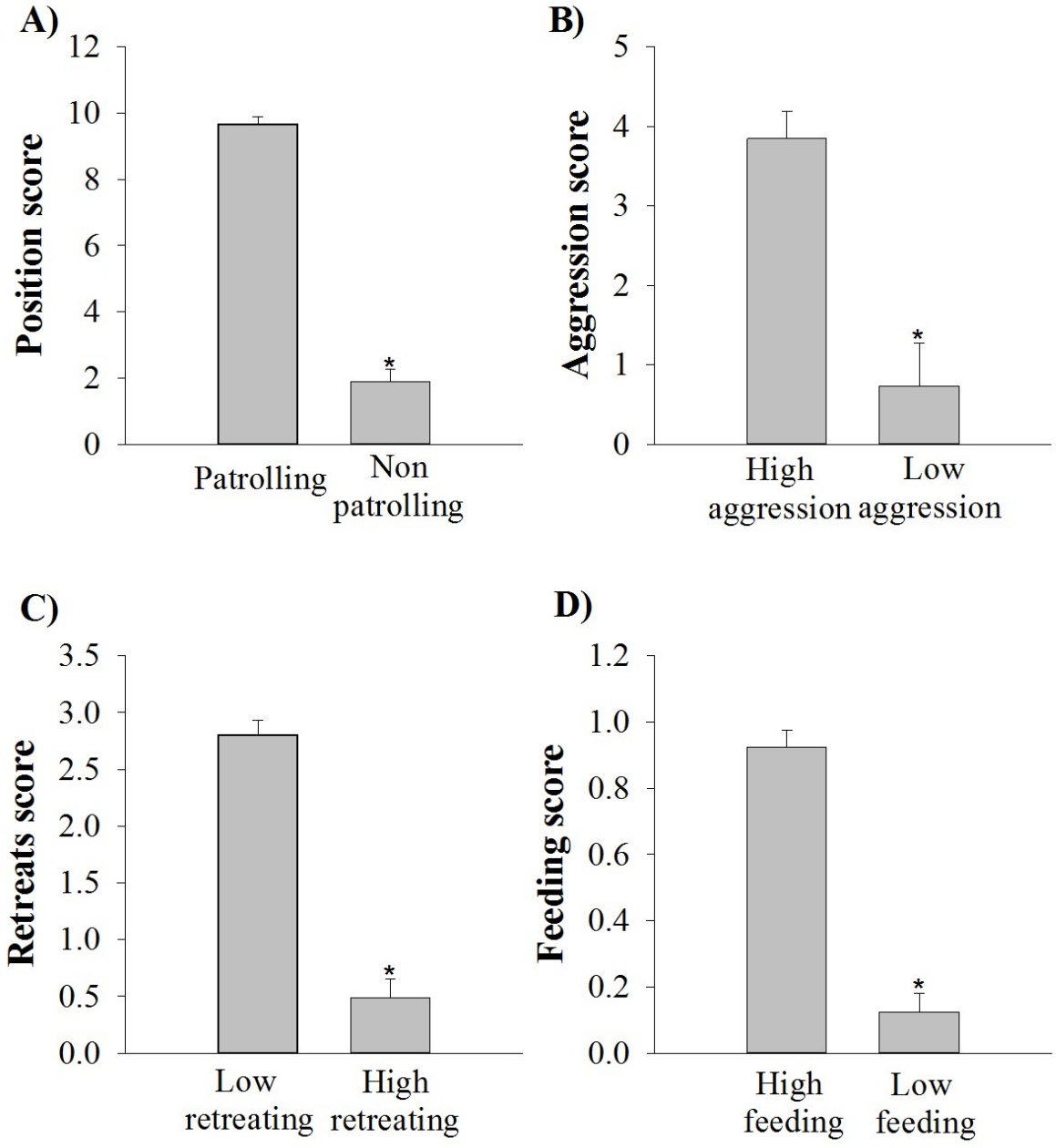

A)
Position score
12
10
8
6
4
2
0
*
Patrolling
Non patrolling
B)
Aggression score
5
4
3
2
1
0
*
High aggression
Low aggression
C)
Retreats score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Low retreating
High retreating
D)
Feeding score
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
High feeding
Low feeding

Figure 4: A comparison of social behaviour in dominant male ($N = 15$) and female ($N = 5$) zebrafish (*Danio rerio*) throughout 96 h of interaction. Values are mean scores and counts over the interaction period for position in the tank (A), aggression (B), retreats (C) and feeding (D). Within each sex, bars that share a letter are not significantly different from one another (one-way RM ANOVA carried out separately for each sex, see text for details).

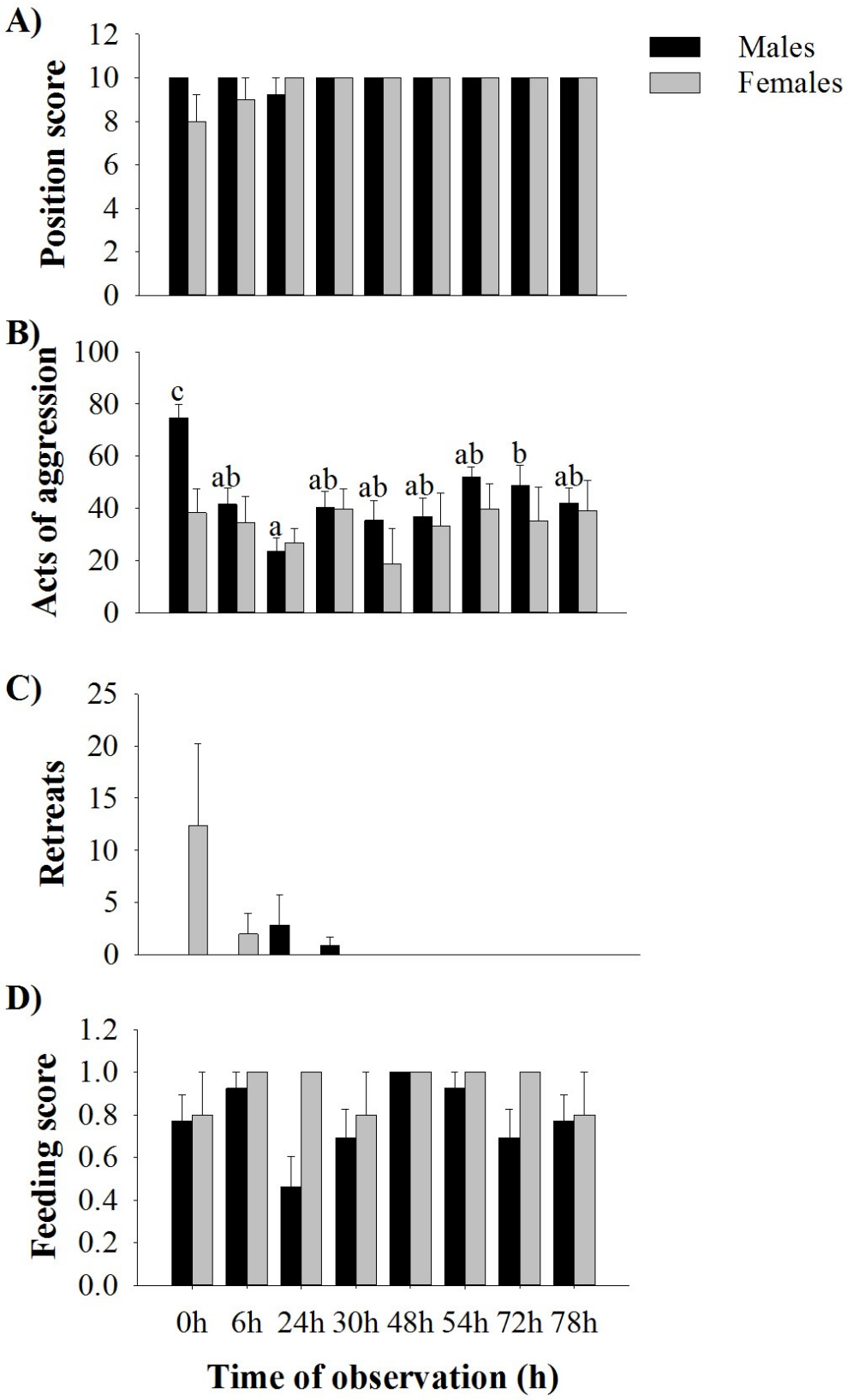

A)
Position score
12
10
8
6
4
2
0
Males
Females
B)
Acts of aggression
100
80
60
40
20
0
c
ab
a
ab
ab
ab
ab
b
ab
C)
Retreats
25
20
15
10
5
0
D)
Feeding score
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0h
6h
24h
30h
48h
54h
72h
78h
Time of observation (h)

Figure 5: Trunk cortisol concentrations (A) and specific growth rates (B) for male dominant and subordinate zebrafish (*Danio rerio*) confined in pairs for interaction periods of 1 to 120 h; specific growth rates of female zebrafish confined in pairs for 96 h are also presented in panel B. Values are presented as means ± SE with $N = 6$ for 1-24 and 80 h, $N = 11$ for 48 h, $N = 12$ for 96 h and $N = 8$ for 120 h in panel A. In panel B, $N = 7$ for 48 h, $N = 8$ for 80-120 h and $N = 13$ for female fish. In panel A, an asterisk represents a significant difference from the control group (one-way ANOVA, see text for details). Interaction periods that share a letter are not significantly different from one another (two-way RM ANOVA, see text for details). For panel B, a significant interaction between social status and interaction period was detected (two-way RM ANOVA, see text for details). Interaction times that share a letter are not significantly different from one another (uppercase letters for dominant fish, lowercase letters for subordinates) and asterisks denote significant differences between dominant and subordinate fish ($N = 9$) for a given interaction period. For female fish, no significant difference in SGR was detected (paired Student's *t*-test, see text for details).

