The effect of *disc1* on the stress response in zebrafish

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Abbreviations

3V	3rd brain ventricle
ACTH	Adrenocorticotropic hormone
Arc	Arcuate nucleus of the hypothalamus
CRF	*Corticotrophin-releasing factor*
DISC1	*Disrupted-In-Schizophrenia-1*
Dlx	*Drosophila distal-less*
DMN	Dorsomedial nucleus of the hypothalamus
DPF	Days post fertilisation
ELISA	Enzyme-linked-immunosorbant-assay
GR	Glucocorticoid receptor
GXE	Gene-environment interaction
HPA	Hypothalamic-pituitary-adrenal axis (mammals)
HPF	Hours post fertilisation
HPI	Hypothalamic-pituitary-interrenal axis (fish)
Lcn2	*Lipocalin2*
LR	Lateral hypothalamic recess
MC2R	Melanocortin 2 receptor
MR	Mineralocorticoid receptor
NaCl	Sodium chloride
NND	Nearest neighbour distance
NPO	Hypothalamic nucleus preopticus (fish)
PDE4B	cAMP-hydrolysing phosphodiesterase 4B
Poly I:C	Polyinosinic:polycytidylic acid
POMC	*Pro-opiomelanocortin a*
PPI	Pre-pulse inhibition
PVN	Paraventricular nucleus of the hypothalamus (mammals)
Rax	*Retina and anterior neural fold homeobox* (mammals)
rx3	*Retinal homeobox gene 3* (zebrafish)
SF-1	*Steroidogenic factor 1*
Shh	*Sonic hedgehog a*
TH	*Tyrosine hydroxylase*

VMN	Ventromedial nucleus of the hypothalamus
ZT	Zeitgeber time; time after lights on

Abstract

Stressful experiences in animals trigger responses that lead to adaptive changes. These changes to physiology, metabolism and behaviour are mediated by the stress axis, which acts to maintain or restore homeostasis [1]. Stress in early life is linked to the development of adult-onset psychiatric disorders in humans [2]. Evidence from animal models has demonstrated that stress can affect behaviour, endocrine function and gene expression. Studies in mice also suggest that the combination of stress and a genetic predisposition can result in abnormal behaviours that resemble psychiatric disease [3].

One such genetic factor is *Disrupted-In-Schizophrenia-1* (*DISC1*). A translocation in *DISC1* segregates with a high prevalence of mental illness in a large human family [4]. The DISC1 protein is implicated in a wide variety of roles in the nervous system [5]. Mouse models of *DISC1* exhibit various behavioural abnormalities that have been likened to anxiety in humans [6]. Recently, it has been demonstrated that mutant *DISC1* mice display abnormal phenotypes in response to stress, thereby opening an avenue to investigate the role of *DISC1* in the stress response [7].

The zebrafish is powerful study system to address such a topic. Zebrafish are genetically tractable, exhibit quantifiable behaviours and have relatively simple brains that offer a good level of functional homology with humans [1]. The work described in this thesis utilises novel zebrafish models of *disc1*, the orthologue of the genetic risk factor in humans, which have not been previously examined with respect to stress responses. I describe studies in which I have analysed *disc1* mutant zebrafish in terms of behavioural and endocrine responses to stress and investigated the expression of genes linked to hypothalamic development and the stress response.

When exposed to an acute chemical stressor, wild type zebrafish modulated their behaviour and upregulate cortisol synthesis. Conversely, *disc1* mutants typically do not modulate their behaviour or cortisol synthesis when exposed to the stressor. Mutants also displayed abnormal expression of a number of genes in the

hypothalamus, which are critical to normal hypothalamic development. These findings suggest that *disc1* alters stress axis function via abnormal hypothalamic development.

1 General introduction

1.1 Introduction

All living organisms experience stress. When homeostasis is threatened by a stressor, animals respond adaptively, by modulating their metabolism, physiology and behaviour. The genes of individuals that adapt successfully will be favoured by natural selection. The system regulating these adaptive responses to stress, the hypothalamic-pituitary-adrenal axis (HPA axis in mammals; HPI, hypothalamic-pituitary-interrenal axis in fish) (Figure 1.1), is controlled by the hypothalamus. There is, however, substantial evidence suggesting that this circuit can become reprogrammed to trigger responses that are seemingly maladaptive. For example, it is well documented that early life stress can result in the development of adult-onset psychiatric disorders in humans [2]. Many psychiatric disorders have been linked with dysregulation of the HPA axis [8] and animal studies suggest that changes to the functioning of the stress axis are ultimately caused by changes in gene transcription, which may be mediated by epigenetic mechanisms [9, 10]. Studies in mice are beginning to investigate the role of genetic factors in modulating stress-induced phenotypes.

1.2 The stress response

Stress is a difficult term to define, given that a broad range of definitions that is often used in different contexts. 'Stress' is often used interchangeably to refer to both the stressor and the stress response. As humans, we often refer to stress as pressure exerted on us from external factors, such as work, exams, relationships, finances, traumatic life events, which alone or in combination might exert emotional unrest. Stress can also be defined on a more basic level for all organisms as a state of perturbation, which is counteracted by the stress response [11]. A stressor and its response may act on many different systems, including an organism's physiology, immunology, behaviour and mental wellbeing. This response should be adaptive and specific to the stressor. Stress responses occur in both complex vertebrates and simple invertebrates, the latter of which includes rapid stimulus-response interactions controlled by the nervous system or nerve nets, as well as endocrine and immune responses, which respond to metabolic

threats [12]. In contrast to stress acclimation, distress can be defined as a state of severe stress, in which the response is insufficient to counteract it. In this case an aversive, negative state emerges, in which coping and adaptation processes fail to return an organism to its physiological and/or psychological 'norm' [11].

In this thesis, I focus on the vertebrate neuroendocrine stress response and define stress as 'an animal's state of threatened homeostasis which triggers the activation of the HPA axis' [13]. Homeostasis is then restored through behavioural, metabolic and endocrine processes which are controlled by the hypothalamus [13]. A diverse array of inputs is brought together at the hypothalamus, from sensory neurons, and local and peripheral circuitry. These are compared to 'ideal set points' and subsequently feedback systems are initiated in order to restore optimal physiology [14]. In response to a changing environment, the hypothalamus can continually re-evaluate these set points in a process known as allostasis. When the stress response is repeatedly or excessively activated, the cost of reinstating homeostasis can become too high, this condition is known as allostatic load [15].

The gene structures, signaling pathways and proteins that underpin the vertebrate HPA axis have been evolutionarily conserved [1] and some stress hormones also act in invertebrates [12]. It is thought that abnormal functioning of these neurobiological mechanisms has evolved in our evolutionary past that would have mediated adaptive avoidance of predators or other harmful stimuli and now contributes to human psychiatric illness [16]. For this reason it is important to study ethologically relevant stimuli and species-specific responses in animal models of stress. The use of natural predator odours in stress research has been successfully utilized in many different animal models [16]. Despite the costs associated with the stress response and its contribution to disease, this complex and carefully regulated system has been maintained by natural selection because it provides selective advantage [17]. The main output of the vertebrate stress response is production of corticosteroids. The genetic sequences for these molecules have been conserved in over hundreds of millions of years and continue to serve closely related defensive functions [17].

In fish, a stressor is detected by sensory neurons and this information is then relayed to a sub-region of the hypothalamus termed the hypothalamic nucleus preopticus (NPO), a region analogous to the mammalian paraventricular nucleus (PVN) (Figure 1.1) [1]. This leads to a cascade of events, which causes the release of corticotrophin-releasing factor (CRF) from the endfeet of the NPO, which project to the rostral pars distalis region of the pituitary gland [1]. Here, CRF binds its receptor [1]. This induces the post-translational cleavage of the protein encoded by the proopiomelanocortin (POMC) gene, causing increased production and secretion of adrenocorticotropic hormone (ACTH).

ACTH then binds to the melanocortin 2 receptor (MC2R) in the interrenal gland, which initiates cortisol synthesis [1]. In fish, rats and humans, the action of corticosteroids is mediated by two ligand-inducible transcription factors, the *mineralocorticoid receptors* (*MR*) and *glucocorticoid receptors* (*GR*). The binding of cortisol to GR contributes to glucocorticoid negative feedback (Figure 1.1). The action of cortisol in the body is wide-ranging and not fully characterised, but one main output of the pathway is the mobilisation of energy for adaptive responses.

1.3 Zebrafish stress research

The zebrafish is a model organism in developmental biology and genetics and in recent years has become an increasingly popular and valuable tool in the study of experimental, behavioural and developmental neuroscience. The model has proved particularly relevant in the field of stress research and strongly complements traditional mammalian models.

Firstly, zebrafish exhibit numerous homologies with humans and other vertebrates including the genome, brain patterning and in particular the stress-regulating axis [1]. Unlike rodents, fish and humans both respond to stress through an increase in cortisol level, whereas in rodents the main corticosteroid is corticosterone [18]. Furthermore, whereas all other teleost fish have duplicates of many genes due to a genome duplication event 350 million years ago, zebrafish, like humans, have only one isoform of the genes responsible for regulating the HPI axis [19].

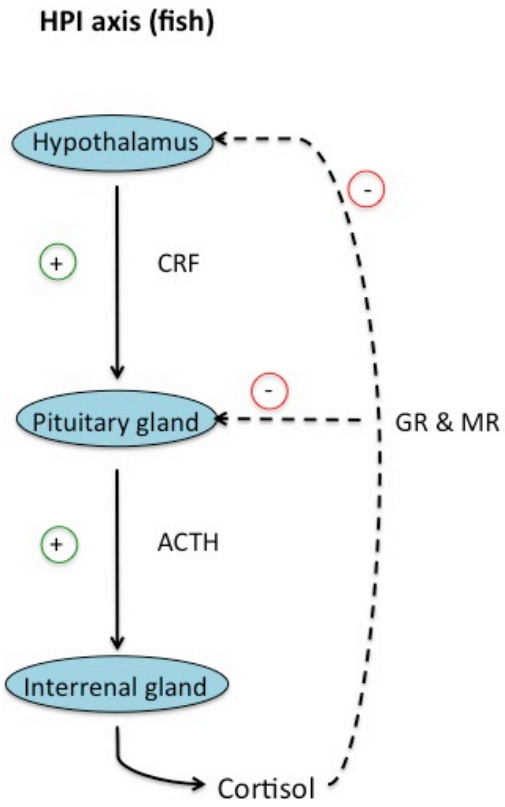

Figure 1.1. Schematic of the Hypothalamic-Pituitary-Interrenal axis in fish. When exposed to a stressful stimulus, CRF released from the hypothalamus binds its receptor at the pituitary gland, initiating the release of ACTH, which in turn stimulates the synthesis and secretion of cortisol from the interrenal gland. The MR and GR receptors then regulate a negative feedback system. CRF, corticotropin releasing factor; ACTH, adenocorticotropic hormone; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; +, positive signals; -, negative feedback. Figure adapted from [1]

In addition, the zebrafish is the only non-primate animal that claims a human-like GRβ isoform, a splice variant of the glucocorticoid receptor gene [1].

There are many practical benefits associated with the zebrafish model. Offspring can be generated with ease in vast numbers, whilst maintenance is relatively cheaper and less labour-intensive than that of mammals. The statistical power gained through large-scale experiments is a clear advantage over mammalian studies. The zebrafish also represents a strong genetic study system, as its genome is sequenced and the ease with which mutant and transgenic fish can be created mediates the identification of genes involved in stress responses [20]. Zebrafish deliver a convenient platform to study developmental processes, in which naturally occurring, confounding stress factors such as parental care are absent. This lack of parental care means that individual differences in stress responses can be more clearly attributed to extrinsic environmental or intrinsic genetic factors [1]. Stresses can also be administered in a non-invasive manner, as substances can be introduced to the swimming water and directly taken up by the fish.

The zebrafish provides a very attractive system to study behaviour when compared to rodent models. Zebrafish allow for extremely high throughput, quantitative measures of behaviour and a simpler system for the study of neurodevelopmental defects [1]. Additionally, the zebrafish lends itself to the development of behaviour-based neurophenotyping and behaviour-based drug screens for psychiatric disorders. These assays can be used to recapitulate the complexity lost in cell assays, whilst reducing the use of mammals and failure rates of clinical trials [1].

1.4 Studying behaviour in zebrafish

Studying behaviour is important when researching the effects of stress in relation to neurological disorders, when diagnoses are typically based on behavioural symptoms. The field of zebrafish behaviour research is developing rapidly and behaviour-based screens are being used in the study of genetic models of psychiatric illnesses. Behavioural phenotypes linked to psychiatric illness in humans are complex and cannot be fully recapitulated in animal models.

Behavioural analysis of animals harbouring mutations linked to mental illness therefore utilises endophenotypes: distinct and stable behaviours that correlate with a given gene or environmental cue [1].

When studying psychiatric disorders linked to stress, it is important to use models that provide good measures of anxiety and other stress-related symptoms. A behavioural response should tell us something about how an animal has perceived the stimulus. It is important to be aware, however, that how we interpret behaviour is difficult, and a particular behaviour may indicate a different response depending on the test situation. A recent study investigated the behavioural responses of zebrafish to the presence of aversive and attractive stimuli [21]. Based on this system, the authors suggest that bottom dwell, jumping, thrashing and proximity to the stimulus are good measures of anxiety in zebrafish, whereas erratic movement, ambulation, freezing and swimming duration are not. The drawback of this system is that it relies on our ability to judge the attractiveness of the stimulus. Other studies have focused on stimuli that we expect to produce a robust behavioural response in nature. Bass studied the responses of zebrafish to various stimulus fish, and found an increased frequency of jumps in response to their natural predator [22], confirming the above result that jumping is a good measure of anxiety or fear. A caveat of this approach is that the behaviour of the stimulus fish cannot be controlled and can vary dramatically between tests [22].

Maximino *et al* argued that defensive distance is a good way to discriminate between anxiety, fear and panic responses [23]. They proposed a theory of discrimination between responses, dependent on the 'perceived immediacy of threat and risk' [23] . Under this system, potential risk should evoke risk assessment-like behaviour, such as change in exploratory behaviour, indicative of anxiety. A distal risk should elicit escape and avoidance responses, thus indicative of fear; whereas proximal risk may induce urgent active or passive behaviour associated with panic.

The study of behaviour in isolated zebrafish is typical when the aim is to assess basic locomotion, or an anxiety response induced by a novel environment.

Nonetheless, the zebrafish is a shoaling species, organised into a social hierarchy [24], and this aspect of behaviour can be measured. The validity of testing an individual zebrafish is questionable in some situations, as the response of an isolated fish may not reflect its natural behaviour. Anomalies were observed when the behavioural response of individual or shoals of zebrafish was analysed whilst exposed to various predatory- stimuli fish [22]. A significant increase in frequency of jumps, a commonly used indicator of stress, was seen in the individual zebrafish in response to their natural predator [22]; however, when shoals of zebrafish were tested, an anxiety or anti-predatory response was not observed.

One stressor that has a reasonably well-characterised response in adult zebrafish is the alarm pheromone. The alarm reaction is a naturally occurring response in which zebrafish respond to the destruction of the epidermal club cells in conspecifics [23]. The release of alarm pheromone, or Schreckstoff, from these cells stimulates fear responses because it can indicate that a predator is nearby. The behavioural characterisation of the response to alarm pheromone in zebrafish has yielded varied results. Upon acute exposure to alarm pheromone, an increase in bottom dwell in a tank diving test was reported in some studies [25-27] but not others [28]. An increase in shoal cohesion was observed in some cases [27, 28]. An increase in the occurrence of erratic swimming was detected by [25, 26, 28]. An increase in the occurrence of freezing was observed by one study [25] but not in a similar analysis by another group [28]. Mathuru and colleagues also reported an increase in the number of slow swim episodes [26]. These differences are likely due to differences in methodology, for example alarm pheromone extraction procedure, exposure concentration, exposure duration, experimental tank size and shape, quantification of behavioural endpoints. The pharmacology of the alarm substance is currently unclear. It has been proposed that hypoxanthine 3-N-oxide is the common compound in all alarm substances and is sufficient to produce the alarm reaction [29]; however this has not been reliably detected in the skin of zebrafish. Mathuru *et al* proposed that the main component of the alarm pheromone is chondroitin, which was detected in the skin and reliably able to produce some components of the alarm response [26].

1.5 Disrupted-In-Schizophrenia-1

Mutation in DISC1 (Disrupted In Schizophrenia 1) is a risk factor for mental illness in humans. A chromosomal translocation was first discovered in DISC1 in a single Scottish family, in which many family members suffered from mental illness, including diagnoses of major depression, schizophrenia and bipolar disorder [30] (Figure 1.2). Interestingly, all relatives carrying the translocation exhibited a defect in their cognitive function during decision-making processes (P300 event-related potential), including those with no psychiatric condition [31].

The DISC1 protein is a large protein, consisting of a globular N-terminal domain, which has been demonstrated to interact with cAMP-hydrolysing phosphodiesterase 4B (PDE4B), and a helical C-terminal domain, which gives it the potential to interact with other protein(s)[4] (Figure 1.3). The translocation disrupts this coiled-coil region, however, no evidence for a truncated protein has been found and reduced *DISC1* transcript levels suggest that the consequence of inheriting the translocation is haploinsufficiency [32]. The protein is thought to act as a scaffold at the center of one or more protein interaction networks [5]. A number of proteins have been identified as interactors with DISC1, indicating a role for DISC1 in cell proliferation, differentiation, neuronal migration and myelination [5]. The relevance of *DISC1* to psychiatric illness in the general population is contentious [33, 34], however, the variety of conditions which manifest in the original human family make *DISC1* a promising candidate for the study of how environmental conditions interact with a genetic component, yielding a variety of phenotypes.