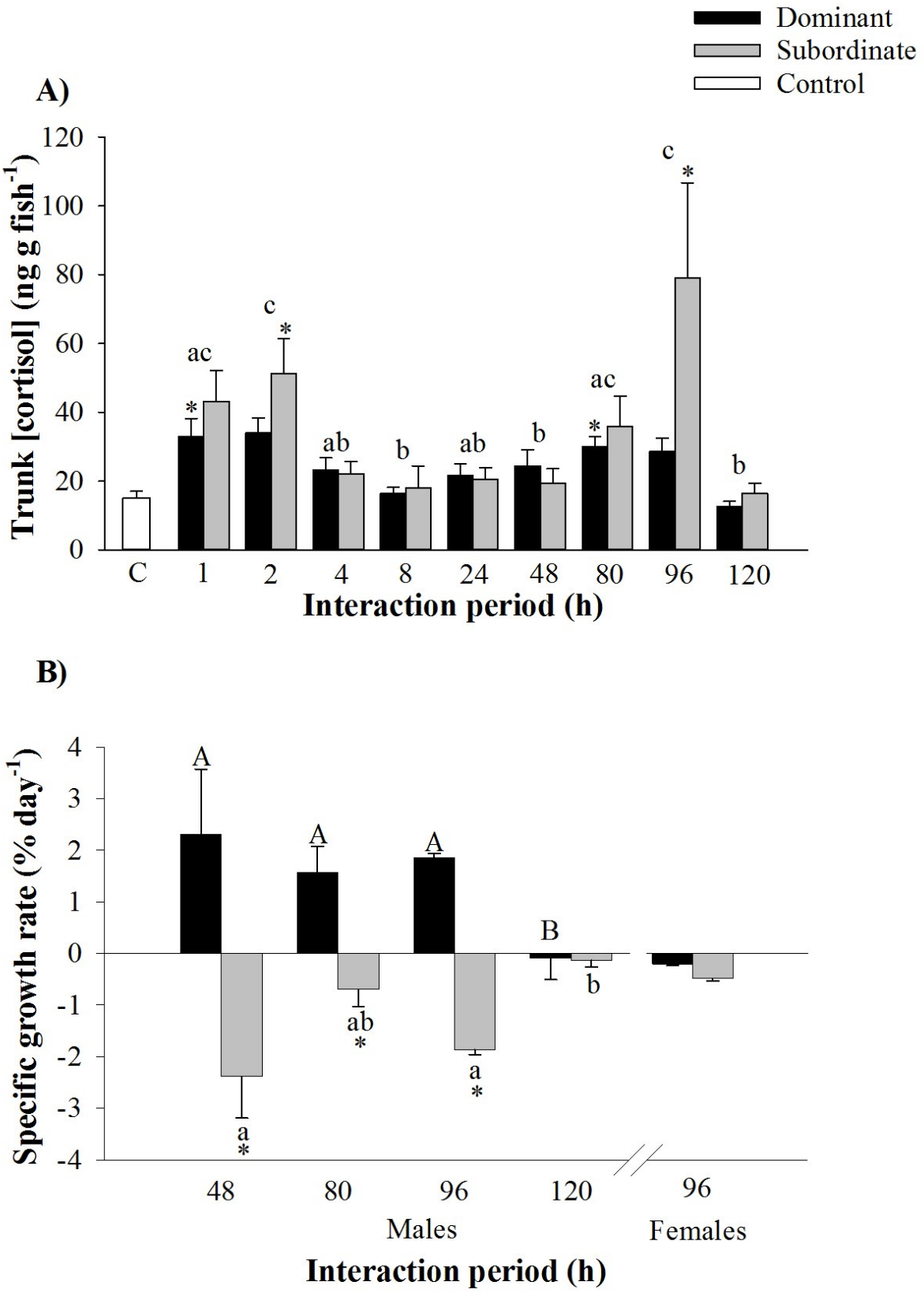

Dominant
Subordinate
Control
A)
Trunk [cortisol] (ng g fish-1)
120
100
80
60
40
20
0
C
1
2
4
8
24
48
80
96
120
Interaction period (h)
ac
c
ab
b
ab
b
ac
c
b
*
B)
Specific growth rate (% day-1)
4
3
2
1
0
-1
-2
-3
-4
A
A
A
B
a
ab
a
b
48
80
96
120
96
Males
Females
Interaction period (h)

Figure 6: Behaviour scores (A) and plasma cortisol concentrations (B) for pairs of male or female zebrafish (*Danio rerio*) that were allowed to interact for 48 h (male only, $N = 7$) or 96 h ($N = 8$ male pairs, $N = 9$ female pairs). Plasma cortisol concentrations are also presented for control male ($N = 7$) and female ($N = 6$) fish that were sampled directly from the holding tank. Behaviour scores were derived from a principal components analysis of mean observations of position in the tank, aggressive behaviour, retreats and feeding behaviour. Values are presented as means ± SE. Asterisks denote significant differences in plasma cortisol concentrations between dominant and subordinate fish (paired Student's *t*-tests, see text for details), whereas a dagger represents a difference from the control group (one-way ANOVA, see text for details).

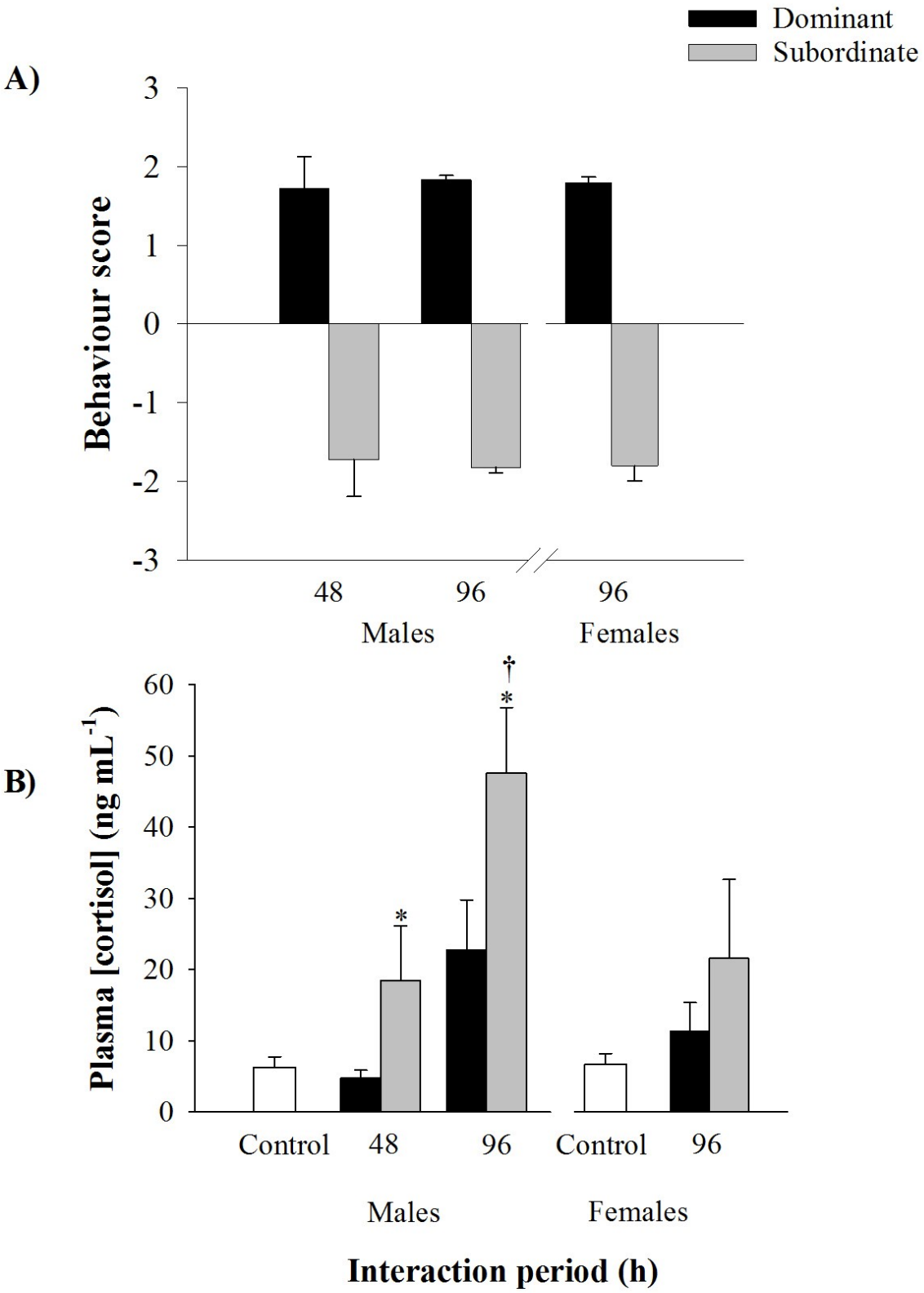
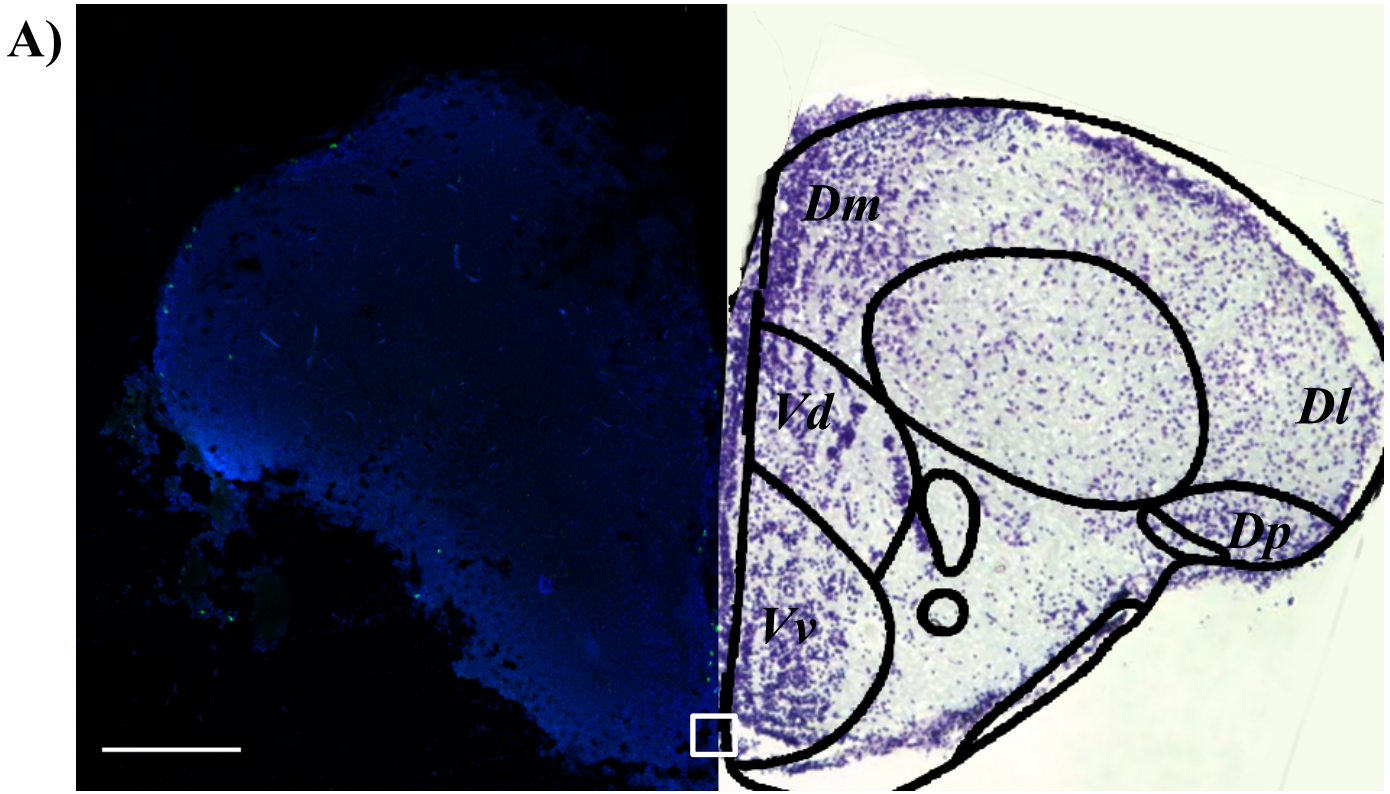
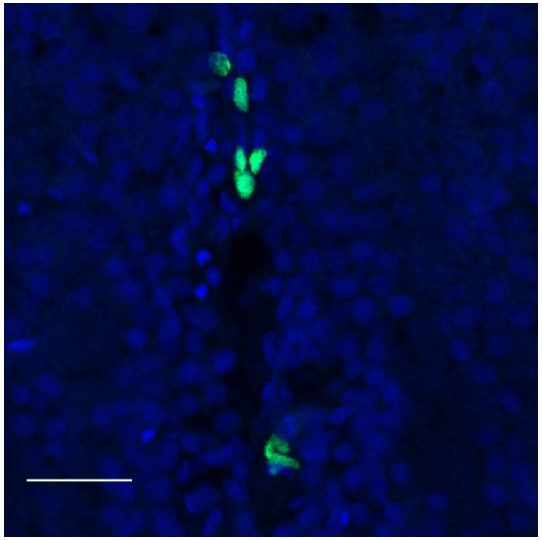

A)
Dominant
Subordinate
Behaviour score
3
2
1
0
-1
-2
-3
48
96
96
Males
Females
B)
Plasma [cortisol] (ng mL-1)
60
50
40
30
20
10
0
*
*
†
Control
48
96
Control
96
Males
Females
Interaction period (h)

Figure 7: Representative images of brain sections prepared for BrdU immunohistochemistry (green fluorescence) on the left and cresyl violet (purple) staining on the right for orientation (A). Panel B presents a higher magnification image of the area marked by the white box in panel A, and panel C illustrates the loss of BrdU immunofluorescence with primary antibody omission. Section 71 of the forebrain in a male zebrafish (*Danio rerio*) is presented (Wullimann et al., 1996). Blue fluorescence represents DAPI. The scale bar represents 200 μm in A and 25 μm for B and C. *V*, Ventral telencephalic area, *D,* Dorsal telencephalic area, *Vv*, Ventral nucleus of V, *Vd*, Ventral telencephalic area of D, *Dm*, Medial zone of D, *Dd*, Dorsal zone of D, *Dl*, Lateral zone of D, *Dp*, Posterior zone of D.


A)
Dm
Vd
Dl
Dp
Vv
?


B)

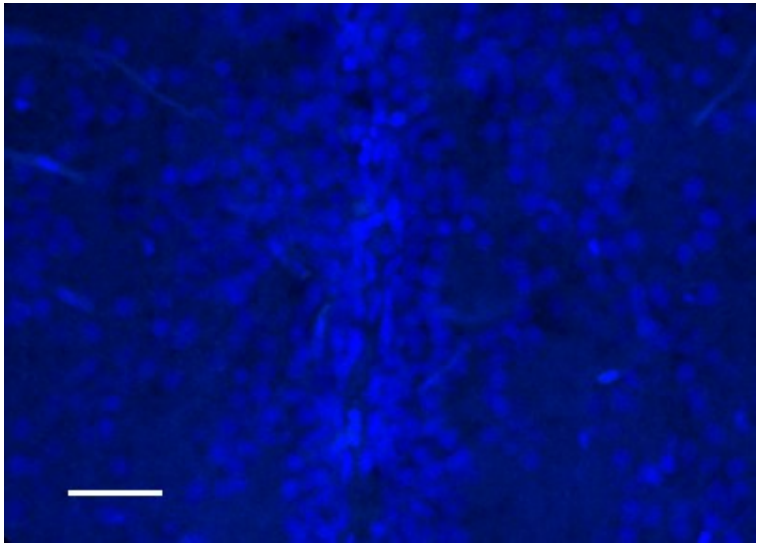

C)

Figure 8: Images of brain sections from male dominant (A, C) and subordinate (B, D) zebrafish (*Danio rerio*) prepared for BrdU (A, B), and BrdU and NeuN (C, D) immunohistochemistry. The images are of section 50 in the forebrain (Wullimann et al, 1996), depicted in the schematic (E) with a box. Panels C and D illustrate co-localization of BrdU-positive and NeUN-positive cells using a slice view into the z-stack on the bottom and right side of the picture. Green immunofluorescence indicates BrdU-positive cells, red immunofluorescence indicates the neuronal marker NeuN, and blue fluorescence indicates cellular nuclei stained with DAPI. Scale bars represent 25 μm.