The zebrafish *disc1* gene is partially annotated on chromosome 13 as gene Q8AV88_DANRE (Ensembl Gene ID ENSDARG00000021895), which shows synteny with human chromosome 1. The full coding sequence of *disc1* is 3190 bp (base pairs) and encodes a 998 amino acid protein (Figure 1.3). The N-terminal half of the protein is poorly conserved, whereas the C-terminal portion shares 35% identity and 53% similarity over 543 residues with human DISC1. A COILS analysis demonstrates that the C-terminal domain has a high probability of forming coiled-coil regions, as does the human protein [30].

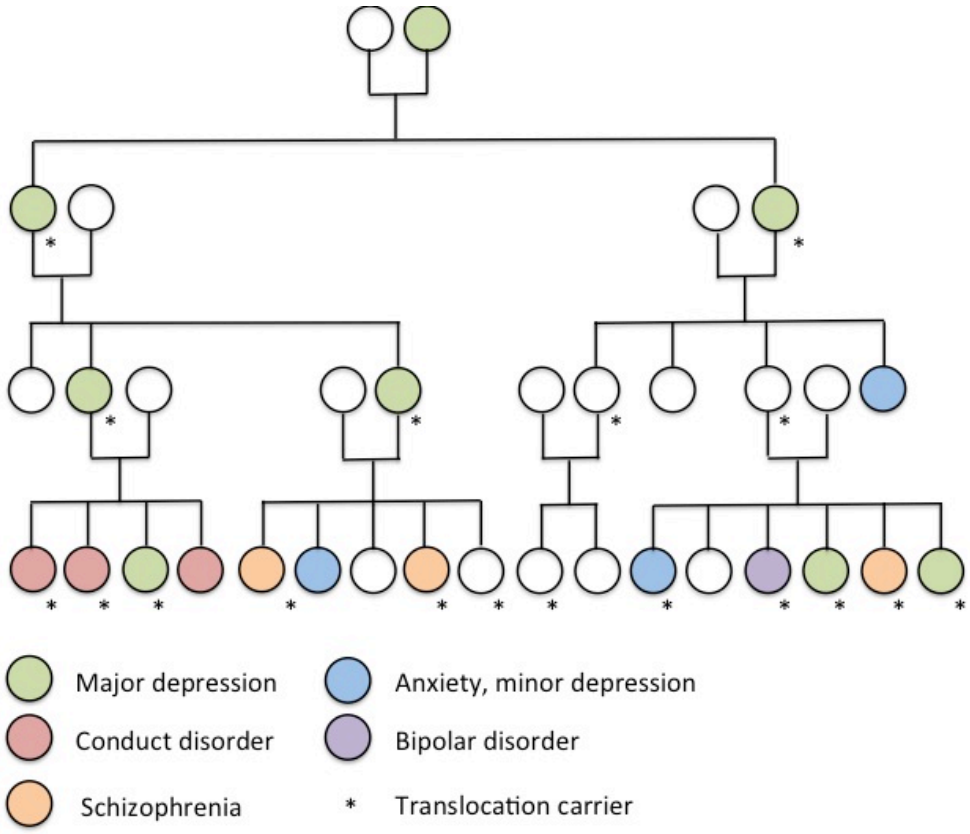

Figure 1.2. Part of the human family with DISC1 translocation. The DISC1 mutation co-segregates with major mental illness. Adapted from [25].

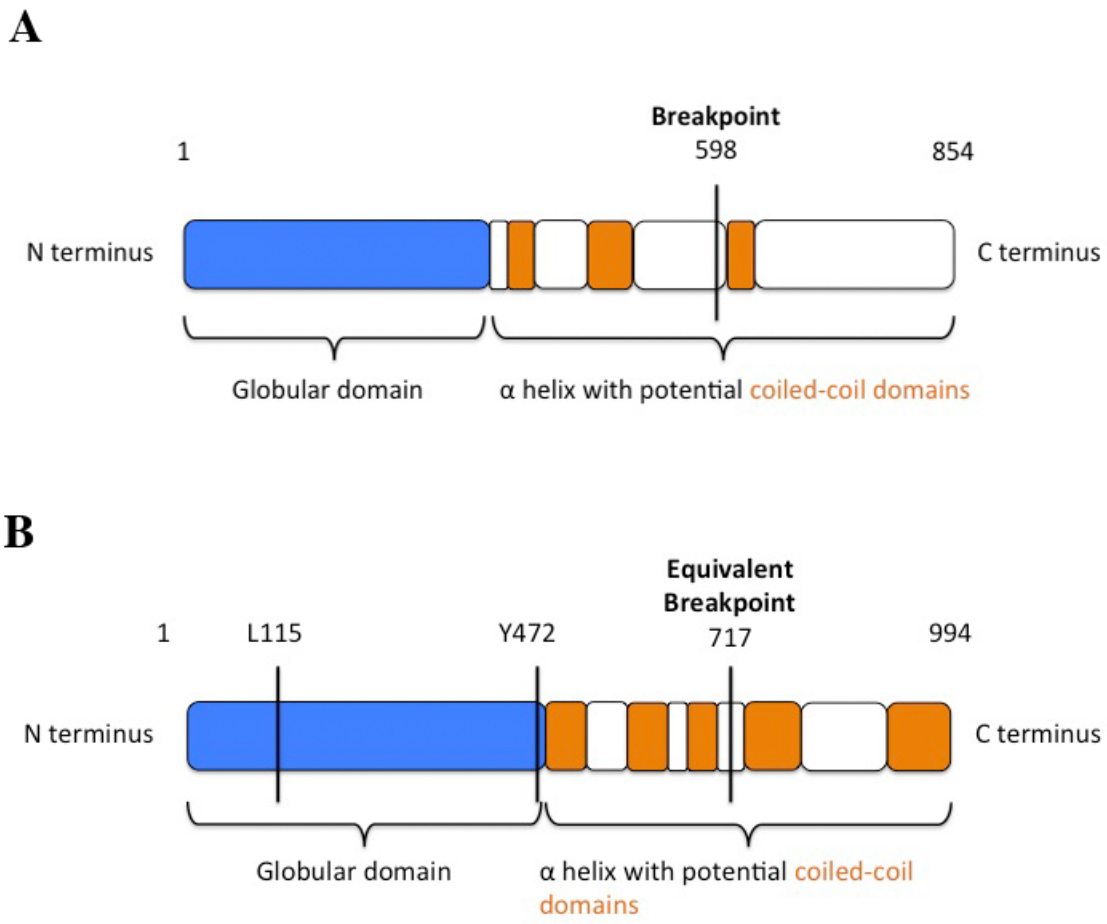

Figure 1.3. Schematic of the DISC1 protein in humans and zebrafish. Globular N-terminal domain in blue, alpha helix C-terminal domain in white with predicted coiled-coil domains in orange.

(A) Human DISC1 protein is 854 amino acids in length. Black line indicates breakpoint caused by the translocation. Adapted from [4].

(B) Zebrafish *disc1* protein is 994 amino acids in length. Black lines indicate the positions of the Y472 and L115 zebrafish mutations, along with the equivalent position of the human translocation breakpoint. [24].

The L115 and Y472 zebrafish lines harbour point mutations in *disc1*, which interrupt the N-terminal domain of the protein and are predicted null alleles, although this is unconfirmed.

Previous work on a number of mouse models has investigated the effect of *DISC1* on physiological, pharmacological, neuroanatomical and behavioural phenotypes and more recently, a link between *DISC1* and the HPA axis has been suggested. [7]. These studies have used either mice with *Disc1* point mutations or transgenic mice that express C'-truncated dominant negative human *DISC1*, as in the original Scottish family.

Strong evidence has come from studies in which the genetic and environmental stress components alone are not sufficient to trigger significant phenotypic changes as this allows thorough investigation into the gene-environment interaction [35]. Depending on the type of mutation and its predicted outcome for protein structure, phenotypes vary between models.

One study investigated the interaction between dominant negative *DISC1* and mild isolation stress during adolescence in mice [7]. The GXE (gene-environment interaction) mice displayed behavioural abnormalities in all of the tests carried out, in comparison with control (C), gene only (G) and environmental stress only (E) groups. These deficits were hyperactivity in a basic motility study and anxiety-related behaviours including increased immobility time in the forced swim test and reduced pre-pulse inhibition (PPI), which is a habituation to a loud noise after pre-exposure to another loud pulse of sound. After behavioural testing, the GXE mice also exhibited higher corticosterone levels, indicating higher levels of baseline stress. When searching for the underlying mechanism, the group found a significant decrease in dopamine levels in the frontal cortex of the GXE mice, but not in other areas of the brain. This was accompanied by a similarly specific decrease in the expression of the *tyrosine hydroxylase* (*TH)* gene. The TH enzyme catalyses the synthesis of a dopamine precursor. Administration of a glucocorticoid antagonist (RU38486) normalised levels of dopamine and the behavioural abnormalities in the GXE mice. The study also found a significant

increase in the level of DNA methylation of the *TH* gene in specific dopaminergic projections. This was maintained into adulthood after return to group housing. This epigenetic effect could also be normalised by the glucocorticoid antagonist.

This study shows how, despite normal brain architecture, functional changes in neurotransmission are sufficient to induce very different phenotypes. This work also names corticosterone as the key mediator in bringing about the downstream behavioural effects and suggests a role for epigenetic mechanisms in this process, although the interaction remains unknown. Despite only measuring the stability of the epigenetic alteration, this study suggests that stress-induced changes in the GXE group are stable into adulthood and leaves open the question of whether such changes might be heritable.

Similarly, mouse models with a point mutation in *Disc1* (L100P) show hyperactivity in the open field and behavioural abnormalities, such as decreased latent inhibition and prepulse inhibition [6], which are linked to schizophrenia in humans [36]. These behaviours could be normalised by treatment with Valproate in early adulthood [37], a drug often used as an anticonvulsant and mood-stabiliser, as well as the anti-psychotic Haloperidol [6]. These studies also identified higher transcript levels of *Lcn2* (*Lipocalin2*) in mutant mice. This transcriptional phenotype could be normalised by Valproic acid treatment and genetic ablation of *Lcn2* normalised behaviour in L100P mutants. This work demonstrates that early pharmaceutical intervention can prevent the onset of psychiatric phenotypes and presents *Lcn2* as a novel drug target [37]. In a subsequent experiment, L100P mice were combined with prenatal Poly I:C (polyinosinic:polycytidylic acid) infection. Poly I;C is a viral mimic which has been shown to induce a cytokine response in maternal serum, the amniotic fluid, placenta, and fetal brain [38] and maternal infection has been previously linked to development of schizophrenia [39]. This L100P: Poly I:C GXE group exhibited exacerbated deficits in PPI and sociability in comparison with L100P controls. This supports the evidence for an interaction between *DISC1* and environmental stressors.

Another study combined prenatal administration of poly I:C with a mouse model expressing mutant human *DISC1* [38]. These GXE mice exhibited neurobehavioural alterations including reduced social interactions, increased anxiety and depressive-like behaviours and a reduced volume of the amygdala and periaqueductal grey matter. Interestingly, the mutant mice exhibited a significant increase in cortisol levels when combined with an acute restraint stress, however this increase was significantly smaller than that seen in wild type mice and levels remained high during a recovery period.

A number of studies have demonstrated that *DISC1* is expressed in the hypothalamus [40, 41], the control centre of homeostasis and the HPA axis. These data further support a role for *DISC1* in the HPA axis.

1.6 Hypothalamus

The hypothalamus is an evolutionary ancient structure and the key regulator of homeostasis in higher vertebrates. Throughout early development the hypothalamus is shaped dynamically and the establishment of several distinct nuclei is crucial for its function. Despite its functional importance, the development of the hypothalamus is not well understood, but expression analysis of genetic markers for distinct regions and cell types can be used to inform our understanding of how early patterning contributes to the emergence of functionally distinct and complex hypothalamic nuclei.

The same neuropeptides and neurotransmitters of the hypothalamus are well conserved in the zebrafish and other vertebrates [14]. This allows for the characterisation of broad neuronal subtypes. In the mouse, the pathways via which hypothalamic progenitor cells are specified into mature neurons in defined nuclei are reasonably well characterized (Figure 1.4)[14]. Recent studies in our lab have analysed HPI axis genes in the hypothalamus of the zebrafish. These, and other published studies, reveal that expression of key hypothalamic genes in the mouse can also be used to define key regions in the zebrafish and that these are broadly similar to domains in other vertebrates. For example, genes implicated in the development of the hypothalamus such as *sf1 (steroidogenic factor 1)*, *fezf1 (Fez*

family zinc finger 1), *hypocretin* [42], *rx3* (*retinal homeobox gene 3*), *pomca* (*pro-opiomelancortin a), shha (sonic hedgehog a)* [43] display similarly restricted patterns of expression in hypothalamic neuronal subpopulations in zebrafish as in rodents. Although the architecture of the hypothalamic nuclei has not been well defined in zebrafish, expression analyses and functional data suggest strong conservation of the neuroendocrine hypothalamus [42]. HPI axis genes are first expressed in the hypothalamus at 24 hpf in the zebrafish, including *crf* (*corticotropin-releasing factor*), *sf1* and *pomca* at 32 hpf [44].

CRF is a conserved key regulator of the HPA axis. CRF is released from the hypothalamus and stimulates the release of ACTH from the pituitary. In the zebrafish, expression of *crf* begins at 6 hpf [45]. By 2 dpf *crf* positive cell clusters can be seen in the preoptic region, two areas of the hypothalamus, ventral telencephalon, posterior tuberculum, thalamus, rhombomeres r1-r4 and the inner nuclear layer of the retina [46]. The abundance of *crf* mRNA increases between 1 dpf and 5 dpf, concurrent with an activation of the HPI axis at 5 dpf [46]. In the adult zebrafish brain, *crf* is expressed in the olfactory bulbs, dorsal and ventral telencephalon, periventricular nucleus of the hypothalamus and the dorsal part of the trigeminal motor nucleus [47]. *CRF* has been previously implicated in locomotion behaviours in fish [48] and in reduced food intake [49].

RAX is a conserved transcription factor, which is expressed in the hypothalamus and retina. Expression of *RAX* is essential for formation of the eye fields in mice and zebrafish [50]. In the mouse, work has shown that *Rax* is also essential for normal development of the hypothalamus. *Rax*-positive progenitor cells give rise to *Pomc* and *Sf-1* neurons in the arcuate nucleus and VMN of the hypothalamus respectively [51]. Rax knock-out results in a loss of both *Pomc* and *Sf-1* cells and a concomitant expansion of *Dlx1* (*Drosophila distal-less)* positive and GABA-ergic neurons in the DMN (Figure 1.4) [51]. The teleost genome contains three *RAX* genes, of which *rx3* is believed to be the orthologue [52]. In the zebrafish, *rx3* is expressed at 8-9 hpf in the anterior neural plate that will give rise to the retina and forebrain and is later restricted to the lateral optic primordia and ventral medial diencephalon [52].

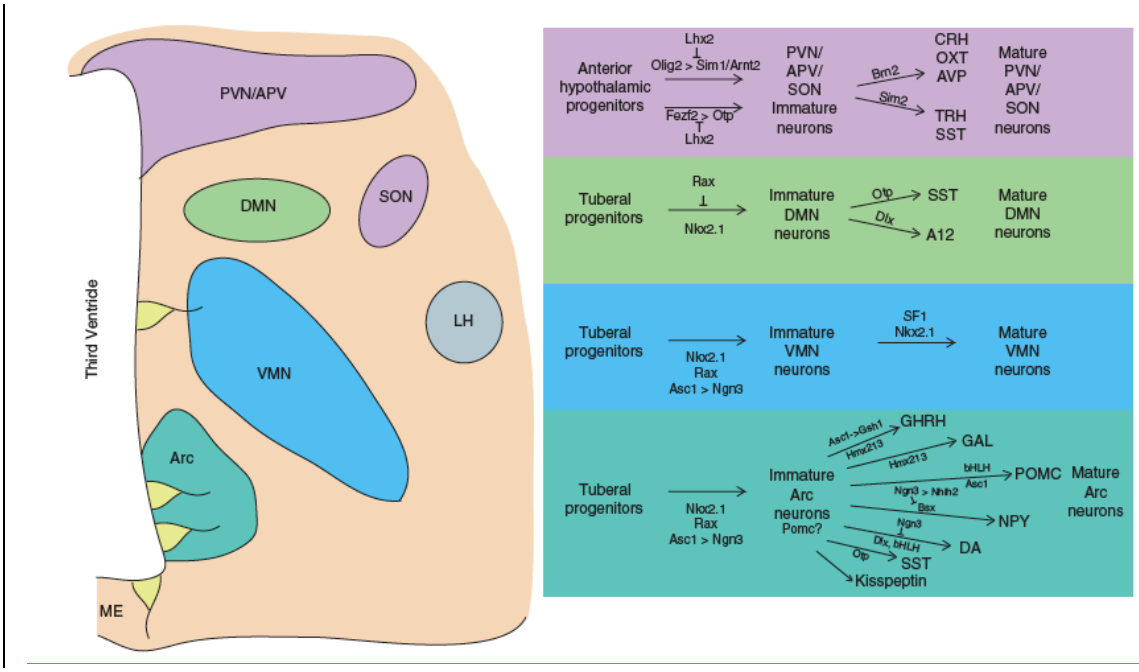

Figure 1.4. Arrangement of hypothalamic nuclei and neuronal differentiation. Figure reproduced with permission from [36]. Left-hand panel shows arrangement of nuclei around the third ventricle of the adult mammalian tuberal/anterodorsal hypothalamus, and shows tanycytes (yellow cells) bordering the third ventricle. Right-hand panel shows how known transcription factors direct anterodorsal hypothalamic and tuberal progenitors into immature and then mature PVN, APV, SON, DMN, VMN, and Arc neurons. PVN, paraventricular nucleus; DMN, dorsomedial nucleus; SON, supra-optic nucleus; LH, lateral hypothalamus; VMN, ventral-medial nucleus; Arc, arcuate nucleus; ME, median eminence.