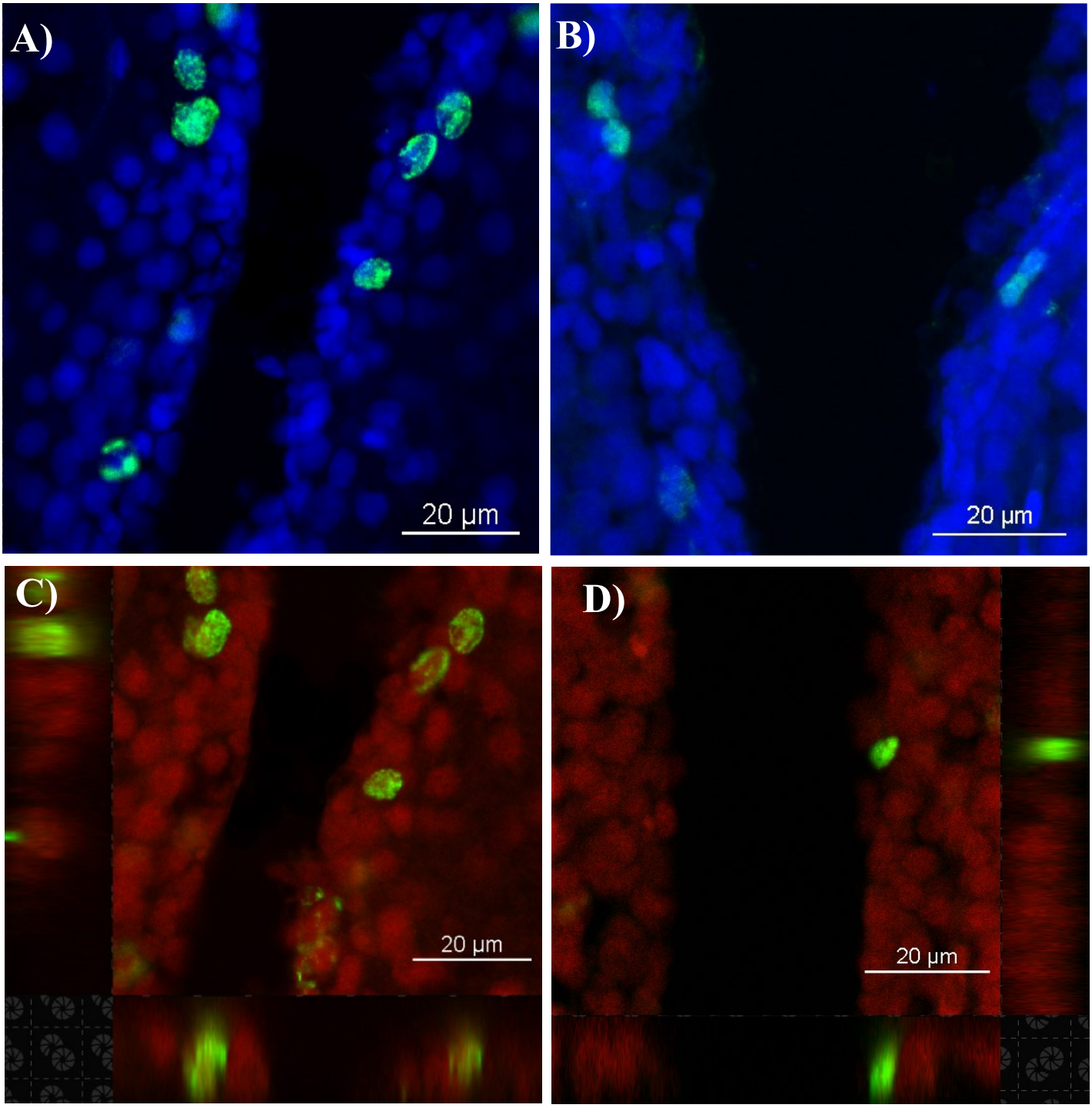
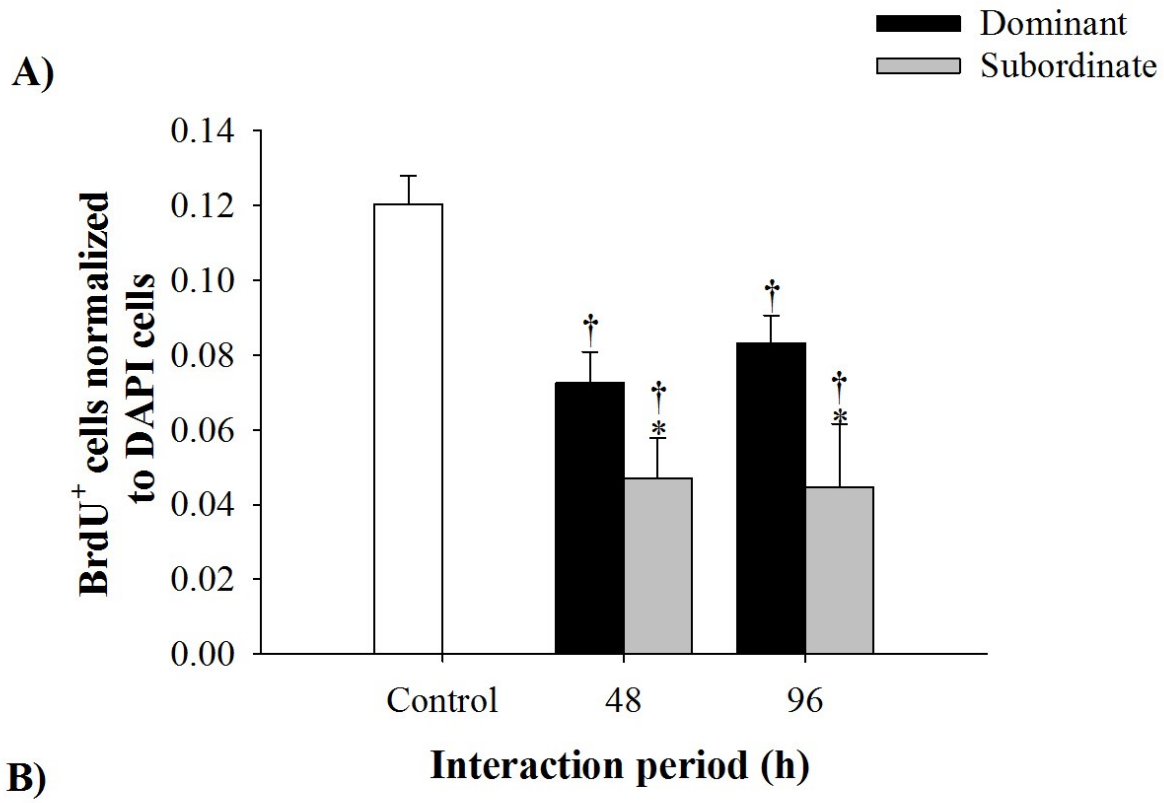

A)
20 µm
B)
20 µm
C)
20 µm
D)
20 µm
E)
D
V
ICL
GL
ECL

Figure 9: BrdU-positive cell counts in pairs of male zebrafish (*Danio rerio*) after 48 ($N = 4$) and 96 h ($N = 4$) of social interaction (A) and in pairs of female zebrafish ($N = 4$) after 96 h of social interaction (B). Counts of BrdU-positive cells are also presented for control ($N = 4$ male and 4 female) fish that were sampled directly from the holding tank. In panel A, counts were averaged across all forebrain regions. Analysis of these data revealed significant effects of social status (two-way RM ANOVA, see text for details) as well as significant effects of social interactions relative to control fish (one-way ANOVA, see text for details). An asterisk indicates a significant difference from the corresponding dominant group, while a dagger represents a significant difference from the control group. In panel B, cell counts were carried out only in the D (refer to legend of Fig. 7 for abbreviations). No significant effect of social status was detected (one-way RM ANOVA; see text for details). In all cases, BrdU-positive cell counts were normalized to counts of DAPI-positive cells. Values are presented as means ± SE.


A)
Dominant
Subordinate
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
BrdU+ cells normalized to DAPI cells
†
†
*
†
†
*
Control
48
96
Interaction period (h)

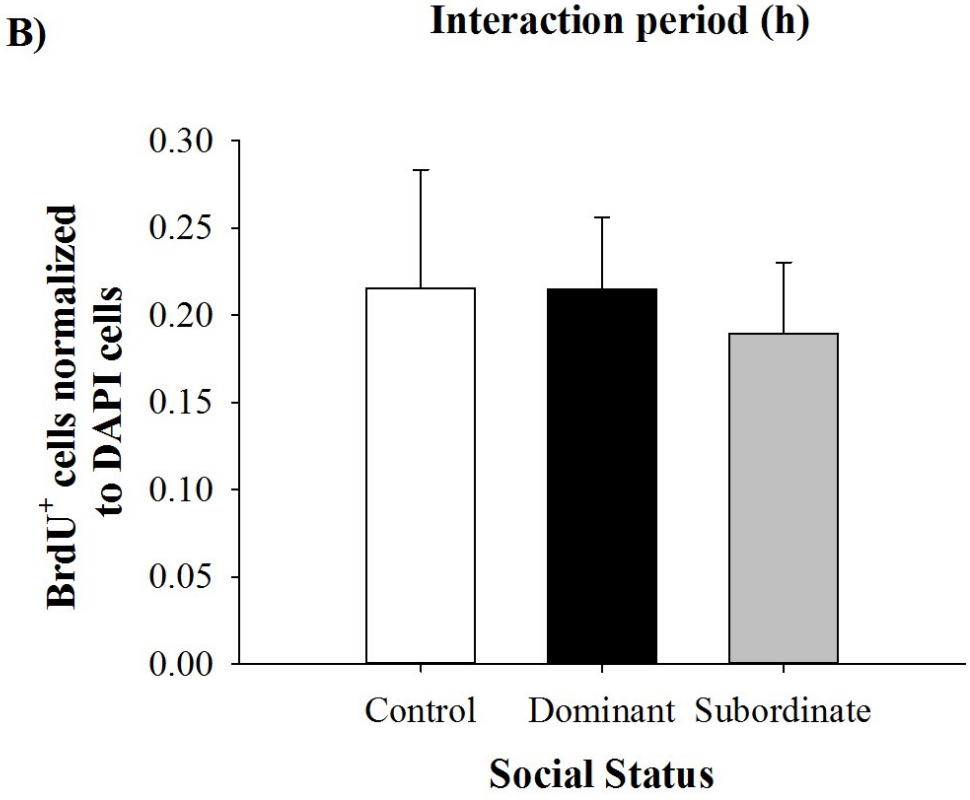

B)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
BrdU+ cells normalized to DAPI cells
Control
Dominant
Subordinate
Social Status

Figure 10: BrdU-positive cell counts in the forebrain of dominant and subordinate male zebrafish (*Danio rerio*) after 48 h (A; $N = 4$) and 96 h (B; $N = 4$) of social interaction. Counts of BrdU-positive cells are also presented for control male zebrafish ($N = 4$) sampled directly from the holding tank; values for the same set of control fish are presented in both panels. In all cases, BrdU-positive cell counts were normalized to counts of DAPI-positive cells. Values are presented as means ± SE. Brain regions that share a letter are not significantly different from one another (different sets of letters used for dominant versus subordinate fish) and asterisks denote significant differences between dominant and subordinate fish for a given brain region (two-way RM ANOVA, see text for details). A dagger represents differences from the control group within a brain region (one-way ANOVAs, see text for details).