From around 48 hpf, expression is restricted to the inner nuclear layer of the retina, the preoptic region and the anterior hypothalamus and is maintained throughout adulthood [52]. The teleost preoptic nucleus is considered to be homologous to the mammalian paraventricular nucleus [53]. In zebrafish, *rx3* mutants have no eyes and an expanded telencephalon, showing that *rx3* is essential in controlling specification of eye and telencephalon fields during early brain patterning [50]. A recent study in our lab has extended the work carried out in the mouse into zebrafish, and shown that zebrafish *rx3* mutant larvae similarly lose *pomc* and *sf1* expression [43].

SF-1 is a nuclear transcription factor, expressed in post-mitotic neurons of the VMH and in steroidogenic cells of the interrenal gland and also the ovaries and testes in mammals [54]. *Sf-1* has a wide variety of roles in the body's systems including reproductive, endocrine and central nervous systems [54]. In the mouse, *Sf-1* knockout leads to adrenal insufficiency, gonadal agenesis, sex reversal and is lethal [55]. Knockouts also have a completely disorganized VMN, and cells that should normally be situated inside the VMN are misplaced [42], suggesting a role for *Sf-1* in neuronal migration. Post-natal VMN-specific knockout shows that *Sf-1* is also essential for energy homeostasis and results in increased body mass via modulation of metabolism and energy expenditure [56]. Knock-out mice also have a number of behavioural abnormalities, including impairments in aggressive and sexual behaviour [57] as a result of impaired gonadal steroid signaling as well as increased anxiety behaviours in a light/dark test and elevated plus maze (a common rodent test for anxiety), and increased activity in an open field test [58]. In the zebrafish, the *Sf-1* homologue *ff1b* or *sf1* is first expressed at 22 hpf in the anterior-most neural tube and in the interrenal tissue from 30 hpf [59]; it later localizes to the ventral medial nucleus (VMN) of the developing hypothalamus and the interrenal gland and expression is maintained into adulthood [60]. Interrenal differentiation requires *sf1* [61] and this transcription factor directly stimulates the expression of cytochrome P450 side chain cleavage (P450scc), which is the rate-limiting step in steroidogenesis [62], as seen in mammals.

The POMC protein is involved in energy balance and is anorexigenic [49]. *POMC* is a precursor for many molecules including α-melanocyte-stimulating hormone (α-MSH) which activates catabolic circuits [49]. POMC is also a crucial component of the HPA axis, when secreted from the anterior pituitary it is cleaved, giving rise to products including adenocorticotrophin (ACTH), which binds its receptor at the interrenal gland and initiates cortisol synthesis [1]. In zebrafish, the *pomc* gene is expressed throughout the life course, in the putative corticotropic cells of the pituitary gland in the early embryo [63] and in the arcuate nucleus of the hypothalamus from 32 hpf [64].

1.7 Aims and objectives

The recent evidence for a link between *DISC1* and the HPA axis opens an avenue to investigate the role of *DISC1* in the stress response. This work is an initial investigation of the role of *disc1* in the stress response in zebrafish. I hypothesised that *disc1* mutation would result in abnormalities in baseline behaviour, and modulate the behavioural and endocrine response to stress in the zebrafish. The expression of *DISC1* in the hypothalamus of other vertebrates and the link with the HPA axis lead me to hypothesise that development of the hypothalamus would be altered by *disc1* mutation. Fish were assessed on the basis of a number of criteria, including: analysis of control and stress-induced swimming behaviour using automated tracking software, measurement of cortisol in control and stress-exposed fish, and expression analysis of genes relevant to the HPI axis in the hypothalamus via *in situ* hybridization. In the long term, this research aims to further our understanding of the role of gene-environment interactions in determining susceptibility to psychiatric disorders.

2 Materials and Methods

2.1 Behavioural zebrafish methods

2.1.1 Adult zebrafish husbandry

Adult zebrafish were maintained in standard conditions under a 14 hour light/ 10 hour dark cycle at 28.5°C, at the University of Sheffield. Adult *disc1* fish were kept in groups of the same genotype, with equal sex ratios, at comparable densities and in the same size of tank (14 fish per 10 l tank). The wild type, heterozygous and homozygous *disc1* mutants used for adult behavioural analysis were siblings produced from in-crossed heterozygotes. Handling of fish was kept to a minimum in order to minimize stress and all fish were experimentally naïve.

2.1.2 Larval zebrafish husbandry

Embryos were staged according to Kimmel [65] and are written in hours post fertilization (hpf) and days post fertilization (dpf). Embryos were obtained using the 'marbling' technique [66]and raised at 28.5 °C until 5 dpf in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$,). Larvae described as 'wild type in-cross' or 'homozygous in-cross' were produced from an in-cross of adult *disc1* wild type siblings or homozygous mutants respectively and thus were not siblings. In contrast, larvae described as 'wild types', 'heterozygotes' and 'homozygous mutants' were siblings produced from an in-cross of heterozygous mutants, which have subsequently been genotyped. Unless otherwise specified, larvae were reared in standard 50 ml/90 mm petri dishes (Thermo Scientific) in groups of approximately 50 per dish, or 21 per dish when fish were raised for shoaling analysis. For the larval housing condition experiment, larvae were raised in standard 48 well plates (Sigma Aldrich) containing E3 medium with one fish per well or in groups on 40 in a standard petri dish. This ensured that both groups were housed within an equal volume and surface area of E3. Larvae that were reared for analysis at 8 dpf and 12 dpf were kept at 26 °C in the same housing (petri dish or multi-well plate) but with daily water changes and feeding from 5 dpf onwards. Disturbance of the developing larvae was kept to a minimum and all fish were experimentally naïve. Larvae were monitored twice daily and dead and

developmentally delayed or abnormal larvae were immediately removed in order to maintain good water quality.

2.1.3 Measurement of larval body size

The *disc1* L115 and Y472 wild type in-cross and homozygous mutant in-cross larvae were measured at 5 dpf using images taken from above, during behavioural analysis. Body length was measured from the anterior most point of the head to the posterior tip of the tail. Head width was measured as the longest distance across the eyes.

2.1.4 Quantification of larval swimming behaviour

On the morning of testing, each zebrafish larva was transferred into one well of a multi-well plate in 1 ml E3 medium using a 3 ml Pasteur pipette, and allowed to acclimate for 1 hour prior to testing (Figure 2.1 A). Individuals of each genotype were distributed randomly across all wells of the plate. Larval swimming behaviour was then quantified using the ZebraLab behavioural tracking software (ViewPoint) (Figure 2.1 B). The multi-well plate was placed into the ZebraBox (ViewPoint) and movement of individual larvae was filmed from above. The test was carried out in the dark over 10 minutes or 1 hour, with measurements taken at 1 minute or 10 minute intervals respectively. Swimming distance (mm), duration (s) and count (n) as well as immobility/freezing duration and count during each time interval were analysed. Swimming speeds were categorized as follows: > 6 mm/s fast swimming; 3-6 mm/s normal swimming; < 3 mm/s slow swimming. Place preference analysis was carried out by analysing movement in the central and peripheral areas of the well of a 12 well plate, which were equal in surface area.

2.1.5 Quantification of light responsiveness

All larvae were acclimated to the lit testing chamber for 10 minutes prior to analysis. The white light was emitted from below (8 banks of 3 white LEDs, 'Nichia' NSDW510GS-K1-B5P9) in a uniform distribution and intensity was set at 2% of maximum. In the light-dark experiment, larvae were exposed to alternating 1 minute intervals of light on/off for 4 minutes.

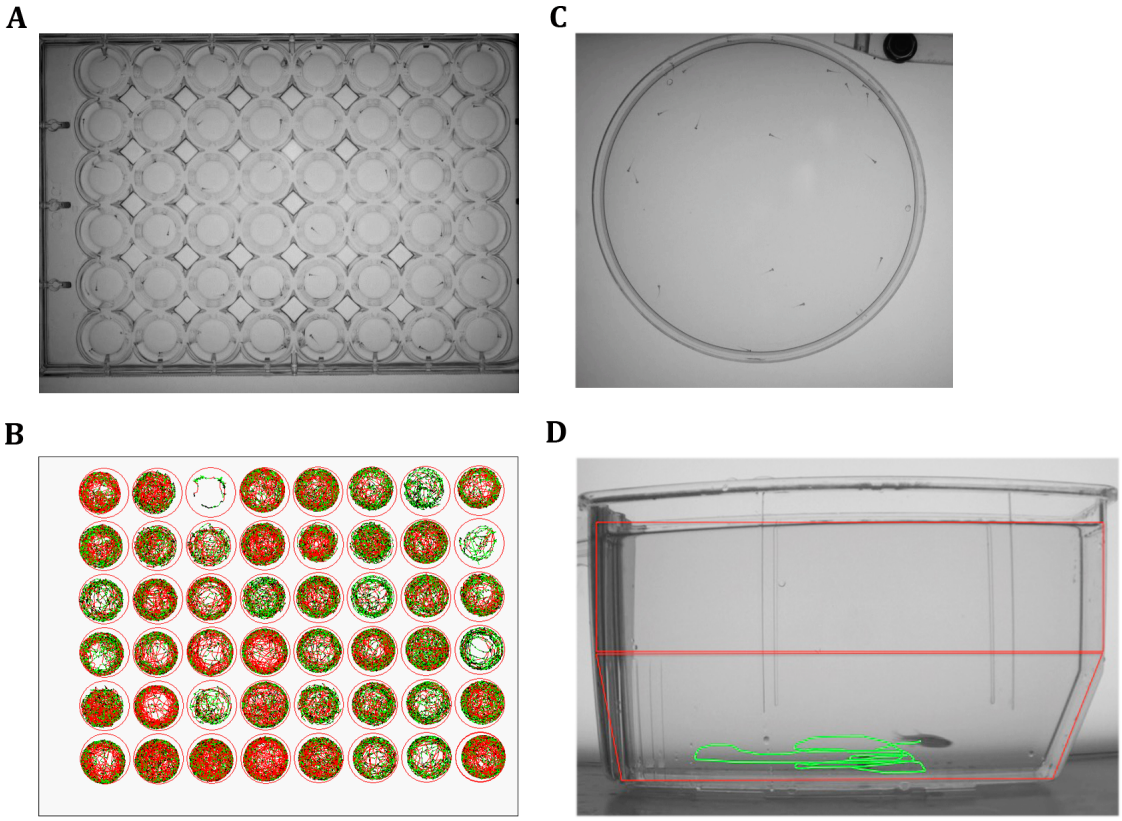

Figure 2.1. Automated behavioural tracking of larval and adult zebrafish.
(A) Basic swimming behaviour in larvae was performed on individual larvae in each well of a multi-well plate.
(B) Tracking lines of larval zebrafish. The ViewPoint automated tracking software tracks movement of each fish in the well and provides quantitative data. Colours represent movement categories: red, fast swimming; green, slow swimming; black, inactive.
(C) Shoaling analysis of larval zebrafish. 21 zebrafish larvae are housed in a petridish. The software provides mean values for the shoaling behaviour of the group.
(D) Analysis of individual adult zebrafish in a tank diving test, showing tracking line. The movement performed in the upper and lower compartment of a novel tank is analysed.

Swimming behaviour and place preference was quantified in each interval. In the dark flash experiment, larvae were exposed to a 1 second dark flash, with 1 minute of light exposure either side. Distance swam pre-, during and post- stimulus was analysed.

2.1.6 Quantification of larval shoaling behaviour

Zebrafish larvae were allowed to acclimate for 1 hour prior to testing in the behavioural analysis room. Shoaling behaviour of the 21 larvae was quantified using the 'Shoaling' behavioural tracking software (ViewPoint) (Figure 2.1 C). The petri dish was placed into the ZebraBox (Viewpoint) and movement of larvae was filmed from above. The test was carried out in the dark for 10 minutes, with mean measurements for all fish taken at 1 minute intervals. Quantification of behavioural parameters by the Viewpoint software was based on the formulae given in [67]. Nearest neighbour distance (NND) was defined as the mean distance between each fish and its nearest neighbour (Figure 2.2 A). Polarization was the magnitude of the mean vector of all the fish (Figure 2.2 B-C). Briefly, this was the distance that the mean group position travels during the experimental period. This means that polarization was not only affected by the movement of the fish in a given direction, but also by how fast the fish were moving.

2.1.7 Adult behavioural analysis

Adult zebrafish were separated into separate sexes and left to acclimatise for a minimum of 16 hours in the behavioural analysis room. Fish were fed in the morning prior to testing and all tests were carried out between 10.00 and 16.00. All behavioural analysis was carried out under controlled and consistent lighting, at 26 °C and within a soundproofed booth. Fish were transferred individually into the experimental tank and the test was started immediately. Swimming behaviour was tracked using automated tracking software (ViewPoint) (Figure 2.1 D). Swimming speeds were categorized as follows: >9 cm/s fast swimming; 2-9 cm/s normal swimming; < 2 cm/s slow swimming.

2.1.7.1 Open field test

In this test, fish were placed into an empty 20 l rectangular tank. The fish was

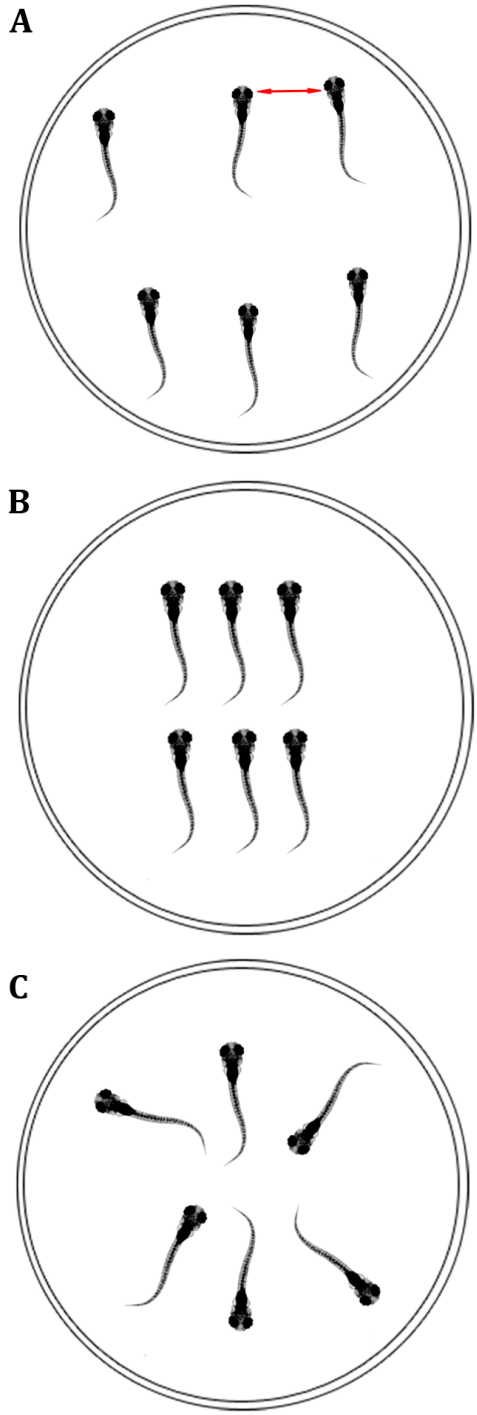

Figure 2.2. Schematic of the group swimming behaviours quantified during larval group swimming behavioural analysis.
(A) The nearest neighbour distance (NND) is the mean distance between each fish and it's nearest neighbour.
(B) A high polarization value suggests that more of the larvae are swimming in the same direction, therefore the mean group position changes a large amount.
(C) A low polarization value suggests that more of the larvae are swimming in different directions, therefore the mean group position changes only a small amount.

filmed from above for a duration of 5 minutes. The movement data was divided into that occurring around the perimeter of the tank and that in the central area, ensuring that both regions have equal surface area (Figure 2.3 A).

2.1.7.2 Scototaxis test

In this test, a fish was placed into a 15 l rectangular tank, which was divided into a light compartment and a dark compartment (Figure 2.3 B). The fish was filmed from above for 5 minutes and the swimming behaviour occurring in the light compartment was recorded, as well as the time spent in each compartment.

2.1.7.3 Tank diving test

This test is similar to the open field test, but filmed from the side, therefore measuring vertical swimming behaviour. The experimental tank was trapezoid and either 20 l or 3 l in experiments where alarm pheromone was used. Upon analysis, the vertical area of the tank was divided into two sections (upper and lower) and swimming behaviour in each section was recorded (Figure 2.3 C).

2.1.8 Application of chemical stressors

Chemical stressors were added to the swimming water of larval zebrafish immediately prior to beginning the behavioural analysis by pipetting the liquid into the centre of the petri dish or well slowly, whilst trying to keep disturbance to the larvae to a minimum. Chemical stressors were added to the water in the experimental tank of adult zebrafish, immediately prior to the addition of the fish. NaCl was added at a final concentration of 250 mM in accordance with [68].

2.1.9 Alarm pheromone extraction (Protocol 1)

Alarm pheromone extraction procedure was adapted from the protocol described by Speedie and colleagues [28]. Alarm pheromone was extracted and prepared on the morning of testing and kept on ice. Adult zebrafish of different sexes were culled, washed in aquarium water and blotted with a paper towel. Ten shallow cuts were made on each side of the trunk of each donor zebrafish using a razor blade, making sure not to contaminate the solution with blood. Each fish was then washed with 10 ml of dH_2O on each side and shaken at 40-50 for two minutes.

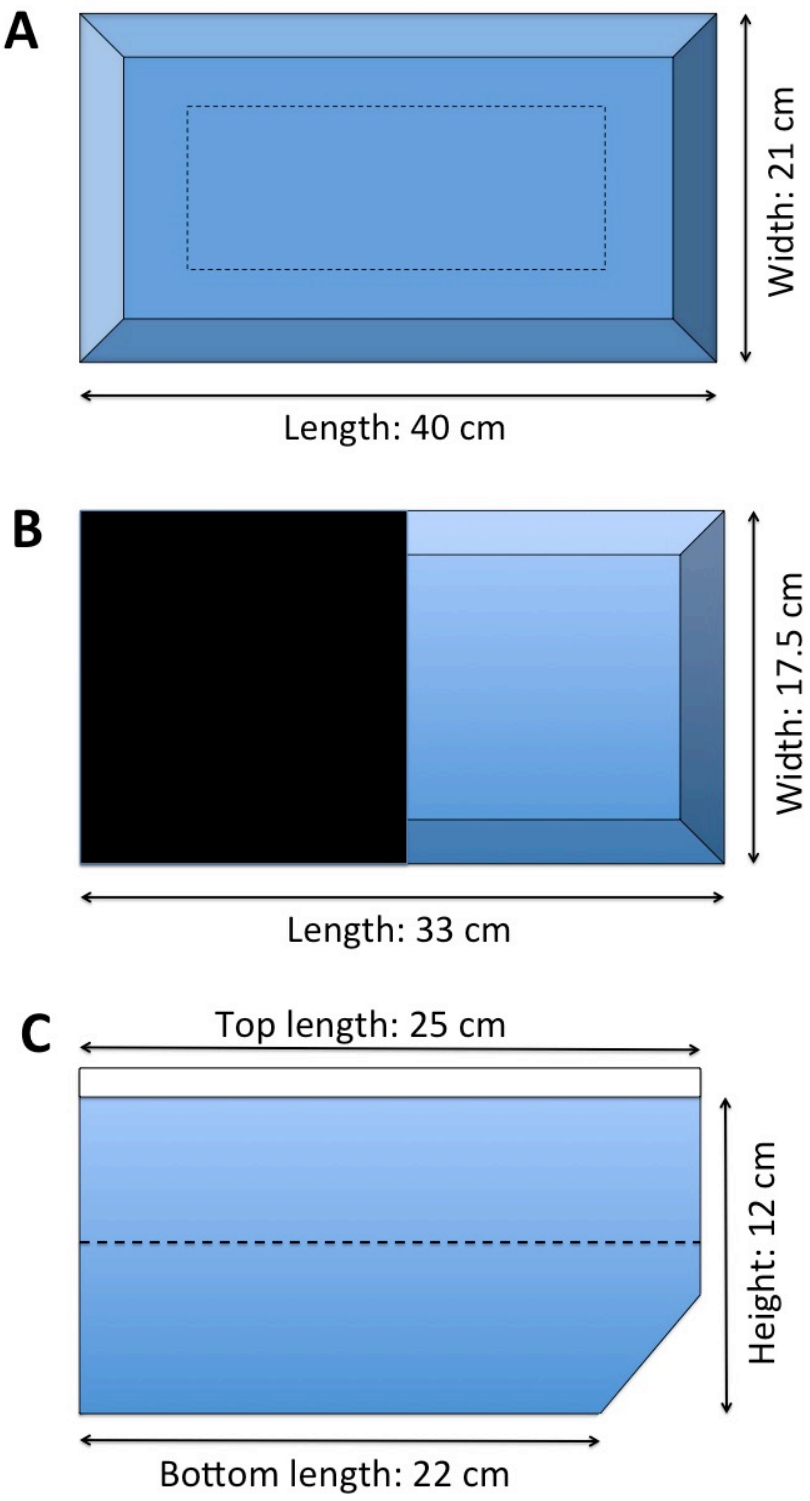

Figure 2.3. Experimental tanks for behavioural analysis of adult zebrafish.
(A) Open field test. Tank as viewed from above. Height is 25 cm. Dotted line represents division of the perimeter and central area.
(B) Scototaxis test tank as viewed from above. Height is 18.5 cm. Dark compartment of tank is covered by a black material on all sides.
(C) Tank Diving test, lateral view. Dotted lines represent divisions of the lower and upper areas. Width is 6 cm.

The solution was then collected in a 50 ml tube and used the same day. Alarm pheromone was used at a final dilution of 1 in 1000 in accordance with [28].

2.1.10 Alarm pheromone extraction (Protocol 2)

Alarm pheromone extraction procedure was adapted from the protocol described by Mathuru and colleagues [26]. Exposure to alarm pheromone extracted using protocol 1 yielded some varied results, with behavioural responses of wild type fish differing between experiments, in which different batches of extract were used (see Chapter 3.2.6). For this reason a new extraction protocol was utilized for future experiments. The extract was prepared from 4-5 euthanized zebrafish, by inducing 10 shallow lesions with a blade (No.5 scalpel blade), being careful not to draw blood. Fish were then immersed into 2 ml aquarium water in a 20 ml tube and rocked for 2 minutes. The 2 ml extract was heated for 4 hours to overnight at 95 °C. Next the extract was centrifuged at 13 000 rpm for 10 minutes. The supernatant was then filtered using a 0.45 μm filter (Minisart syringe filter, sartorius stedim biotech) and 5 ml syringe and kept at 4 °C for up to 10 days. Alarm pheromone was used at a final dilution of 1 in 5000 in accordance with [69].

2.1.11 Data analysis

Technical replicates indicate independent experiments. The N numbers indicates the number of biological replicates, within each experiment/technical replicate. Technical replicates were combined after significant differences between replicates were ruled out. This factor was then removed from the model for subsequent analyses. All data analyses were carried out using Microsoft Excel 2011 and R (Version 3.0.1, The R Foundation). The Shapiro-Wilks test was used to test for a normal distribution of data and data sets were tested for all assumptions of any statistical test to be carried out. If the data set did not fulfil the assumptions of a statistical test then a data transformation may have been applied or a non-parametric test was used. Paired or two-sample t-tests were used to test for significant differences between means of measurements between two samples for paired and non-paired samples respectively. Where data were ranks or non-normally distributed, a Wilcoxon test or Mann-Whitney U-test was used. A One-way or two way ANOVA was used to test for significant differences between means

of measurements between more than two samples, classified by one or more factors. Where data were ranks, non-normally distributed or variance within the factors was not equal a Kruskal-Wallis test was used with pairwise comparisons using Tukey and Kramer (Nemenyi) test. A Tukey Multiple Comparison test was used post-ANOVA to identify between which means a significant difference occurred. Repeated measures analysis was used for data collected at multiple time points on the same individuals and post-hoc analysis was carried out using pairwise comparisons. Time was considered as a continuous variable.

2.2 Molecular methods

2.2.1 Genotyping of *disc1* mutants

2.2.1.1 Tissue preparation

Adult zebrafish used for producing embryos and for behavioural were genotyped at 3 months of age. Fish were anaesthetized, a clip of no more than a third of the caudal fin was taken and fish were then stored in individual tanks until identification. Larvae were culled post behavioural analysis and either the whole body or tail only was used for extraction of DNA.

2.2.1.2 DNA extraction

The tissue was added to 100 µl of 50 mM NaOH, heated at 98 °C for 5 minutes and cooled on ice for 10 minutes. 10 µl of 1 M Tris pH 8 was added and samples were vortexed and then centrifuged at 13 000 rpm for 10 minutes. DNA extractions were stored long-term at -20 °C.

2.2.1.3 Genotyping of *disc1* Y472 fish

Y472 DNA extracts were then amplified using a nested PCR (Polymerase chain reaction) and sequenced at the University of Sheffield Core Genomics Facility. The Y472 primers (Box 1) and genotyping protocol were obtained from Jon Wood (University of Sheffield). In a 10 µl reaction volume containing a PCR ready-mix (FirePol, Solis Biodyne), 1 µl of DNA was amplified. An 800 bp fragment of DNA containing the Y472 mutation was amplified using primers 2C-1 and 2C-4 using

PCR programme 1 (Box 2). One microliter of this template was then used in a 20 µl PCR reaction volume to amplify a 600 bp region with primers 2C-2 and 2C-3 using PCR programme 2 (Box 2).

Prior to sequencing, unwanted contaminants such as unbound dNTPs (deoxynucleotides) and primers were removed by adding 5 µl of PCR product to 1 µl Shrimp Alkaline Phosphatase (NEB), 0.05 µl Exonuclease I (NEB) and 3.95 µl H_2O and heated at 37 °C for 45 minutes then 80 °C for 15 minutes. Final products were then sequenced using the m13 reverse primer. Sequence data were analysed using the Finch TV (Geospizer, Perkin Elmer) programme and genotypes were allocated by identifying the nucleotide indicated in Box 3.

2.2.1.4 Genotyping of *disc1* L115 fish

L115 DNA extracts were amplified using a single PCR and restriction digest. DNA was amplified as before in a 10 µl reaction volume containing a PCR ready-mix (Readymix, Sigma Aldrich) using PCR programme 3 (Box 2). Primers which amplify a 202 bp region containing the L115 mutation were used (Box 1). A 20 µl volume of digest solution containing 5 units DdeI was added to the PCR reaction following amplification and DNA product was digested for 4 hours or overnight at 37 °C. Products were run on a 2 % agarose TAE gel. DdeI cuts the mutant DNA, giving bands of 172 bp and 30 bp length.

2.2.2 Anesthesia and euthanasia

Tricaine (1.53 mM Ethyl 3-aminobenzoate methanesulfonate salt, Sigma; 21 mM Tris, pH 7) was diluted to 4.2 % to anaesthetize adult fish prior to fin clipping for genotyping. Adult zebrafish and larvae over 5 dpf were culled by an overdose of Tricaine (100 %) and decapitation when brain tissue was required. Adult zebrafish were culled via destruction of the cranium when tissue was required for endocrine analysis.

2.2.3 Fixation of tissues

Adult zebrafish brains were dissected out on ice immediately following culling post- behavioural analysis, whilst larval zebrafish were collected in a 1.5 ml tube

Box 1: **Zebrafish *disc1* genotyping primers**

Y472-2C-1	CCTCCATCTGCTTTAATGAATC
Y472-2C-2	TGTAAAACGACGGCCAGTAATGAACCAATCAGAAACCAG
Y472-2C-3	AGGAAACAGCTATGACCATAAGATAAACTTGATCCTCACTTAAAAC
Y472-2C-4	GGCGAGTATATAACAACCCTGATATTG
L115-FWD	ACTCATCAAAGTCTTCAAATAAACACCCTT
L115-REV	GACTTCAGCTGGTGCCCTGA

Box 2: **Zebrafish *disc1* genotyping PCR profiles**

Programme 1: 94 °C for 2 mins 20 s
35 cycles of 94 °C for 45 s
53 °C for 45 s
72 °C for 90 s
72 °C for 7 mins

Programme 2: 94 °C for 3 mins
35 cycles of 94 °C for 45 s
58 °C for 45 s
72 °C for 90 s
72 °C for 7 mins

Programme 3: 94 °C for 2 mins 30 s
35 cycles of 94 °C for 30 s
60 °C for 30 s
72 °C for 90 s

Box 3: Zebrafish *disc1* sequence analysis:

L115: 5'-CAT TT(A)G CAT GAT-3' TTG = Leu, TAG = Stop
3'-GTA AA(T)C GTA CTA-5'

Search for ATCATGC – followed by A = WT
followed by T = Mutant
followed by A/T = Het

Y472: 5'-AAG TAT(A) GAG GAT-3' TAT = Tyr, TAA = Stop
3'-TTC ATA(T) CTC CTA-5'

Search for ATCCTC - followed by A = WT
followed by T = Mutant
followed by A/T = Het

(Eppendorf) using a 3 ml Pasteur pipette. Adult fish brains and whole larval zebrafish were then fixed in 4 % paraformaldehyde (1.33 M paraformaldehyde (Sigma), 0.12 M phosphate buffer, pH 7) overnight at 4 °C. When necessary for whole-mount imaging, larvae were then bleached in 3 % H_2O_2, 0.5 % KOH in PBS for approximately 20 minutes until the pigmentation was removed. Larval zebrafish and adult brains were then dehydrated in methanol and stored at -20 °C at least overnight prior to whole-mount *in situ* hybridization.

2.2.4 Synthesis of DIG-labelled probe

2.2.4.1 Plasmid DNA constructs

RNA probes for *in situ* hybridization were made for the detection of the following genes: *sf1 (nr5a1a, ff1b), pomca, rx3, crf, disc1*. The plasmids containing the DNA constructs were obtained from the following: *sf1*, Vincent Laudet lab, University of Lyon; *pomca*, Vincent Cunliffe lab, University of Sheffield; *rx3,* Jarema Malicki lab, University of Sheffield; *crf*, Vincent Cunliffe lab, University of Sheffield; *disc1*, Jon Wood lab, University of Sheffield.

2.2.4.2 Bacterial subcloning and plasmid extraction

Plasmid DNA contained the construct of interest was suspended in 30 µl dH_2O and stored long-term at -20 °C. The plasmid DNA was subcloned into E.coli cells (DH5α competent cells, Invitrogen) by mixing 1-10 ng of DNA with 50 µl of cells and incubating on ice for 30 minutes. The cells were then heat-shocked for 20 seconds in a 42 °C water bath and then placed on ice for 2 minutes. Pre-warmed SOC medium (Super Optimal Catabolite Medium, ThermoFisher Scientific) was then added and the cells were incubated for 1 hour at 37 °C at 225 rpm. Between 50 µl and 150 µl of the transformation was spread on pre-warmed selective LB plates (*Luria Bertani*) and incubated overnight at 37 °C. The bacterial colonies were then propagated in LB culture broth as described in Molecular Cloning: A Laboratory Manual [70]. The amplified plasmid was then extracted from the bacterial culture and purified using the Maxi-Prep Plasmid extraction kit (Qiagen).

2.2.4.3 Template preparation

Linearization of the plasmid was achieved by cutting 10-20 µg with the appropriate restriction enzyme. DNA was then purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v; Invitrogen). The organic phase was collected and back-extracted with an equal volume DEPC-treated water to increase yield. The DNA was then precipitated with 1/10 volume 3 M sodium acetate pH 5.2 and 2.5 volumes EtOH at -20 °C for 1 hour to overnight. The precipitate was then centrifuged and re-suspended in 10-20 µl DEPC-treated water. DNA yield was assayed using a NanoDrop spectrophotometer (Thermo).

2.2.4.4 Transcription reaction

The DNA was transcribed *in vitro* in a reaction volume of 20 µl, containing 1 µg DNA, 1 x transcription buffer (NEB), 1 x DIG labelling mix (Roche), 2 µl RNA polymerase (NEB) and RNase inhibitor (NEB). The reaction solution was mixed gently, spun down briefly in the centrifuge and incubated for 2-5 hours at 37 °C.

2.2.4.5 Probe purification

After successful transcription reaction, DNase buffer and 5 units RNase-free DNase I were added and the reaction was incubated for a further 15 minutes at 37 °C. The probe was then precipitated with 2.5 µl 4 M LiCl and 75 µl ethanol at -80 °C for one hour to overnight. The precipitate was then centrifuged at 13 000 rpm, at 4 °C for 20 minutes and the pellet was washed with 100 µl RNA grade 70 % ethanol and re-centrifuged for 15 minutes at 4 °C. The pellet was then air-dried for 15 minutes at room temperature and re-suspended in 50 µl DEPC-treated water. The purified probe was analysed via electrophoresis on a 1 % agarose gel, alongside samples taken after the synthesis and template digestion reactions. When a clear band of expected size was seen on the gel, 50 µl deionised formamide was added to the probe and it was stored long-term at -20 °C.

2.2.5 Whole-mount *in situ* hybridization of larval zebrafish

Standard *in situ* hybridization is a three day protocol and was performed on whole fixed zebrafish larvae that had been stored in methanol in 1.5 ml tubes, using the desired RNA probe. All washes were 1 ml unless otherwise specified and larvae

were rocked gently during each wash. On day one, zebrafish larvae were rehydrated through a methanol: PBS series, washed five times in PTW (PBS with 0.1 % Tween 20) and then incubated in 10 µg/ml Proteinase K (Sigma) in PTW for the following time periods: 24 hpf, 10 minutes; 48 hpf, 20 minutes; 3 dpf, 30 minutes; 4 dpf, 40 minutes; 5 dpf, 50 minutes. Fish were then re-fixed for 20 minutes at room temperature, washed five times with PTW and incubated in 250 µl hybridization solution (50 % Formamide, 5x SSC, 9.2 mM Citric acid, 0.1 % Tween 20, 50 µg/ml Heparin (Heparin sodium salt from porcine intestinal mucosa, Sigma), 0.5 mg/ml tRNA (tRNA from baker's yeast, Sigma)) for 2-4 hours at 65-70 °C. Finally, larvae were hybridized overnight in 200 µl pre-heated hybridization solution with 1:200 DIG-labelled probe at 65-70 °C.