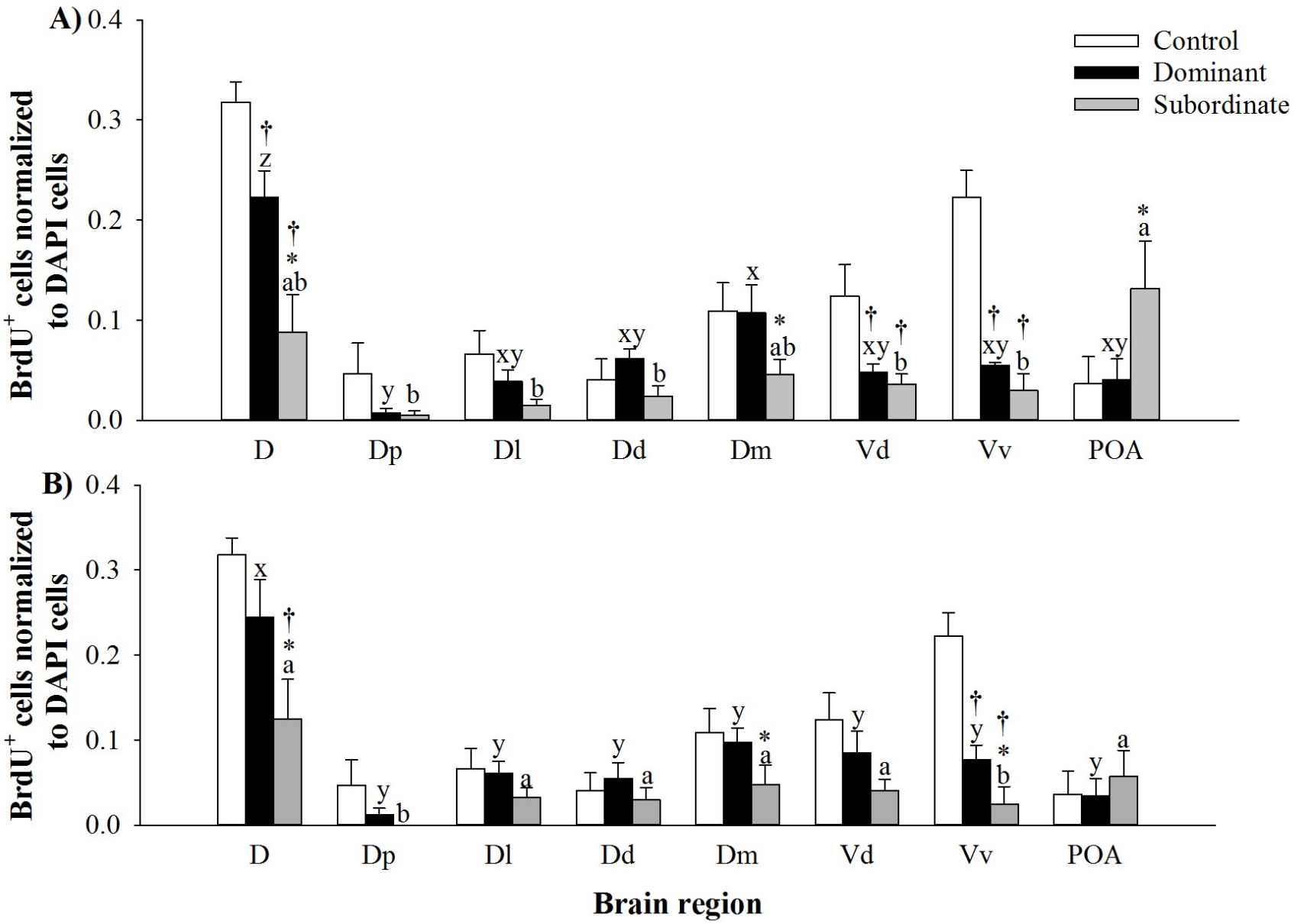

A)
B)
BrdU+ cells normalized to DAPI cells
0.0
0.1
0.2
0.3
0.4
Control
Dominant
Subordinate
D
Dp
Dl
Dd
Dm
Vd
Vv
POA
Brain region

Figure 11: Percentage of $BrdU^+$ cells that expressed NeuN as a function of forebrain region (see legend of Fig. 7 for details) for dominant and subordinate male zebrafish (*Danio rerio*) after 48 h of interaction. Values are presented as means ± SE, $N = 4$ pairs. An asterisk indicates a significant difference between dominant and subordinate fish (two-way RM ANOVA; see text for details).

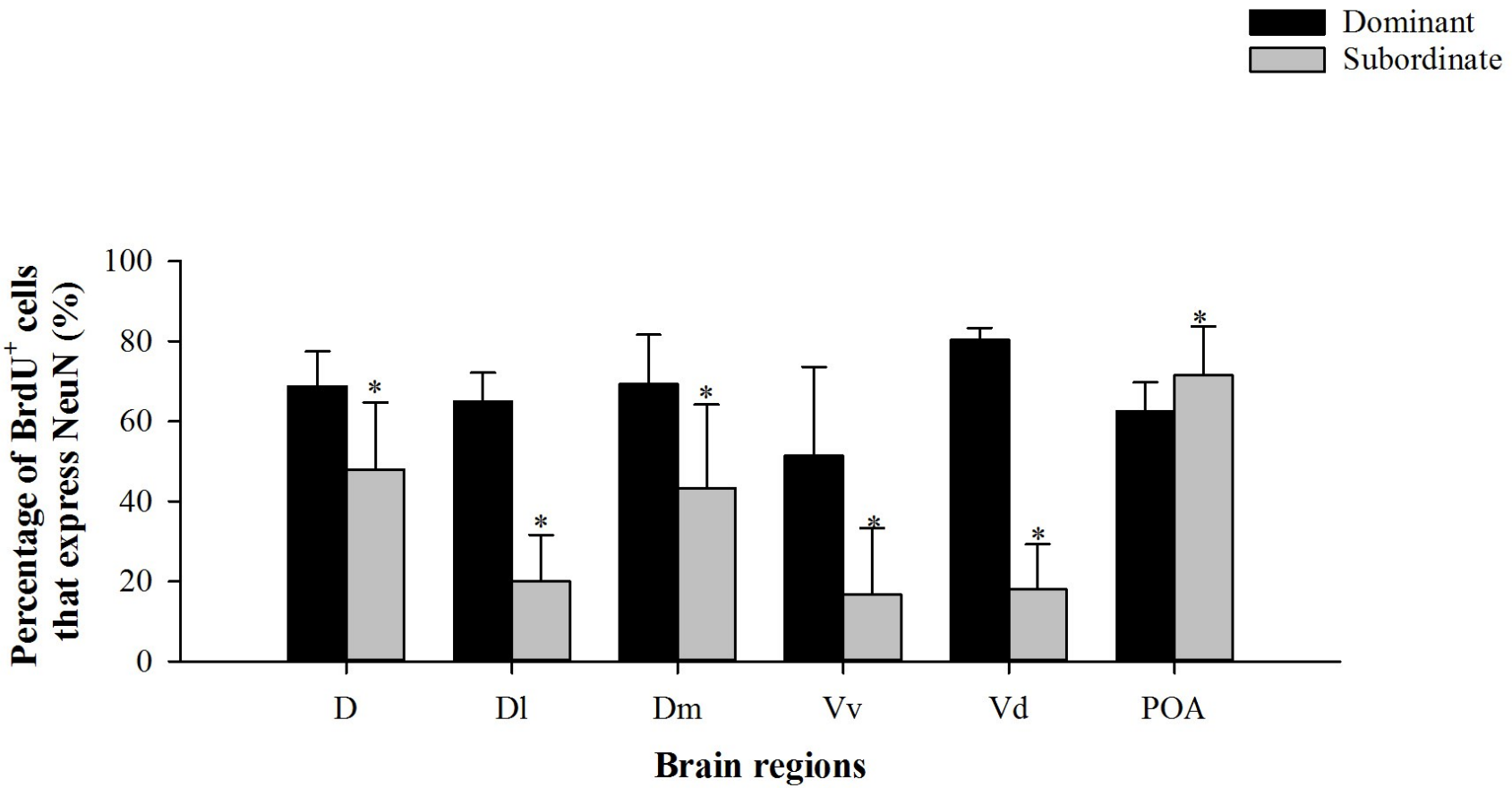

Dominant
Subordinate
Percentage of BrdU+ cells that express NeuN (%)
100
80
60
40
20
0
D
Dl
Dm
Vv
Vd
POA
Brain regions

Figure 12: Baseline and stress-induced plasma cortisol concentrations for male zebrafish (*Danio rerio*) after being fed food laced with metyrapone ($N = 6$ per group) at various doses (mg g^{-1}) or exposed to metyrapone via the water ($N = 6$). Values are presented as means ± SE. An asterisk indicates a significant difference between post-stress and baseline values (Student's *t*-tests, 0 mg g^{-1} $P < 0.001$, 0.03 mg g^{-1} $P < 0.001$, 0.05 mg g^{-1} $P < 0.001$, 0.5 mg g^{-1} $P < 0.001$, 1 mg g^{-1} $P = 0.002$, waterborne metyrapone $P = 0.978$).