On day two, the probe solution was removed and larvae were transferred through a dilution series into pre-warmed SSC solutions at 65-70 °C: 20 minutes in 50:50 hybridization solution (lacking the Heparin and tRNA): 2xSSC; 20 minutes in 2xSSC; two times 1 hour in 0.2xSSC. Larvae were then transferred through a dilution series into PBT solutions (PTW with 2 % sheep serum and 0.2 % BSA (albumin, from bovine serum, Sigma)) at room temperature: 10 minutes in 50:50 0.2XSSC:PBT; 10 minutes in PBT; then blocked for 2-4 hours in PBT. Finally, larvae were incubated overnight at 4 °C in PBT with 1:2000 anti-DIG-AP (Roche).

On day three, the larvae were washed six times for 20 minutes in PTW at room temperature and then equilibrated four times for 10 minutes in staining buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1 % Tween 20). Larvae were then transferred to a multi-well plate for staining and incubated in staining buffer with 3.5 µl/ml BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt, Roche), and 4.5 µl/ml NBT (nitro blue tetrazolium chloride, Roche) in the dark until signal was developed. Larvae were periodically monitored under a dissecting microscope for signal development. Development was stopped by washing three times for 5 minutes in PTW and fish were then fixed overnight at 4 °C.

2.2.6 Whole-mount *in situ* hybridization of adult zebrafish brain

The protocol used for whole-mount *in situ* hybridization of adult zebrafish brains was identical to that used in the larval assay unless otherwise stated. This was again a three day protocol and was performed on whole fixed zebrafish brains that had been stored in methanol in 1.5 ml tubes, using the desired RNA probe. All washes were 1 ml unless otherwise specified and brains were rocked gently during each wash. On day one the rehydration steps and PTW washes were carried out as previously described. Brains were then digested with 100 µg/ml Proteinase K in PTW for 30 minutes. The fixation, PTW washes and hybridization steps were then carried out as previously described. On day two, brains were transferred through a more gradual dilution series from hybridization solution (lacking Heparin and tRNA) into SSC solution at 65-70 °C: 75 % hybridization solution: 25 % 2xSSC for 15 minutes; 50:50 hybridization solution: 2xSSC for 15 minutes; 25 % hybridization solution: 75 % 2XSSC for 15 minutes; 2xSSC for 15 minutes; and 0.2XSSC for two times 40 minutes. Next, brains were transferred through a more gradual dilution series into PBT at room temperature: 75 % 0.2xSSC: 25 % PBT for 10 minutes; 50:50 0.2xSSC: PBT for 10 minutes; 25 % 0.2xSSC: PBT for 10 minutes; PBT for 10 minutes. Samples were then blocked in PBT for 3-4 hours at room temperature and then incubated in antibody overnight as before. Day three of the protocol was carried out as previously described.

2.2.7 Cryosectioning of zebrafish tissue

After whole-mount *in situ* hybridization, zebrafish tissues were washed twice in PBS and then transferred to 30 % sucrose at 4 °C for at least overnight to prepare for cryosectioning. Tissue was then mounted in OCT medium (VWR) onto a cryostat chuck on dry ice. The chuck was stored in the cryostat for at least 20 minutes prior to sectioning to allow for temperature adjustments. Transverse sections were cut through larval zebrafish at 12 µm and 15 µm through adult brain tissue on the cryostat (Bright). Sections were collected on Superfrost Plus microscope slides (Thermo Scientific) and dried for at least an hour at room temperature. Slides were then washed briefly with PBS to remove OCT and coverslips (22 x 64 mm, thickness No.1, VWR) were mounted on the slides using Glycergel mounting medium (Dako) or Vectashield mounting medium with DAPI

(Vector). Coverslips mounted with Vectashield were then secured at the edges with clear nail varnish (Boots). Slides were then dried overnight before imaging. Sections from the anterior, mid and posterior hypothalamus of the larval zebrafish were compared and positioning was determined using markers such as the shape of the 3rd ventricle and position relative to the optic nerve (Figure 2.4).

2.2.8 Image acquisition

Standard DIC and fluorescent images of sections and whole-mount zebrafish were captured using an Olympus BX60 and QCapture programme (QImaging). Whole-mount larval zebrafish were transferred into glycerol after *in situ* hybridization for imaging. On microscope slides (Super Premium, VWR), small wells were crafted by layering 1-3 pieces of electrical tape, depending on the developmental stage, and cutting a rectangular well in the centre. The larva was then placed in the well in glycerol and mounted with a coverslip (22 x 22 mm # 1, VWR) for imaging. Images were organised into composites using Adobe Photoshop CS5. Illustrations were constructed using Adobe Illustrator CS or Microsoft PowerPoint 2011.

2.2.9 Cell counting

The number of *pomc*-positive cells in the hypothalamus and pituitary (Figure 2.5) was counted in cryosections after labelling via whole-mount *in situ* hybridization. The number of labelled cells within the hypothalamus and pituitary was counted in each section by eye. Merging of adjacent sections determined only a small amount of overlap of the cells labelled in each section, and that these could be easily distinguished in order to avoid duplication (Figure 2.6). The anterior-posterior length of each region was determined using the thickness and number of sections between morphological markers. Density was determined by dividing the total number of cells in each region by the region length.

2.3 Endocrine methods

2.3.1 Preparation of samples

Post behavioural analysis, processing of tissues was done as quickly as possible, so as to capture a view of the physiology of the animals at the end of the assay and

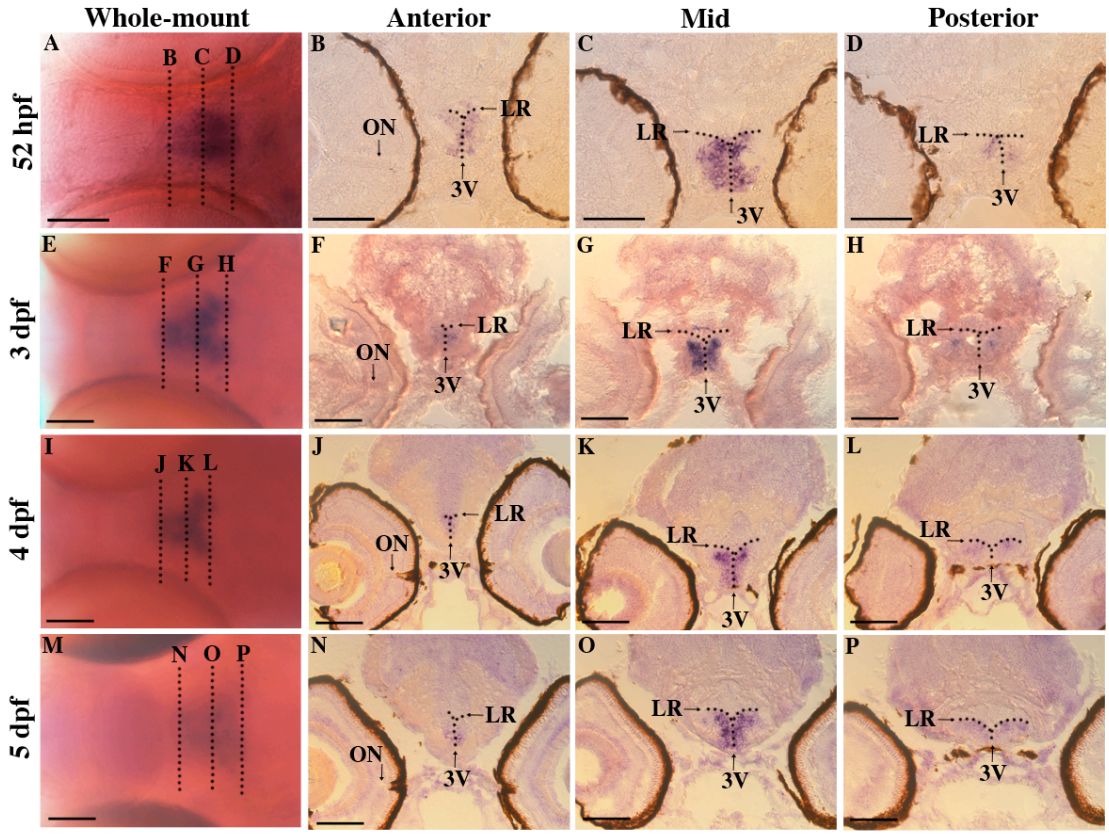

Figure 2.4. Planes of sectioning through the larval zebrafish hypothalamus. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Dotted lines in whole-mounts indicate planes of sectioning; dotted line in sections indicates position of the 3rd ventricle. Scale bar is 100 µm.

(A,E,I,M) Expression of *sf1* in the hypothalamus in a whole-mount view showing planes of sectioning through the anterior, mid and posterior hypothalamus of 52 hpf, 3, 4 and 5 dpf larvae respectively. ON, optic nerve; 3V, 3rd ventricle; LR, lateral recess of the hypothalamus.

(B-D) Expression of *sf1* in the anterior, mid and posterior hypothalamus of 52 hpf larvae.

(F-H) Expression of *sf1* in the anterior, mid and posterior hypothalamus of 3 dpf larvae.

(J-L) Expression of *sf1* in the anterior, mid and posterior hypothalamus of 4 dpf larvae.

(N-P) Expression of *sf1* in the anterior, mid and posterior hypothalamus of 5 dpf larvae.

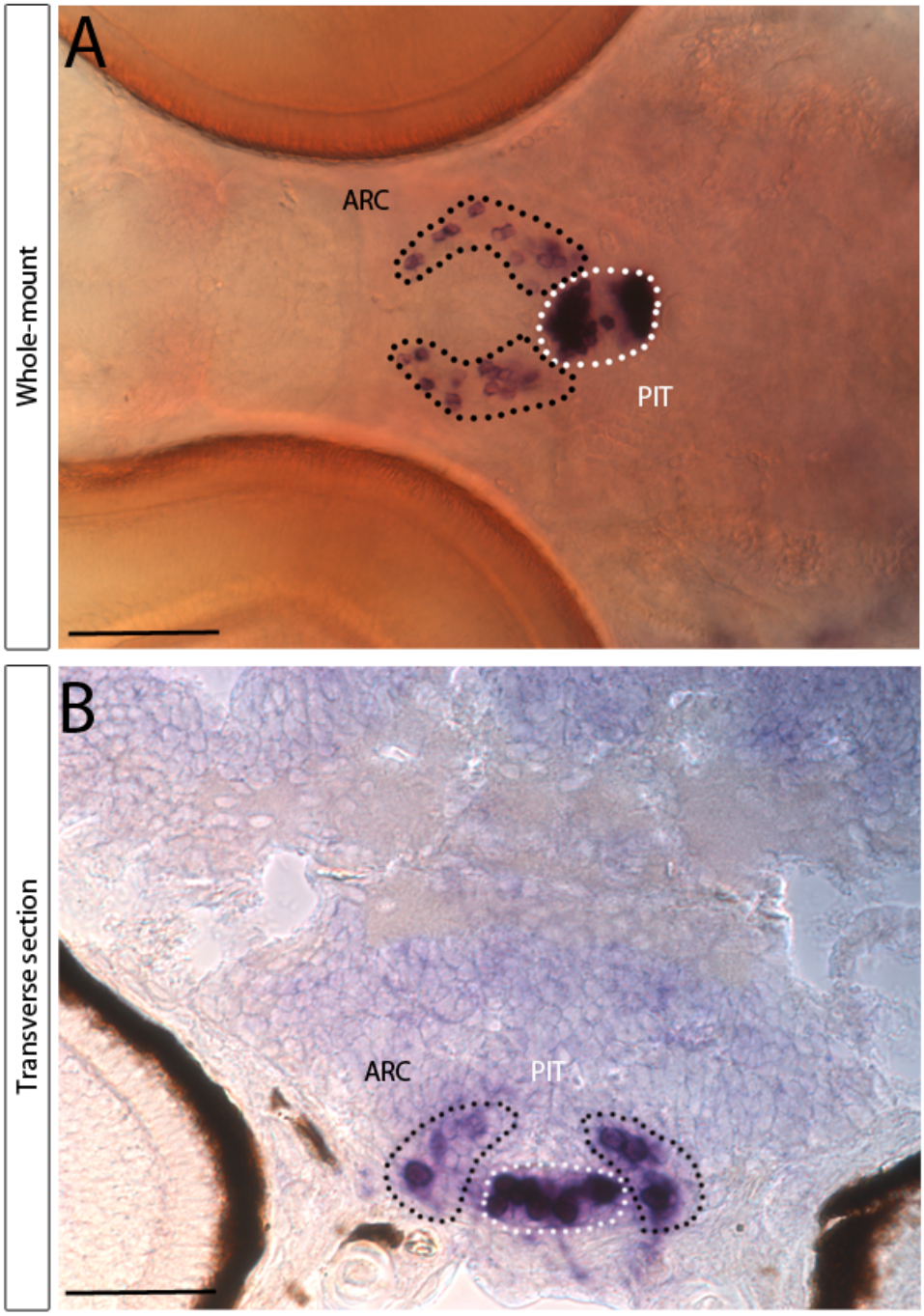

Figure 2.5. *In situ* hybridisation showing expression of *pomc* in the arcuate nucleus of the hypothalamus and the pituitary gland in the zebrafish larva. Black dotted line outlines the arcuate nucleus of the hypothalamus (ARC). White dotted line outlines the pituitary gland (PIT). Scale bar is 50 µm.

(A) Ventral view of a 4 dpf whole-mount zebrafish larva. Anterior is to the left.

(B) Transverse section through the posterior hypothalamus and pituitary gland of a 3 dpf zebrafish larva. Dorsal is upper-most.

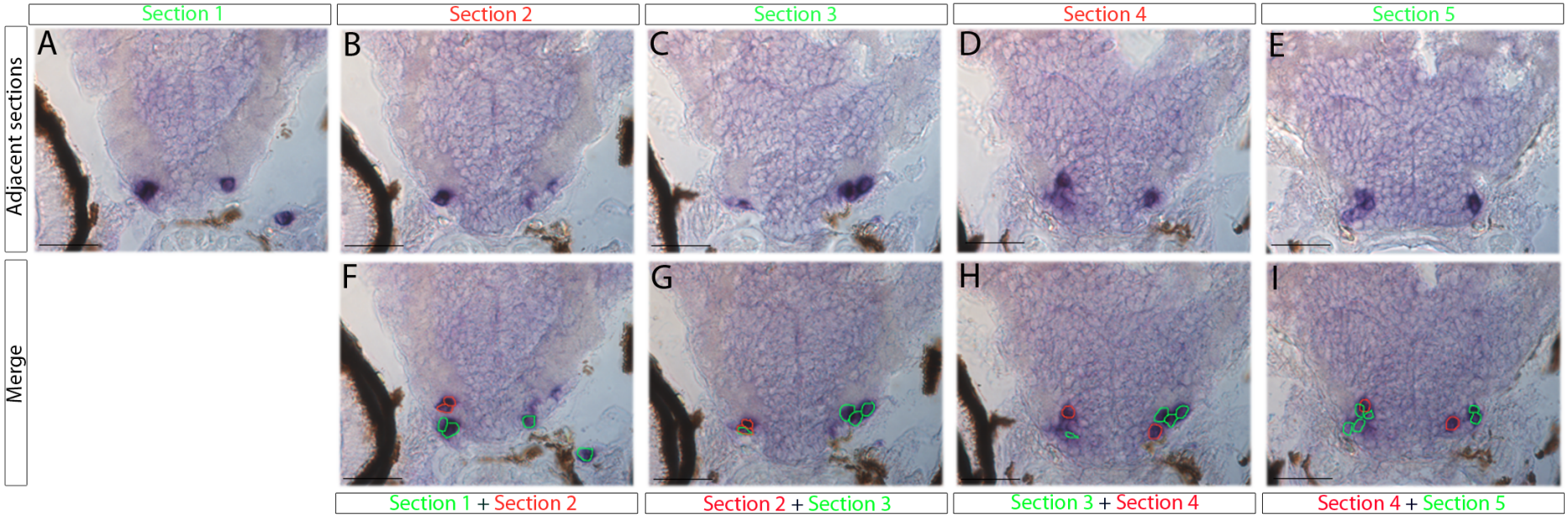

Figure 2.6. Methodology of counting *pomc* positive cells.
(A-E) Adjacent transverse sections through the hypothalamus of a 3 dpf larval zebrafish after whole-mount *in situ* hybridisation for *pomc*, showing expression in neurons of the arcuate nucleus. Section 1 is anterior-most; dorsal is upper-most.
(F-I) Overlayed images of sections A-E in the hypothalamus. The *pomc*-positive cells that were counted are outlined with red or green lines in alternate sections. This analysis revealed minimal overlap of the *pomc*-positive cells between sections and identified that these cells could be easily identified in order to avoid duplication. Scale bar is 50 μm.

avoid any degradation of hormones. After the larval shoaling assay, the 21 zebrafish larvae were collected into a 1.5 ml tube using a Pasteur pipette, excess water was removed and the sample was snap-frozen in an ethanol/dry ice bath. After the adult behavioural analysis, individuals were culled via the quickest possible method (destruction of the cranium), placed into a 1.5 ml tube and snap frozen in liquid nitrogen. All tissue samples were stored long-term at -20 °C prior to cortisol extraction.

2.3.2 Extraction of cortisol from larval zebrafish samples

Cortisol was extracted from larval zebrafish samples according to the protocol described by Yeh and colleagues [68]. Samples were thawed completely and 150 µl H_2O was added to each tube. Samples were then homogenised for 20 seconds each using a pellet mixer (VWR). One ml of ethyl acetate (Sigma) was added to each tube and the sample was vortexed for 10 seconds. The samples were then centrifuged for 5 minutes at 3000 x g at 4 °C. The solvent layer was then removed carefully with a pipette and transferred to a clean 1.5 ml tube. The solvent was then evaporated off, by spinning in a speed vacuum concentrator (DNA Speed Vac DNA110, Savant) for 30 minutes on the high heat setting. The cortisol extract was then dissolved in 60 µl sample buffer (0.2 % BSA in PBS) and stored long-term at -20 °C.

2.3.3 Extraction of cortisol from adult zebrafish samples

Cortisol was extracted from adult zebrafish according to the protocol described by Canavello and colleagues [25]. Zebrafish samples were partially thawed, weighed and then dissected on ice into small pieces to aid homogenization. The sample was homogenized in 500 µl ice cold PBS using a glass homogenizer (Dounce tissue grinder, 7 ml, Sigma). The glass pestle was washed with an additional 500 µl ice cold PBS and the homogenate was collected in a glass centrifuge tube which was kept on ice wherever possible. Five ml diethyl ether (Fisher Scientific) was added to the homogenate and the sample was vortexed for 20 seconds. The sample was then centrifuged at 3500 rpm for 5 minutes. Following centrifugation, the organic layer containing cortisol was collected from each sample and transferred to a clean glass tube. Samples were then stored overnight in the fume hood to allow for

evaporation of ether. Once the ether had evaporated the cortisol was reconstituted in 1 ml PBS and stored long-term at -20 °C.

2.3.4 Cortisol ELISA

Cortisol in larval and adult zebrafish samples was quantified according to the protocol described by Yeh and colleagues [68]. Each well of the ELISA plate (96-well Immulon 2HB, VWR) was coated with 100 µl of cortisol mAB-solution at 1.6 µg/ml in PBS (anti-cortisol monoclonal antibody, EastCoast Bio) and incubated for 16 hours at 4 °C. Each well was then washed three times with 300 µl PBST (0.05 % Tween 20 in PBS) and then incubated for 30 minutes with 300 µl blocking buffer (0.1 % BSA in PBS) at room temperature. Wells were washed again with three PBST washes. Fifty µl of standards and samples were added to each well of the plate in a random distribution. Prior to use, samples and standards were thawed, vortexed for 10 seconds and centrifuged for 5 seconds in the centrifuge. Standards used were 0, 0.5, 1, 2.5, 7.5, 20, 50 ng/ml cortisol (Hydrocortisone, Sigma) in PBS. Fifty µl cortisol-HRP (1:1600 dilution, Cortisol-HRP antigen, EastCoast Bio) was then added to each well and the plate was incubated for 2 hours at room temperature on an orbital shaker (40-50 rpm). Each well was washed again three times with PBST and then 100 µl staining solution (TMB liquid substrate system, Sigma) was added to each well and incubated for 20 minutes at room temperature on an orbital shaker. The reaction was stopped by adding 100 µl 1 M sulphuric acid (Sigma). The plate was shaken briefly and absorbance was read at 450 nm in a photometric plate reader (Varioskan, Thermo Scientific).

2.3.5 Analysis of absorbance readings

Cortisol standards were run in duplicate and so an average absorbance for each standard was taken. The percentage bound for each cortisol standard and sample was calculated by dividing the absorbance for each well by the average absorbance for the zero standard. A 4-parameter non-linear regression curve was created to fit the average absorbance readings to the cortisol standard concentrations. Cortisol concentrations for the samples were then determined via interpolation from the standard curve.

3 Behavioural analysis of *disc1* zebrafish larvae

3.1 Introduction

Behavioural models utilising adult zebrafish are reasonably well established, thus there are behaviours that are accepted as indicative of anxiety or biomarkers for symptoms of psychiatric disease. The behaviour of the developing zebrafish has been less widely studied to-date, but is a rapidly growing area of research, as the advantages of the larval zebrafish become more widely recognised. Larval zebrafish provide an extremely high-throughput system in which simple behaviours can be studied. Tests such as the open field test and place preference tests, initially established in rodents and then used with adult zebrafish, are easily adapted for use with zebrafish larvae and have shown that larvae display some of the same anxiety-like behaviours as adult zebrafish, such as thigmotaxis and dark avoidance [1].

It has been reported that some level of shoaling behaviour is apparent soon after hatching in zebrafish [71]. The young larvae are situated closer to each other than a random distribution would predict, and so already appear to display some affinity to one another. Shoal cohesion, as determined by distance between individuals, continues to increase throughout development and has been found to be almost the same in juveniles at 76 dpf as adult fish [72]. To study the natural behaviour of a group of early larvae, as well as their individual locomotion, allows assessment of how their social development might be affected by genetic and environmental factors.

The behavioural response to stress in adult zebrafish has been fairly well documented in the scientific literature and a small number of studies have also investigated the behavioural responses to stress in zebrafish larvae. Thus, it has been established that early larvae of 3-5 dpf can respond to stress, both in terms of short-term changes in stress hormone levels [45, 73] and long-term changes in development [74]. Exposure to stressors such as dexamethasone, cortisol, and deoxycorticosterone or rearing in isolation resulted in a significantly blunted locomotor response to a sudden pulse of darkness [1]. Exposure to ethanol at 1.5%

induced hyperactivity and thigmotaxis in 7 dpf zebrafish larvae, whilst a higher concentration (3%) induced hypoactivity [75]. In another study, zebrafish larvae avoided sodium chloride-treated water in a place preference test, but post-exposure locomotion was unaffected [76]. Sodium chloride has been established as an effective stressor in developing larvae, in that it induces a significant increase in cortisol levels [68]. The effect of sodium chloride on larval swimming behavior during exposure is not known, other than the avoidance response [76].

The behavioural response of adult zebrafish to the alarm pheromone has been reasonably well characterised. Reported effects are increased bottom dwell in a tank diving test [25-27], increased shoal cohesion [27, 28], increased occurrence of erratic swimming [25, 26, 28], increased freezing occurrence [25] and increased frequency of slow swim episodes [26]. To date, only one study has investigated the effect of exposure to alarm pheromone on the development of early zebrafish larvae, observing an accelerated physiological development [74]. As yet, no study has investigated early behavioural responses to the alarm pheromone.

Given reports that stress responses and behaviour can be investigated in larval zebrafish, I set out to test the hypothesis that mutation in the zebrafish *disc1* gene would affect larval behaviour. Previous work on a number of mouse models has investigated the effect of *DISC1* on behavioural phenotypes and more recently, evidence for a link between *DISC1* and the HPA axis has been suggested. Mouse models with a point mutation in *Disc1* (L100P) show hyperactivity in the open field and anxious behaviours [6]. When L100P mice were combined with prenatal Polyl:C infection, this GXE group exhibited exacerbated anxiety behaviours. In another DISC1 mouse study, mutants exposed to a mild isolation stress during adolescence displayed hyperactivity in the open field and anxiety-related behaviours [7].

These data open an avenue to investigate the effect of a mutation in *disc1* on early zebrafish behaviour and the role of *disc1* in the behavioural stress response. The experiments described here utilised two lines of zebrafish carrying point mutations in the *disc1* gene: L115 and Y472. The swimming behaviour of the

mutants was compared to wild types in a basic locomotion assay at a number of developmental stages. The behavioural response to a number of stressors (isolation, alarm pheromone and sodium chloride) was also analysed, in terms of basic locomotion and shoaling behaviour. The way in which zebrafish larvae respond to these stressors had not been previously described, nor was it clear as to which tests might be most useful in identifying a response. For this reason, I tested two developmental stages: 4-5 dpf (a stage when Home Office licencing is not required, but the HPI is developed) and 8 dpf (a stage when free-feeding larval zebrafish might show more significant behaviours). Different behavioural tests and stressor methodologies were also tested.

3.2 Results

3.2.1 Analysis of the baseline swimming behaviour of larval *disc1* zebrafish: performed on single larvae in individuals wells of a 48 well plate

Y472: 4 & 8 dpf

There was no significant difference between Y472 wild types and mutants at 4 dpf or 8 dpf in any of the behavioural measures in the 10 minute swimming assay (Table 3.1). Subsequent baseline swimming assay tests utilised a 1 hour duration to allow for acclimation and give a more accurate overview of baseline swimming behaviour.

L115: 4dpf

In a 1 hour test, 4 dpf L115 wild type larvae displayed a high initial level of activity, which decreased throughout the test, whilst mutants maintained a constant lower level of activity (Figure 3.1A,C; Table 3.2). Mutants also exhibited significantly fewer freezing occurrences than wild types (Figure 3.1D; Table 3.2). Swimming duration decreased over the course of the test and did not differ between wild types and mutants (Figure 3.1B, Table 3.2).

Table 3.1. Statistical analysis of *disc1* Y472 larval swimming behaviour. The results of two-sample Welch t-tests comparing mean values for wild type in-cross and homozygous mutant in-cross larvae at 4 dpf, and 8 dpf for various behavioural parameters are shown. SEM; standard error of the mean. d.f; degrees of freedom (varied due to adjustment for unequal variance). N= 20-141 per genotype, 2 technical replicates.

	Behavioural parameter	**Wild type in-cross mean ± SEM**	**Homozygous in-cross mean ± SEM**	**t value**	**d.f**	**p value**
Y472 4 dpf	Distance swam (cm)	181 ± 8	207 ± 15	1.54	366	0.123
	Swimming duration (mins)	9.0 ± 0.2	9.3 ± 0.1	1.51	424	0.132
	Fast swimming distance (cm)	70 ± 3	90 ± 12	1.62	294	0.106
	Freezing occurrence	150 ± 11	126 ± 7	1.84	409	0.066
Y472 8 dpf	Distance swam (cm)	199 ± 17	182 ± 23	-0.62	260	0.536
	Swimming duration (mins)	8.0 ± 0.2	8.0 ± 0.2	0.24	260	0.807
	Fast swimming distance (cm)	106 ± 17	92 ± 23	-0.51	260	0.612
	Freezing occurrence	758 ± 40	754 ± 39	-0.07	260	0.946

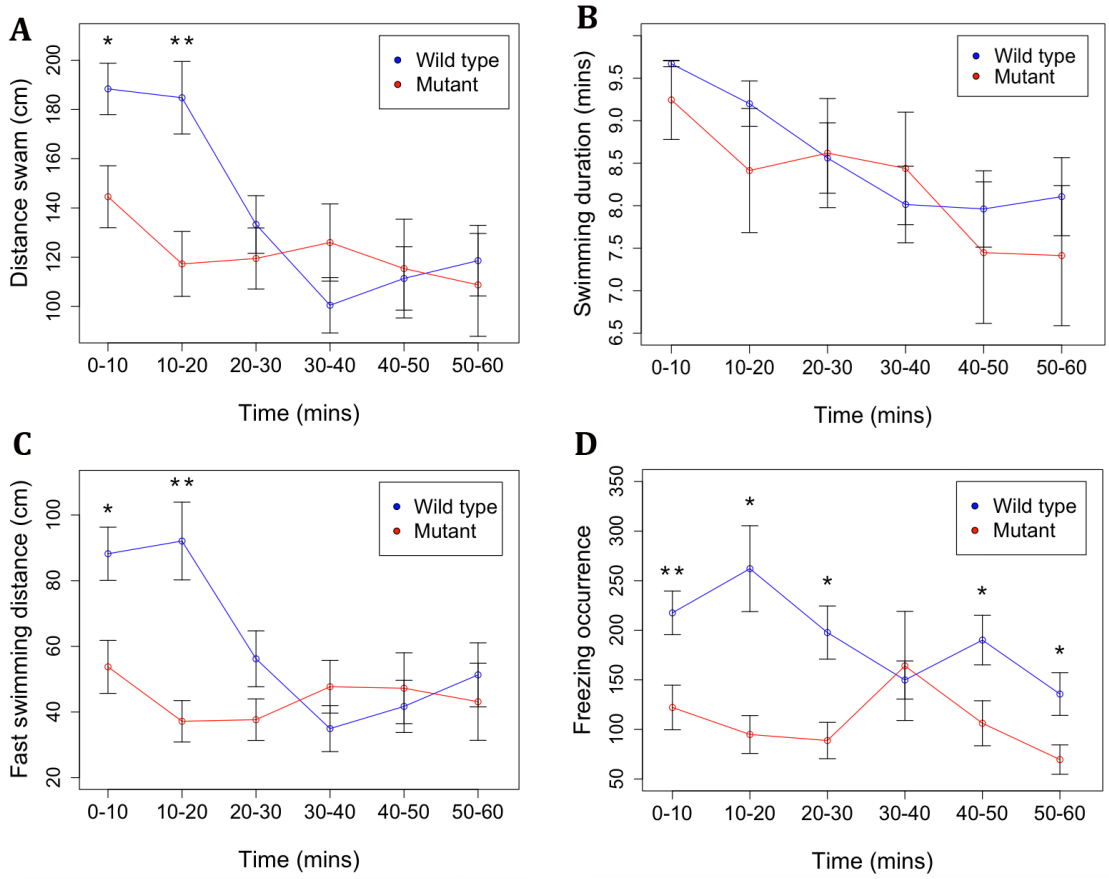

Figure 3.1. Behavioural analysis of 4 dpf *disc1* L115 wild type and mutant zebrafish in a 1 hour test. Points and bars represent mean ± standard error. ** indicates a statistically significant difference between wild type and mutant means at $p < 0.001$. Time, time interval during experiment; wild type, wild type in-cross larvae (non-sibling); mutant, homozygous mutant in-cross larvae. N= 270 per genotype, 3 technical replicates. See table 3.2 for statistics.

(A) Mean distance swam by wild type and mutant larvae.

(B) Mean duration of swimming by wild type and mutant larvae during each 10 minute period of the test.

(C) Mean duration of fast swimming (> 6 mm/s) by wild type and mutant larvae during each 10 minute period of the test.

(D) Mean number of freezing occurrences by wild type and mutant larvae.

Table 3.2. **Statistical analysis of *disc1* L115 4 dpf larval swimming behaviour.** The results of two-way ANOVA tests with repeated measures for the factor time, carried out on various behavioural parameters are shown. d.f; degrees of freedom. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 270 per genotype, 3 technical replicates. See figure 3.1 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam	Genotype	1.16	1, 72	0.285	
	Time	65.49	1, 320	<0.0001	***
	Genotype: time	11.84	1, 320	0.0007	***
Swimming duration	Genotype	0.39	1, 72	0.532	
	Time	33.27	1, 320	<0.0001	***
	Genotype: time	0.02	1, 320	0.904	
Fast swimming distance	Genotype	2.17	1, 72	0.145	
	Time	41.19	1, 320	<0.0001	***
	Genotype: time	18.12	1, 320	<0.0001	**
Freezing occurrence	Genotype	8.44	1, 72	0.005	**
	Time	12.12	1, 320	0.0005	***
	Genotype: time	3.26	1, 320	0.072	

L115: 8dpf

In a 1 hour test, 8 dpf L115 mutants swam significantly less distance, did less fast swimming and exhibited fewer freezing occurrences than wild types (Figure 3.2 A, C, D; Table 3.3). Swimming distance and duration generally decreased throughout the hour, with total distance and fast swimming reducing more rapidly for mutants (Figure 3.2 A-C, Table 3.3). There was no effect of genotype on swimming duration and no genotype: time interaction (Figure 3.2 B, Table 3.3).

L115 sibling: 4dpf

Zebrafish *disc1* siblings are the offspring of a heterozygous in-cross, which were genotyped post-experimentation. In a 1 hour test, the behavioural parameters measured did not differ between L115 wild type and mutant 4 dpf larvae (Figure 3.3, Table 3.4). In this experiment, swimming distance, duration, fast swimming and freezing occurrence decreased throughout the test in all larvae (Figure 3.3, Table 3.4).

L115 sibling: 8 dpf

In a 1 hour test, there was no significant effect of genotype on swimming distance, duration, fast swimming distance or freezing occurrence in the 8 dpf L115 larvae (Figure 3.4, Table 3.5). All genotypes showed a significant reduction in swimming distance, duration, fast swimming distance and freezing occurrence over the course of the test, whilst freezing occurrence decreased most rapidly in the wild type larvae.

3.2.2 Analysis of place preference in *disc1* zebrafish larvae

In a 10 minute open field test, 5 dpf Y472 larvae showed a preference for the perimeter of the well (Figure 3.5 A). Perimeter duration did not differ between wild type and mutant larvae and decreased over time for both genotypes (Figure 3.5 A, Table 3.6).