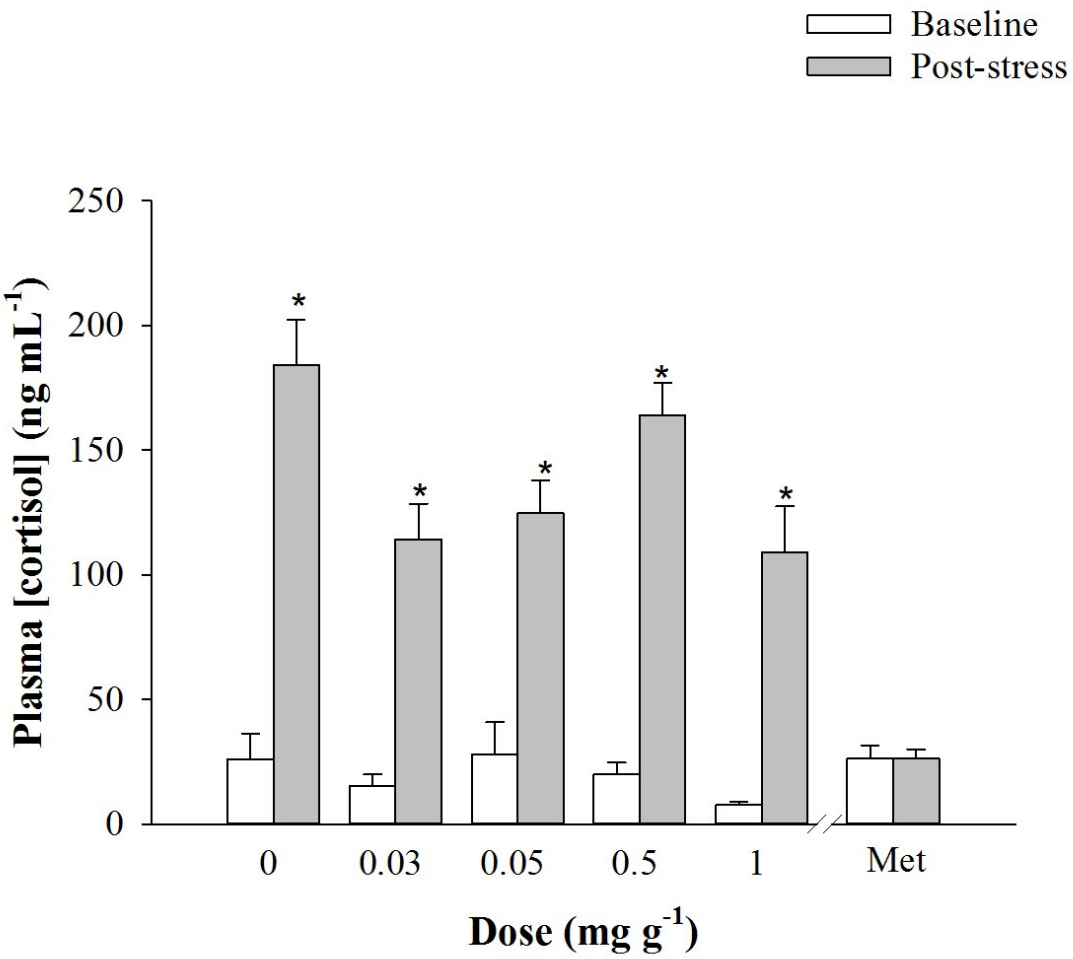
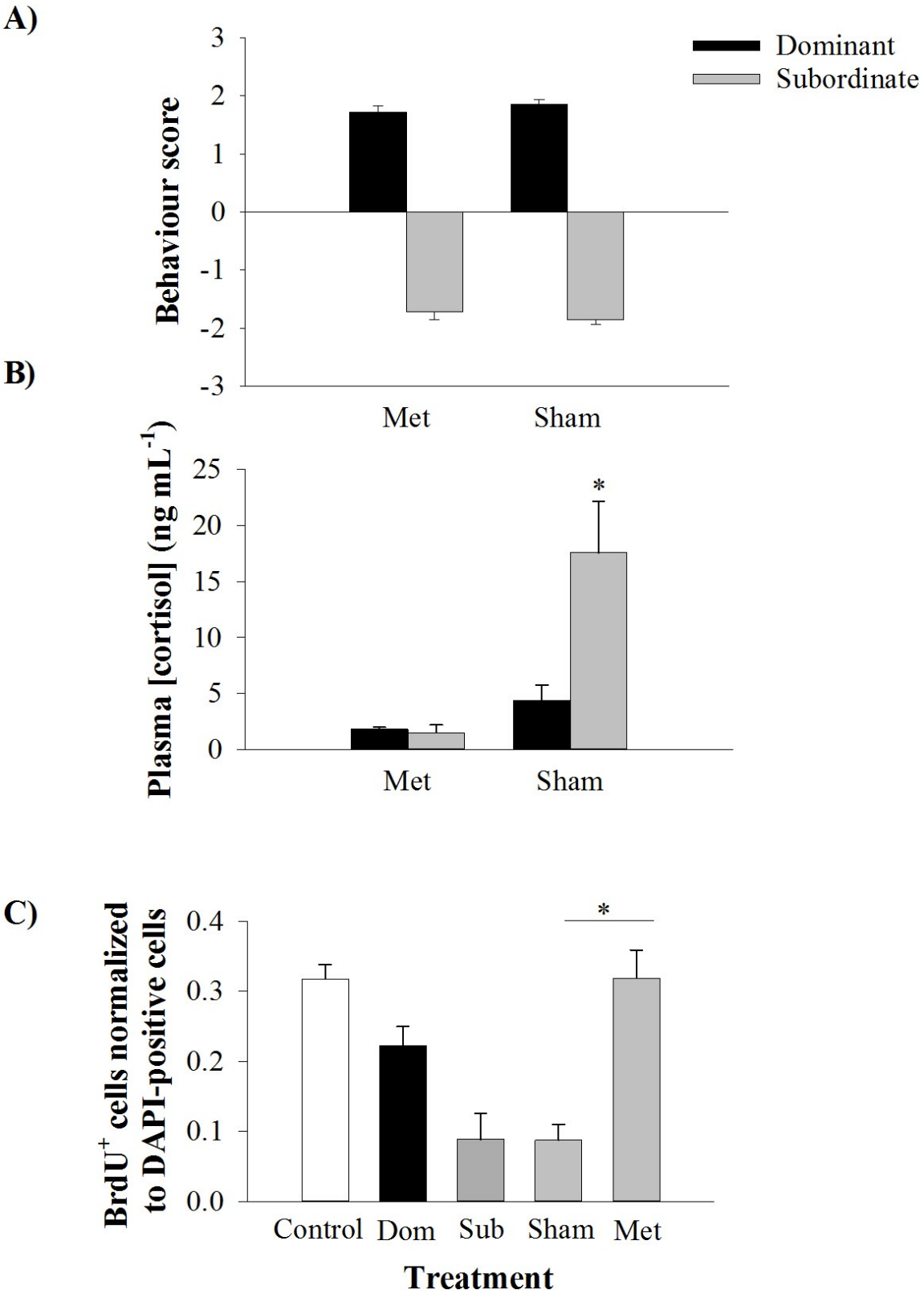

Baseline
Post-stress
Plasma [cortisol] (ng mL-1)
250
200
150
100
50
0
*
*
*
*
*
0
0.03
0.05
0.5
1
Met
Dose (mg g-1)

Figure 13: Effects of metyrapone treatment on behaviour scores (A), plasma cortisol concentrations (B) and counts of BrdU-positive cells in the forebrain (C) of male zebrafish (*Danio rerio*) after 48 h of social interaction. Behaviour scores presented in panel A were derived from a principal components analysis of mean observations of position in the tank, aggressive behaviour, retreats and feeding behaviour. Counts of BrdU-positive cells (normalized to the total number of cells based on DAPI) in panel C are for the D region of subordinate fish only and are compared with corresponding values for control, dominant and subordinate fish replotted from Fig. 10A. Values are presented as means ± SE, with $N = 2$ metyrapone-treated pairs and $N = 7$ sham-treated pairs for the measurement of plasma cortisol concentrations, and $N = 3$ metyrapone-treated pairs and $N = 4$ sham-treated pairs for the measurement of cellular proliferation. Behaviour scores are presented for all pairs ($N = 5$ for metyrapone, $N = 7$ sham-treated pairs). Paired Student's *t*-tests were used to analyze the effects of social status on plasma cortisol concentrations (panel B), and a Student's *t*-test was used to analyze the effects of metyrapone treatment versus sham treatment on cell counts in the forebrain of subordinate fish (panel C). Asterisks indicate significant differences between social status or treatments (see text for details).


A)
Dominant
Subordinate
Behaviour score
3
2
1
0
-1
-2
-3
Met
Sham
B)
Plasma [cortisol] (ng mL-1)
25
20
15
10
5
0
*
Met
Sham
C)
BrdU+ cells normalized to DAPI-positive cells
0.4
0.3
0.2
0.1
0.0
*
Control
Dom
Sub
Sham
Met
Treatment

Chapter 4: Discussion

The findings of the present thesis (Fig. 4.1) suggest that chronic social stress reduces cellular proliferation and neurogenesis in the forebrain of subordinate male, but not female, zebrafish. Specifically, the number of $BrdU^+$ cells, indicative of cellular proliferation, was lower across multiple proliferative zones in the forebrain of subordinate male zebrafish, as was the percentage of $BrdU^+$ cells that were positive for the neuronal marker NeuN, indicative of neurogenesis. Male and female subordinate zebrafish also differed in the effect of chronic social stress on circulating levels of the glucocorticoid stress hormone cortisol, which were elevated in male but not female subordinate fish. This observation implicated elevation of circulating cortisol levels during chronic social stress as a causal factor in the reduction of cellular proliferation and neurogenesis. Confirming this hypothesis, inhibition of cortisol synthesis by means of metyrapone treatment restored cellular proliferation to control levels in subordinate male zebrafish.

4.1 Social behaviour during dyadic interactions

As in previous work (e.g. Larson et al., 2006; Dahlbom et al., 2011b; Pavlidis et al., 2011; Oliveira et al., 2016), same-sex pairs of male or female zebrafish in the present study formed dominant-subordinate social hierarchies as indicated by differences in aggression, retreats from aggression, position in the tank and likelihood of feeding. Male fish required more time to establish a hierarchy than female fish, and this difference was accompanied by higher rates of aggressive behaviour during the initial observation period for male dominant fish. Once formed, hierarchies were typically stable throughout the interaction period, as indicated by the general lack of significant variation with time in behavioural indices such as acts of aggression.

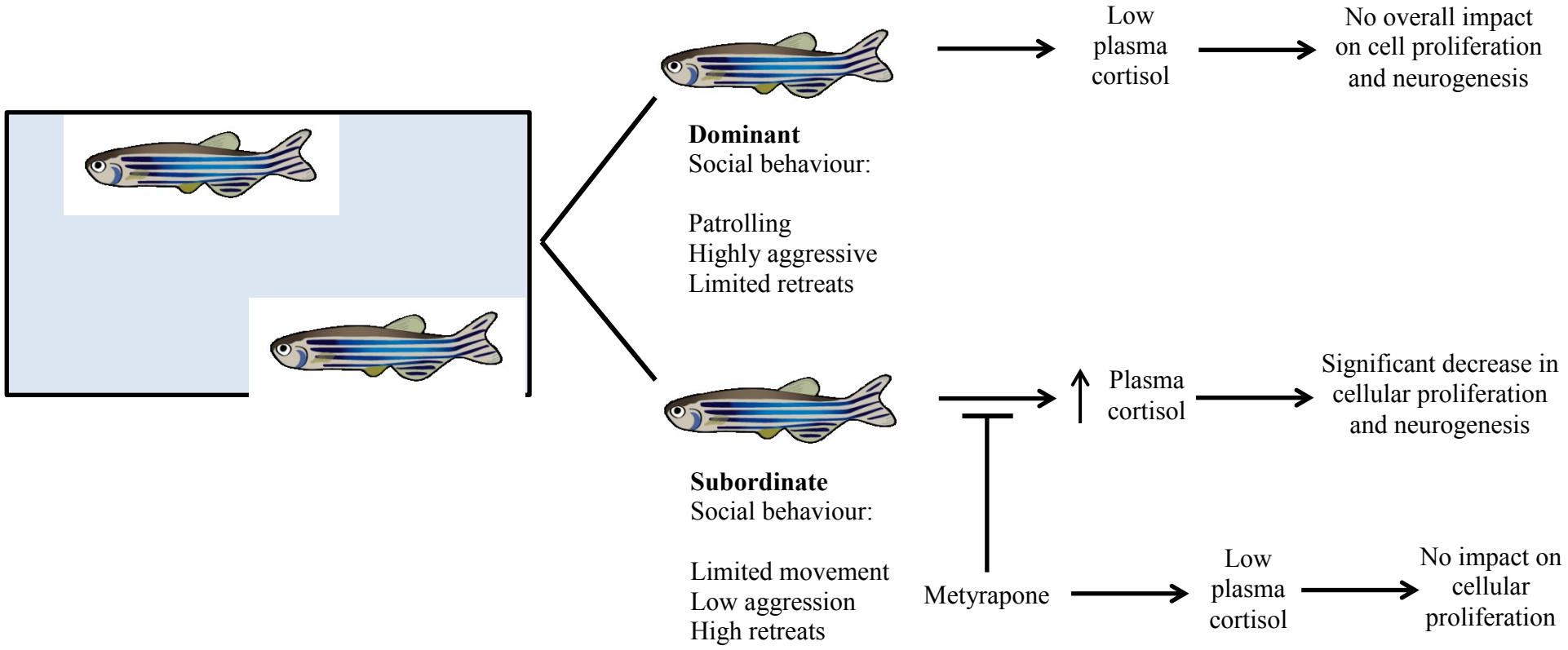


Figure 4.1: Schematic illustrating the effects of chronic social stress on cellular proliferation and neurogenesis in male zebrafish (*Danio rerio*), and the likely role of cortisol in eliciting these effects.

Similar consistency of social behaviour throughout interaction periods of up to 5 days was reported by Pavlidis et al., (2011), whereas Paull et al., (2010) found differences between morning and afternoon observation periods. However, the fish studied by Paull et al., (2010) were held in groups of two female plus two male fish to encourage reproduction, a factor that may have contributed to differences in behaviour between morning and afternoon observation periods. The intensity of social interactions in the present study was enhanced by a 24 h period of social isolation prior to commencing interactions, as observed previously (Larson et al., 2006; Oliveira et al., 2016). Dominant fish that are able to monopolize food resources induce more bouts of social aggression (Roy and Bhat, 2015). The use of this feeding strategy in the present study, together with the period of isolation prior to social interaction, likely contributed to the sustained aggression observed throughout the interaction period.

4.2 Physiological consequences of social interaction

Among salmonid fish, in which the physiological consequences of social interaction arguably have received most attention, social stress results in chronic elevation of circulating cortisol concentrations and reduced growth rates (reviewed by Gilmour et al., 2005; Johnsson et al., 2006; Sørensen et al., 2013). Similarly, plasma cortisol concentrations and specific growth rates in the present study differed between dominant and subordinate male zebrafish at both 48 and 96 h of social interaction. However, neither plasma cortisol concentrations nor specific growth rates differed between dominant and subordinate female zebrafish, despite the robust differences in behaviour that were observed. Filby et al., (2010b) also detected a significant difference in plasma cortisol levels between dominant and subordinate zebrafish for fish held in

groups of two female and two male fish. Although these researchers were unable to detect an effect of fish sex on the relationship between social status and circulating cortisol levels, cortisol concentrations after five days of social interaction were virtually identical in dominant and subordinate female fish, consistent with the present study. Moreover, examination of transcript abundance for HPI axis genes including CRF and GR revealed social status-associated differences in male but not female fish (Filby et al., 2010a). Status-associated differences were also detected in growth rates for male but not female fish (Filby et al., 2010b). Collectively, these data suggest that the physiological costs of subordinate social status are more severe for male than female zebrafish.

Although measurement of plasma cortisol concentrations consistently revealed status-associated differences for male zebrafish in the present study, these differences were not apparent in measurements of trunk cortisol concentrations. Similarly, Pavlidis et al., (2011) reported that trunk cortisol concentrations did not differ between dominant and subordinate male zebrafish, although values for fish that had experienced social interactions were significantly higher than those of control fish, a trend that was apparent for some interaction periods in the present study, as well. These observations suggest that measurements of trunk cortisol concentrations lack sensitivity in comparison to plasma measurements, perhaps reflecting factors such as the need to extract cortisol from tissues, a step that can be avoided with use of plasma. Although collection of blood from zebrafish is challenging owing to their small size, the use of low centrifugal force following caudal severance (Babaei et al., 2013) was helpful in this regard. Importantly, Babei et al. (2013) demonstrated that blood collected by low centrifugal force was indistinguishable in composition from blood collected directly from the caudal vessel (in both cases, following caudal severance).