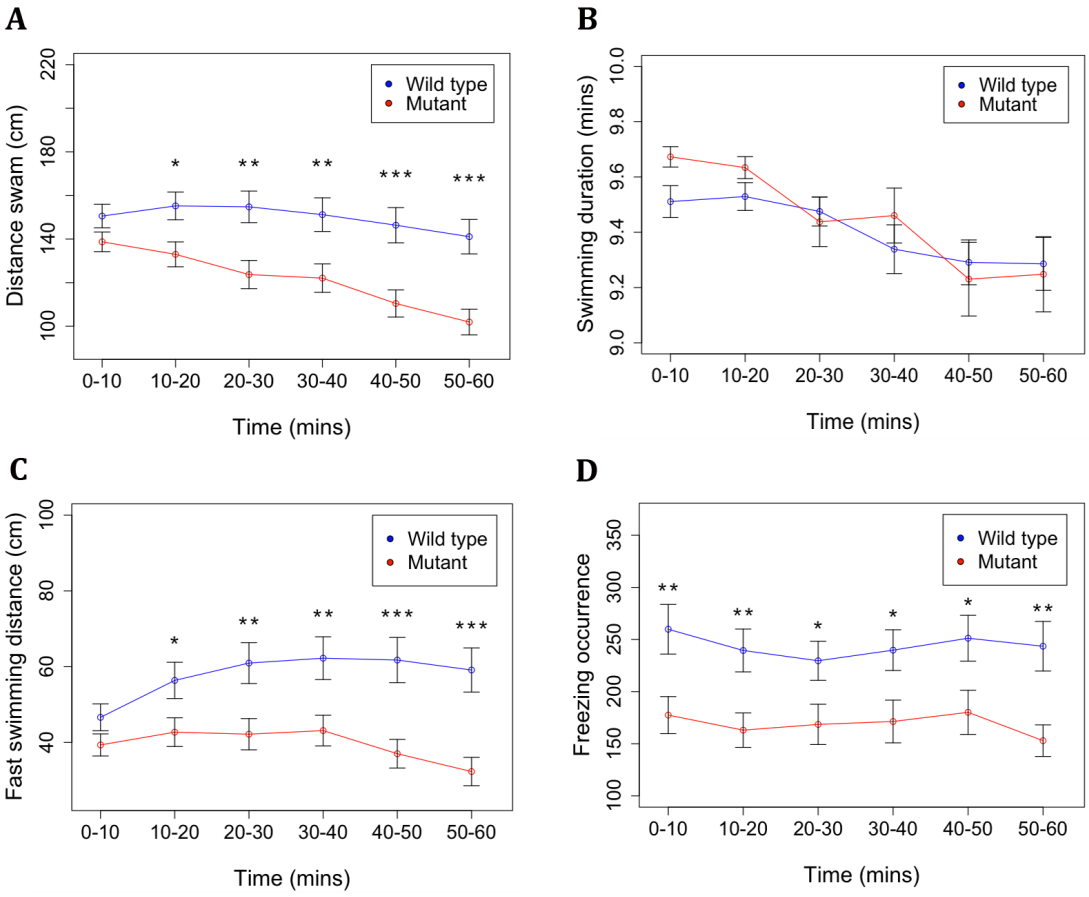

Figure 3.2. Behavioural analysis of 8 dpf *disc1* L115 wild type and mutant zebrafish in a 1 hour test. Points and bars represent mean ± standard error. * indicates a statistically significant difference between wild type and mutant means at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. Time, time interval in the experiment, wild type, wild type in-cross (non-sibling), mutant, homozygous mutant in-cross. N= 80 per genotype, 2 technical replicates. See Table 3.3 for statistics.

(A) Mean distance swam by wild type and mutant larvae.

(B) Mean duration of swimming by wild type and mutant larvae during each 10 minute period of the test.

(C) Mean duration of fast swimming (> 6 mm/s) by wild type and mutant larvae during each 10 minute period of the test.

(D) Mean number of freezing occurrences by wild type and mutant larvae.

Table 3.3. **Statistical analysis of *disc1* L115 8 dpf larval swimming behaviour in a 1 hour test.** The results of two-way ANOVA tests, with repeated measures for the factor time, carried out on various behavioural parameters are shown. d.f; degrees of freedom. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. Time, time interval during experiment. N= 80 per genotype, 2 technical replicates. See Figure 3.2 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam	Genotype	11.51	1, 159	0.0008	***
	Time	75.91	1, 803	<0.0001	***
	Genotype: time	21.32	1, 803	<0.0001	***
Swimming duration	Genotype	0.21	1, 159	0.651	
	Time	53.22	1, 803	<0.0001	***
	Genotype: time	3.38	1, 803	0.066	
Fast swimming distance	Genotype	10.03	1, 159	0.002	**
	Time	1.15	1, 803	0.285	
	Genotype: time	25.42	1, 803	<0.0001	***
Freezing occurrence	Genotype	8.94	1, 159	0.003	**
	Time	0.75	1, 803	0.388	
	Genotype: time	0.07	1, 803	0.789	

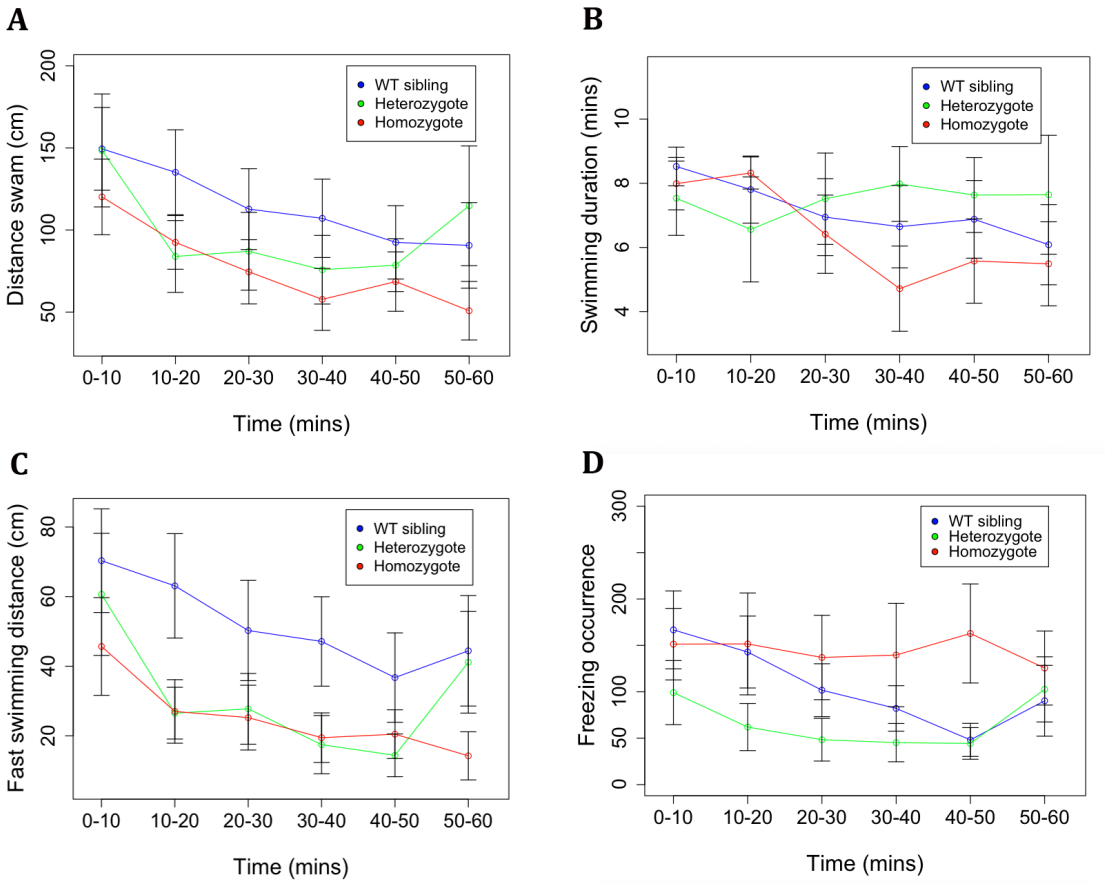

Figure 3.3. Behavioural analysis of 4 dpf *disc1* L115 wild type sibling, heterozygous and and homozygous mutant zebrafish in a 1 hour test. Points and bars represent mean ± standard error. Time, time interval in the experiment. N= 9 per genotype, 2 technical replicates. See Table 3.4 for statistics.

(A) Mean distance swam by wild type, heterozygous and homozygous mutant larvae.

(B) Mean duration of swimming by wild type, heterozygous and homozygous mutant larvae during each 10 minute period of the test.

(C) Mean duration of fast swimming (> 6 mm/s) by wild type, heterozygous and homozygous mutant larvae during each 10 minute period of the test.

(D) Mean number of freezing occurrences by wild type, heterozygous and homozygous mutant larvae.

Table 3.4. Statistical analysis of swimming behaviour of *disc1* L115 4 dpf sibling larvae from a heterozygous in-cross in a 1 hour test. The results of two-way ANOVA tests, with repeated measures for the factor time, carried out on various behavioural parameters are shown, including pairwise comparisons for significant differences between genotypes. d.f, degrees of freedom. WT, wild type; het, heterozygous mutant; mut, homozygous mutant. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 9 per genotype, 2 technical replicates. See Figure 3.3 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam (mm)	Genotype	0.99	2, 30	0.383	
	Time	48.12	1, 163	<0.0001	***
	Genotype: time	0.01	2, 163	0.994	
Swimming duration (s)	Genotype	0.31	2, 30	0.735	
	Time	18.46	1, 163	<0.0001	***
	Genotype: time	2.13	2, 163	0.122	
Fast swimming distance (mm)	Genotype	2.09	2, 30	0.142	
	Time	3.12	1, 163	<0.0001	***
	Genotype: time	0.29	2, 163	0.748	
Freezing occurrence	Genotype	1.29	2, 30	0.290	
	Time	10.15	1, 163	0.002	**
	Genotype: time	2.97	2, 163	0.054	

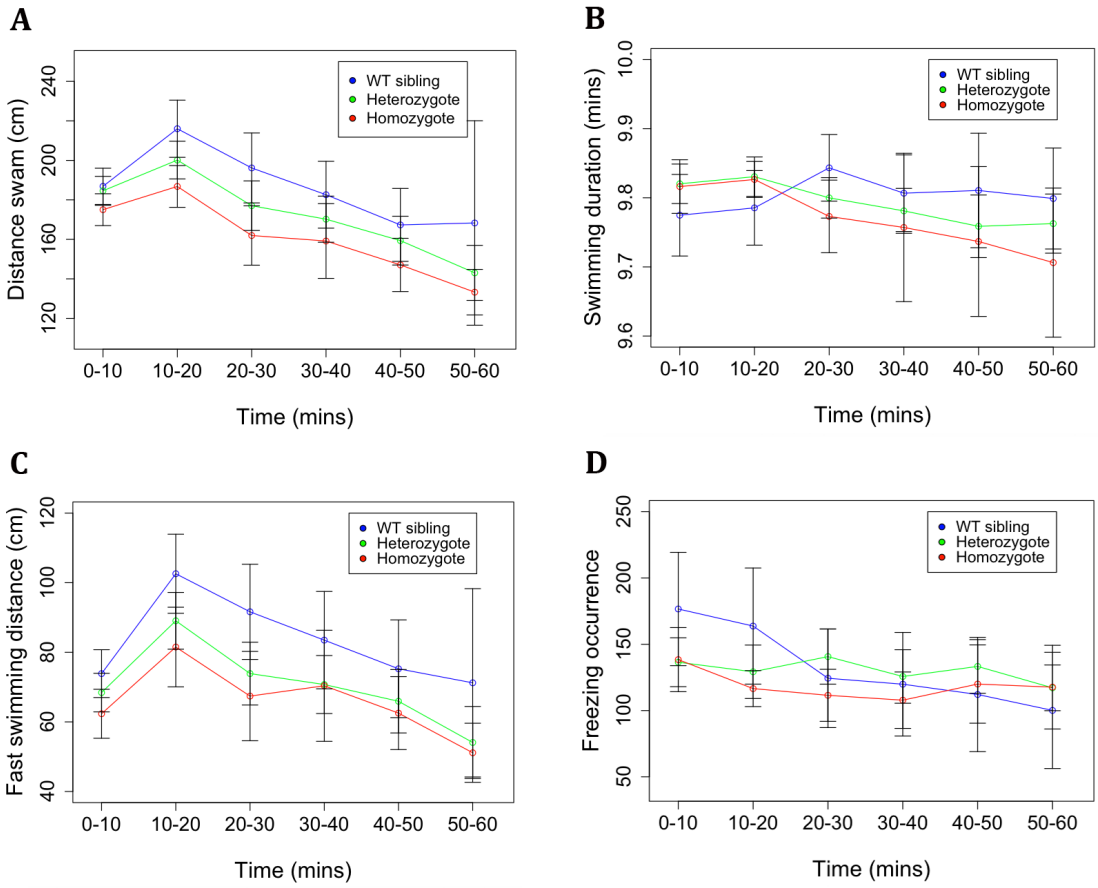

Figure 3.4. Behavioural analysis of 8 dpf *disc1* L115 wild type sibling, heterozygous and and homozygous mutant zebrafish in a 1 hour test. Points and bars represent mean ± standard error. Time, time interval in the experiment. N= 8-12 per genotype, 2 technical replicates. See Table 3.5 for statistics.

(A) Mean distance swam by wild type, heterozygous and homozygous mutant larvae.

(B) Mean duration of swimming by wild type, heterozygous and homozygous mutant larvae during each 10 minute period of the test.

(C) Mean duration of fast swimming (> 6 mm/s) by wild type, heterozygous and homozygous mutant larvae during each 10 minute period of the test.

(D) Mean number of freezing occurrences by wild type, heterozygous and homozygous mutant larvae.

Table 3.5. Statistical analysis of swimming behaviour of *disc1* L115 8 dpf sibling larvae from a heterozygous in-cross in a 1 hour test. The results of two-way ANOVA tests, with repeated measures for factor time, carried out on various behavioural parameters are shown, including pairwise comparisons for differences between genotypes. d.f, degrees of freedom. Time, time interval in experiment. WT, wild type; het, heterozygous mutant; mut, homozygous mutant. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 8-12 per genotype, 2 technical replicates. See Figure 3.4 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam	Genotype	0.98	2, 37	0.386	
	Time	36.25	1, 197	<0.0001	***
	Genotype: time	0.19	2, 197	0.823	
Swimming duration	Genotype	0.11	2, 37	0.895	
	Time	8.50	1, 197	0.004	**
	Genotype: time	2.23	2, 197	0.111	
Fast swimming distance	Genotype	0.81	2, 37	0.455	
	Time	10.68	1, 197	0.001	**
	Genotype: time	0.12	2, 197	0.890	
Freezing occurrence	Genotype	0.11	2, 37	0.898	
	Time	7.16	1, 197	0.008	**
	Genotype: time	3.21	2, 197	0.042	*

3.2.3 Analysis of light responsiveness in *disc1* zebrafish larvae

Y572 5 dpf larvae were exposed to a 1 second dark flash, which initiated a sharp peak in activity (Figure 3.5 B). There was no difference in pre-, during and post-stimulus locomotor response to the dark flash of wild type or mutant larvae (Figure 3.5 C, Table 3.6). Distance swam by wild type and mutant larvae increased in response to the stimulus, in comparison to pre-stimulus levels. Wild type larval post-stimulus activity was significantly lower than during the stimulus, but higher than pre-stimulus; whilst mutant post-stimulus levels returned to pre-stimulus levels more quickly.

In a four minute test, L115 5 dpf larvae were exposed to alternating 1 minute intervals of light and dark. There was no effect of genotype on distance swam, swimming duration or fast swimming, whilst mutants spent significantly less time in the perimeter of the well (Figure 3.6, Table 3.7). Light exposure affected total distance and fast swimming distance and caused a significant increase in time spent in the perimeter of the well (Figure 3.6, Table 3.7). The difference between wild types and mutants was larger when the light was off.

3.2.4 The effect of isolation on *disc1* larval swimming behaviour

Y472 + isolation: 8dpf

Zebrafish were raised in isolation in individual wells of a multi-well plate, or in groups in a petridish from 0 dpf and swimming behaviour was analysed at 8 dpf (in a multi-well plate). In a 1 hour test, there was no effect of housing conditions on the behavioural parameters measured in either wild type or mutant larvae (Figure 3.7, Table 3.8).

The 8 dpf Y472 mutants swam significantly less distance and did less fast swimming than wild types, whilst distance also decreased more rapidly over time in the mutants (Figure 3.7 A-F, Table 3.8). There was no effect of genotype on swim duration or freezing occurrence and no genotype: time interactions (Figure 3.7 G-H, Table 3.8). Both genotypes displayed a reduction in swimming duration throughout the test and a significant increase in number of freezing occurrences.