4.3 Effects of chronic social stress on forebrain cellular proliferation and neurogenesis

Exposure to social stress for 48 or 96 h significantly inhibited cellular proliferation (based on the abundance of $BrdU^+$ cells) and neurogenesis (based on the abundance of $BrdU^+$ cells that also expressed NeuN) in the forebrain of subordinate male zebrafish. Similarly, Sørensen et al. (2012) found reduced cellular proliferation in the forebrain of subordinate juvenile rainbow trout that had been confined in pairs for 96h with a dominant conspecific, and cell proliferation across multiple brain regions was significantly lower in subordinate than dominant male *A. burtoni* that were held in social groups with four female fish (Maruska et al., 2012). In both rainbow trout and the African cichlid *A. burtoni*, cell proliferation in dominant and control animals was comparable and greater than that in subordinate fish (Maruska et al., 2012; Sørensen et al., 2012), indicating that the suppression of cell proliferation in subordinate animals was a product of chronic social stress rather than social interaction *per se* (Dunlap et al., 2016). A similar pattern was detected in the present study, with dominant and control male zebrafish being comparable and subordinate fish exhibiting lower cellular proliferation. The overall impact of subordinate social status on cellular proliferation in male zebrafish was not affected by the length of the interaction period (for 48 vs 96 h of interaction), suggesting that the suppression of cellular proliferation occurred early in the interaction period and was maintained as the period of social interaction was extended. Aggressive behaviour and other behaviours indicative of dominance (patrolling the tank, monopolizing food resources) were similarly maintained throughout the interaction period for 96 h of social interaction, although aggressive attacks were highest (significantly so) in the initial period after removal of the divider separating the members of a pair.

Overall, male subordinate zebrafish exhibited lower cellular proliferation and consistency in this response between 48 and 96 h of social interaction, but examination of regions within the forebrain revealed the POA as an interesting exception to this general trend. After 48 h of social interaction, cellular proliferation in the POA was significantly higher in subordinate than dominant or control fish, an effect that was lost by 96 h of social interaction. It is tempting to speculate that this effect was associated with activation of the HPI axis in response to social stress in subordinate fish, because neurons that originate in the POA are responsible for the synthesis and release of CRF onto the corticotropes of the pituitary (Flik et al., 2006). In support of this possibility, increased cellular proliferation associated with an increase in functional importance was observed in the midbrain of the brown ghost knifefish in response to social interactions (Dunlap et al., 2006; Dunlap and Chung, 2013; Dunlap et al., 2016). Brown ghost knifefish confined in pairs increase production of a specific electrocommunication signal termed a chirp (Dunlap et al., 2013). Chirping is controlled by the diencephalic prepacemaker nucleus (PPn-c), and after 7 days of social interaction, an increase in cellular proliferation and neurogenesis was detected in the periventricular zone adjacent to the PPn-c; the periventricular zone contributes cells to the PPn-c (Dunlap et al., 2006). Whether elevated neurogenesis in the POA of subordinate male zebrafish is functionally associated with increased HPI axis activity is a possibility that requires further investigation.

Apart from the POA, variation in levels of cellular proliferation and neurogenesis across brain regions was similar to that reported previously (Lindsey et al., 2012). The highest density of $BrdU^+$ cells was found in the D, with somewhat lower but still substantial densities in the Vv, Vd and Dm. The Vv was notable in having a higher density of $BrdU^+$ cells in control than dominant or subordinate fish after 48 or 96 h of interaction, suggesting an effect of social

interactions (rather than social status) on cellular proliferation in this region. Limited numbers of $BrdU^+$ cells were detected in the Dp and Dd, in agreement with previous reports (Lindsey et al., 2012). The fate of progenitor cells labelled with BrdU during social interactions was examined at two weeks post-BrdU injection, a time at which all proliferating zones of the PVZ should have reached their maximum number of newborn neurons, with the exception of the Vd, where neuron numbers continue to increase beyond two weeks (Lindsey et al., 2012). Using NeuN as a neuronal marker, ~51% of proliferating cells in the forebrain of fish that had engaged in social interactions for 48 h differentiated into neurons, with minor but not statistically significant variation across brain regions. Similarly, Lindsey et al., (2012) reported that, depending on region, 45-80% of $BrdU^+$ cells in the forebrain of zebrafish had differentiated into neurons two weeks post BrdU injection. Differences between the studies may reflect the use of different neuronal markers, Hu C/D (Lindsey et al., 2012) versus NeuN (present study). For example, some types of neurons, such as Purkinje cells, do not exhibit NeuN immunoreactivity (Mullen et al., 1992).

Although the mechanisms underlying the effects of social stress or social interactions on brain cellular proliferation and neurogenesis remain uncertain, cortisol has received attention as a potential endocrine mediator of these effects (Dunlap et al., 2016). To date, however, studies of the potential role played by cortisol have yielded mixed results. In rainbow trout, subordinate fish that exhibited reduced forebrain cellular proliferation also experienced significant elevation of circulating cortisol levels (Sørensen et al., 2012). Dietary cortisol administration to fish that did not experience dyadic interactions resulted in significant but not dose-dependent decreases in cellular proliferation (Sørensen et al., 2011). Brown ghost knifefish held with a conspecific exhibited elevated circulating cortisol concentrations relative to isolated fish (Dunlap, 2002), as

well as higher rates of cell addition in the PVZ adjacent to the PPn-c (Dunlap et al., 2006). Again, cortisol treatment mimicked the effects of social interaction – isolated knifefish given a cortisol-containing implant exhibited increased cellular proliferation specifically in the PVZ adjacent to the PPn-c, and not in surrounding areas, after 7 d (Dunlap et al., 2006). Moreover, the effect of social interaction on cellular proliferation in the PVZ adjacent to the PPn-c was partially blocked by treatment of knifefish with the GR antagonist RU-486 prior to and during social interaction (Dunlap et al., 2011b). Similarly, the present study adopted a manipulative approach to provide evidence of a causal link between circulating cortisol concentrations and suppression of forebrain cellular proliferation and neurogenesis in subordinate male zebrafish. In behaviourally subordinate fish in which cortisol synthesis was inhibited via waterborne exposure to metyrapone, circulating cortisol levels as well as cellular proliferation in the D were restored to dominant or control values. This finding argues compellingly for a role for elevated cortisol levels in suppressing forebrain cellular proliferation during chronic social stress in male zebrafish. The lack of differences in cellular proliferation in the D of subordinate female zebrafish, which also failed to exhibit elevated circulating cortisol concentrations, provides further support for this conclusion. However, the context in which circulating cortisol concentrations are elevated appears to be important. Elevation of circulating cortisol concentrations by repeated injection of cortisol over 3 d did not impact forebrain cell proliferation in zebrafish held in groups or held in isolation for 2 weeks prior to cortisol treatment (Lindsey and Tropepe, 2014), suggesting that cortisol levels must rise in conjunction with other cues indicative of social subordination to suppress forebrain cellular proliferation. Interestingly, serotonin (5-hydroxytryptamine, 5-HT) has been proposed to regulate neurogenesis in the telencephalon of fish (Lillesaar, 2011), and both social status-related and sex-related

differences in brain serotonergic activity were reported in zebrafish (Dahblom et al., 2012). These observations suggest that it would be useful to probe the relationships among cortisol levels, serotonergic activity and neurogenesis during social interactions in zebrafish. In addition, whether cortisol mediates its effects by binding to GR or to MR requires further research.

4.4 Study limitations

The present study used BrdU incorporation as a marker of cell cycling and hence cellular proliferation, an approach used previously in zebrafish (Lindsey and Tropepe, 2014) as well as other fish species (e.g. Dunlap et al., 2006; Maruska et al., 2012; Sørensen et al., 2012; reviewed by Dunlap et al., 2016). However, the administration of BrdU involves exposure of fish to an acute stressor. Therefore, it would be useful to confirm the results of the present study using other immunohistochemical markers of cellular proliferation, such as proliferating cell nuclear antigen (PCNA), Ki67 (Scholzen and Gerdes, 2000; Welker et al., 2016) or minichromosome maintenance complex component 2 (mcm2; Wharton et al., 2001), that do not require handling of the animal. Assessment of transcript abundance for one or more markers of cellular proliferation and/or neurogenesis could also serve to reinforce the findings of the present study.

A second limitation reflects the difficulty of establishing a causal link between cellular proliferation/neurogenesis and social stress in the absence of prior knowledge of which fish will become subordinate or dominant. A reliable marker of ultimate social status would allow cellular proliferation and neurogenesis to be assessed prior to social interaction to investigate whether differences in neurogenesis strictly reflect the outcome of social interactions or play a role in determining the outcome of social interactions. Research into factors that are divergent within

social hierarchies, such as arginine vasotocin (Larson et al., 2006), histamine (Norton et al., 2011), or behaviour (Dahlbom et al., 2011b) shows promise in this regard.

The identification of appropriate holding conditions for control fish in the present study also was of concern. The use of group-held fish as controls, as opposed to 'sham' fish that were handled/treated in the same way as fish paired with a conspecific but held in isolation provided a challenge in making comparisons between control fish and those that had experienced social interactions. However, social isolation was linked to changes in cellular proliferation in adult zebrafish (Lindsey and Tropepe, 2014), rendering sham-treated fish problematic as a control for the present study. Thus, group-held fish were used as a control, as in previous work (Lindsey and Tropepe, 2014).

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