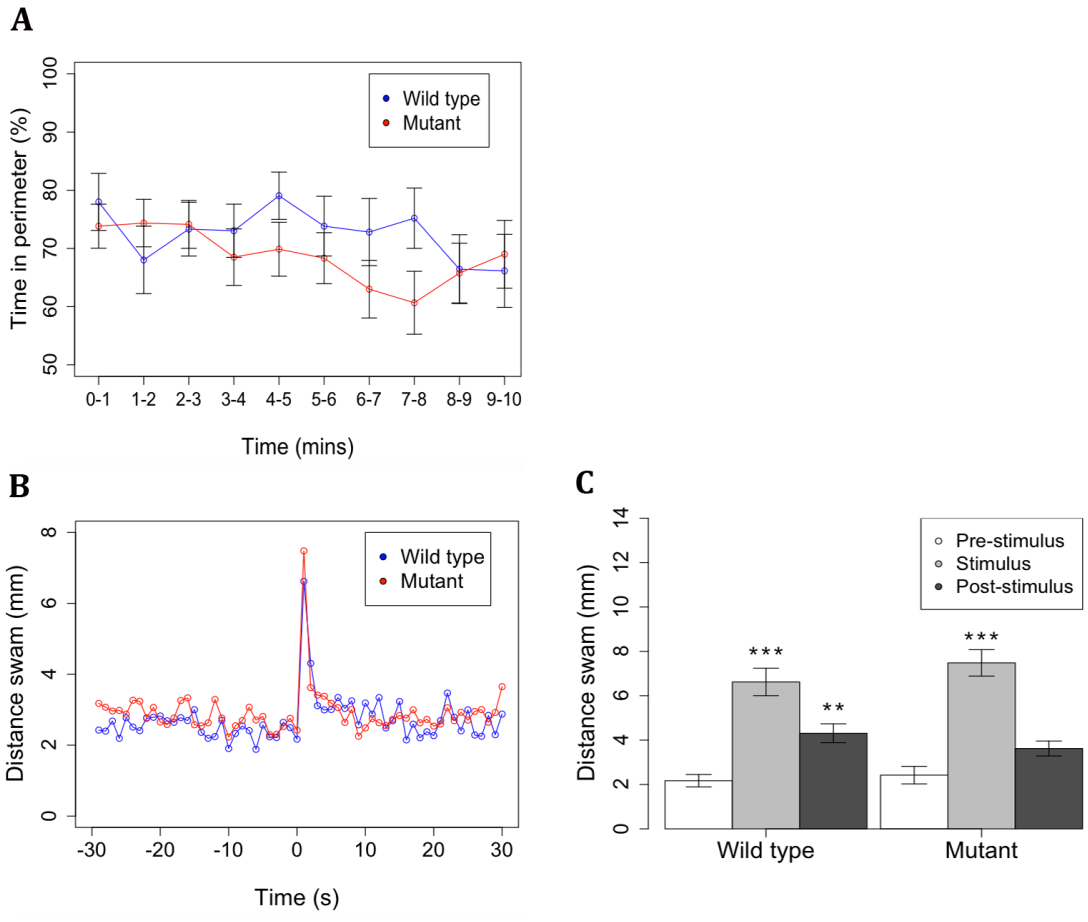

Figure 3.5. Place preference and response to a dark flash in 5 dpf *disc1* Y472 wild type and mutant zebrafish. Points and bars represent mean ± standard error. Wild type, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross. * indicates statistically significant difference from pre-stimulus levels for within genotype comparisons. **, p<0.01; ***, p<0.0001. N= 36 biological replicates, 1 technical replicate. See Table 3.6 for statistics.

(A) Mean time spent in the perimeter of the well by wild type and mutant larvae during each 1 minute interval during a 10 minute test.

(B) Mean distance swam by wild type and mutant larvae in the 30 seconds either side of exposure to a dark flash. Larvae were exposed to a dark flash at 0 s, time is relative to dark flash exposure.

(C) Mean distance swam by wild type and mutant larvae in the 1 second pre-stimulus, during the dark flash stimulus, and post-stimulus.

Table 3.6. Statistical analysis of responsiveness to a dark pulse and place preference in a 10 minute open field test in *disc1* Y472 5 dpf larvae. The results of two-way ANOVA tests with repeated measures for time and genotype; pairwise comparisons for within period and one way ANOVA for within genotype are shown. d.f; degrees of freedom. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N = 36 per genotype, 1 technical replicate. See Figure 3.5 for plot.

Behavioural parameter	Factor	F /t value	d.f	p value	
Distance swam in light response test	Genotype	0.09	1, 70	0.765	
	Time	9.45	1, 142	<0.0001	***
	Genotype: time	0.75	1, 142	0.389	
Place preference in open field test	Genotype	0.61	1, 69	0.439	
	Time	9.86	1, 645	0.002	**
	Genotype: time	0.57	1, 645	0.453	

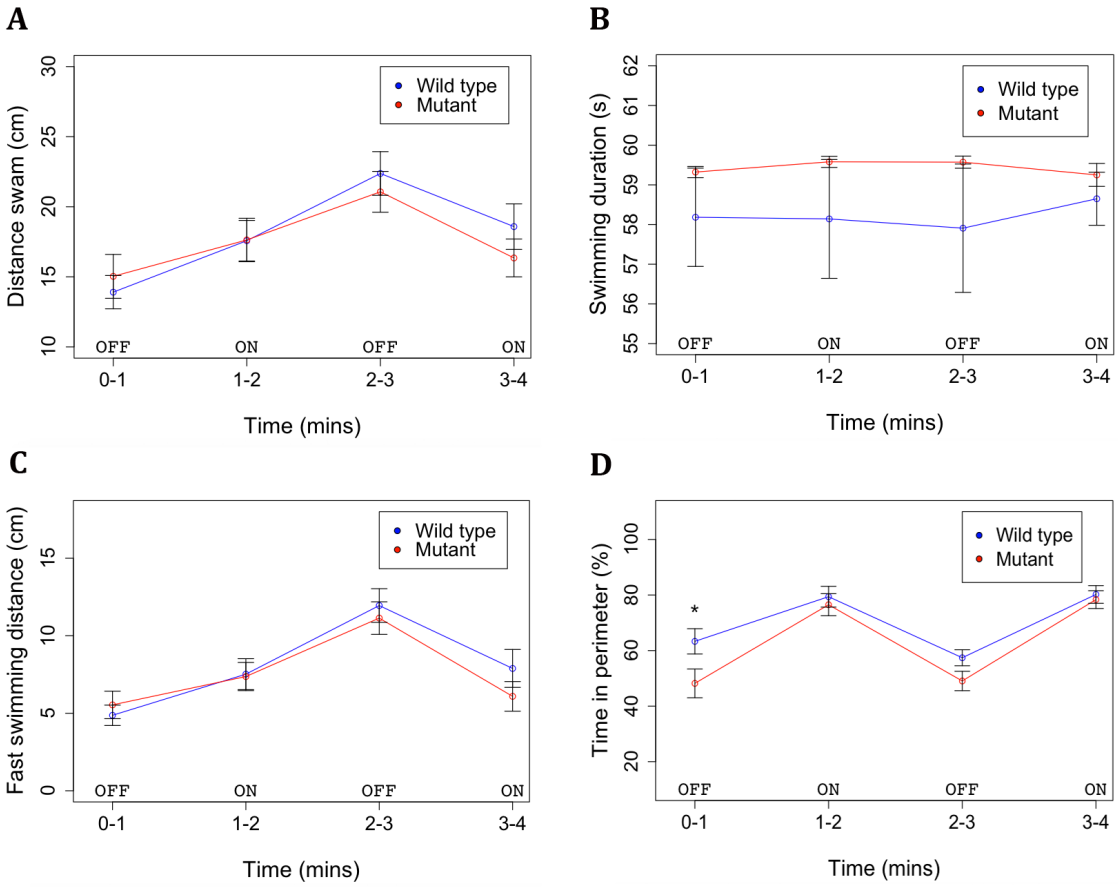

Figure 3.6. Behavioural responses of 5 dpf *disc1* L115 wild type and homozygous mutant zebrafish to light in a four minute test. Points and bars represent mean ± standard error. Time, time interval in the experiment; wild type, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; OFF/ON indicates light status in each one minute interval. * indicates statistically significant difference between wild types and mutants at $p<0.05$. N = 36 per genotype, 1 technical replicate. See Table 3.7 for statistics.

(A) Mean distance swam by wild type and mutant larvae during each minute of the test.

(B) Mean swimming duration by wild type and mutant larvae during each minute of the test.

(C) Mean distance of fast swimming (> 6 mm/s) by wild type and mutant larvae during each minute of the test.

(D) Mean time spent in the perimeter of the well by wild type and mutant larvae during each minute of the test.

Table 3.7. Statistical analysis of light responsiveness in *disc1* L115 5 dpf larvae in a four minute test. The results of two-way ANOVA tests with repeated measures for factors time and light and pairwise comparisons are shown. d.f; degrees of freedom. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N = 36 per genotype, 1 technical replicate. See Figure 3.6 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam	Genotype	0.11	1, 69	0.736	
	Time	36.86	1, 210	<0.0001	***
	Light	4.93	1, 69	0.030	*
	Genotype: time	2.71	1, 210	0.101	
	Genotype: light	0.29	1, 210	0.593	
Swimming duration	Genotype	0.92	1, 69	0.342	
	Time	0.03	1, 210	0.862	
	Light	0.00	1, 69	0.955	
	Genotype: time	0.10	1, 210	0.752	
	Genotype: light	0.62	1, 210	0.433	
Fast swimming distance	Genotype	0.21	1, 69	0.647	
	Time	47.71	1, 210	<0.0001	***
	Light	4.82	1, 69	0.040	*
	Genotype: time	2.36	1, 210	0.126	
	Genotype: light	0.62	1, 210	0.431	
Time in perimeter	Genotype	4.10	1, 70	0.047	*
	Time	0.06	1, 210	0.805	
	Light	100.31	1, 210	<0.0001	***
	Genotype: time	0.66	1, 210	0.418	
	Genotype: light	3.78	1, 210	0.053	

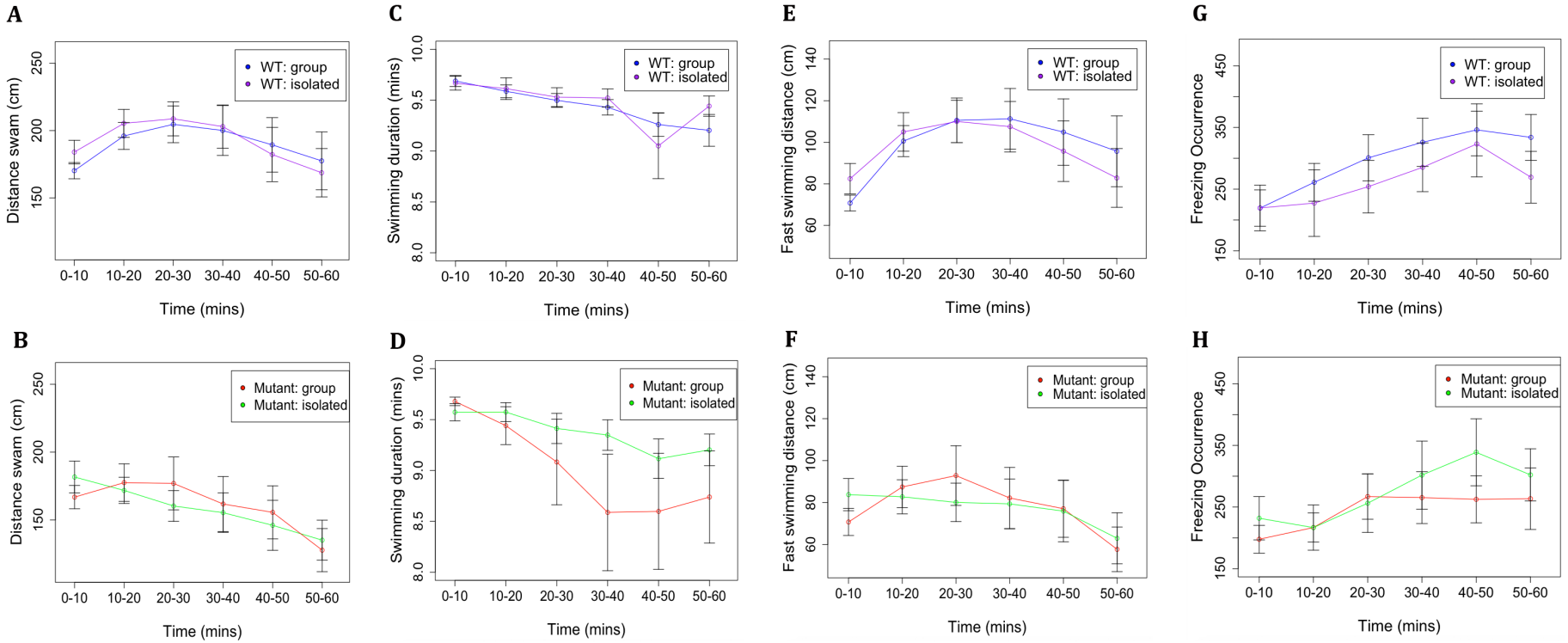

Figure 3.7. Analysis of the effect of rearing in groups vs isolation on swimming behaviour in 8 dpf *disc1* Y472 wild type and mutant larvae in a 1 hour test. Points and bars represent mean ± standard error. Time, time interval in the experiment; wild type, wild type in-cross; mutant, homozygous mutant in-cross. N= 22 per group, 2 technical replicates. See Table 3.8 for statistics.

(A) Mean distance swam by wild type larvae, when reared in groups or in isolation.
(B) Mean distance swam by mutant larvae, when reared in groups or in isolation.
(C) Mean swimming duration by wild type larvae, when reared in groups or in isolation.
(D) Mean swimming duration by mutant larvae, when reared in groups or in isolation.
(E) Mean duration of fast swimming (> 6 mm/s) by wild type larvae, when reared in groups or in isolation.
(F) Mean duration of fast swimming (> 6 mm/s) by mutant larvae, when reared in groups or in isolation.
(G) Mean number of freezing occurrences by wild type larvae, when reared in groups or in isolation.
(H) Mean number of freezing occurrences by mutant larvae, when reared in groups or in isolation.

Table 3.8. Statistical analysis of the effect of rearing in groups or isolation on the swimming behaviour of *disc1* Y472 8 dpf larvae in a 1 hour test. The results of two-way ANOVAs, with repeated measures carried out on various behavioural parameters are shown. d.f; degrees of freedom. N= 22 per group, 2 technical replicates. See Figure 3.7 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Distance swam	Genotype	6.22	1, 88	0.015	*
	Housing	0.00	1, 88	0.986	
	Time	19.78	1, 456	<0.0001	***
	Genotype: housing	0.04	1, 88	0.840	
	Genotype: time	7.55	1, 456	0.006	**
	Housing: time	1.45	1, 456	0.230	
	Genotype: housing: time	0.57	1, 456	0.451	
Swimming duration	Genotype	1.88	1, 88	0.174	
	Housing	1.04	1, 88	0.311	
	Time	39.37	1, 456	<0.0001	***
	Genotype: housing	0.74	1, 88	0.391	
	Genotype: time	2.78	1, 456	0.096	
	Housing: time	3.49	1, 456	0.062	
	Genotype: housing: time	1.86	1, 456	0.172	
Fast swimming distance	Genotype	4.87	1, 88	0.030	*
	Housing	0.01	1, 88	0.905	
	Time	0.87	1, 456	0.351	
	Genotype: housing	0.00	1, 88	0.950	
	Genotype: time	6.51	1, 456	0.011	*
	Housing: time	1.80	1, 456	0.181	
	Genotype: housing: time	1.20	1, 456	0.274	
Freezing occurrence	Genotype	0.29	1, 88	0.589	
	Housing	0.00	1, 88	0.956	
	Time	74.75	1, 456	<0.0001	***
	Genotype: housing	0.71	1, 88	0.402	
	Genotype: time	0.40	1, 456	0.529	
	Housing: time	0.01	1, 456	0.933	
	Genotype: housing: time	3.61	1, 456	0.058	

3.2.5 The effect of alarm pheromone on larval swimming behaviour

WT + alarm: 4 & 5 dpf

A 10 minute exposure to alarm pheromone extract (protocol 1) had no effect on the behavioural parameters measured in the 4 or 5 dpf wild type larvae (Table 3.9).

WT + alarm concentrations: 4 dpf

There was no effect of either concentration of alarm extract (protocol 1) on any of the behavioural parameters measured in the 4 dpf wild type larvae (Table 3.10).

Y472 + alarm: 4 dpf

In a 10 minute test, there was no effect of genotype or alarm treatment (protocol 1) on the behavioural parameters measured in the 4 dpf Y472 larvae (Table 3.11).

3.2.6 Analysis of alarm pheromone exposure on shoaling behaviour in larval zebrafish: performed on groups of larvae in a petridish

3.2.6.1 Alarm pheromone extraction protocol 1

WT + alarm: 5dpf

In a 10 minute test, larvae treated with the alarm pheromone (protocol 1) swam significantly slower and had a lower polarization than controls (Figure 3.8 B-C, Table 3.12). There was no effect of alarm treatment or time on NND (Figure 3.8 A, Table 3.12). Speed and polarization decreased throughout the test, and this was accelerated with alarm treatment.

L115 + alarm: 4dpf

In a 10 minute test, exposure to alarm pheromone (protocol 1) resulted in a significant reduction in swimming speed and polarization in both wild type and mutant larvae. There was no effect of genotype on NND, swim speed or polarization and no genotype: time interactions (Figure 3.9, Table 3.13). Speed decreased throughout the test in both genotypes, whilst NND increased.

Table 3.9. Statistical analysis of the effect of alarm pheromone (protocol 1) on wild type larval swimming behaviour in a 10 minute locomotion assay. The results of two-sample t-tests carried out on various behavioural parameters for larvae at 4 and 5 dpf are shown. SEM; standard error of the mean. d.f; degrees of freedom. N = 24 per group, 1 technical replicate.

	Behavioural parameter	Control mean ± SEM	SS-treated mean ± SEM	t value	d.f	p value
4 dpf	Distance swam (cm)	110 ± 25	135 ± 23	0.75	46	0.455
	Swimming duration (mins)	5.7 ± 0.9	6.7 ± 0.9	0.79	46	0.436
	Fast swimming distance (cm)	36 ± 12	55 ± 15	0.96	46	0.340
	Freezing occurrence	12 ± 4	18 ± 4	0.97	46	0.337
5 dpf	Distance swam (cm)	175 ± 13	181 ± 11	0.36	90	0.716
	Swimming duration (mins)	9.2 ± 0.4	9.4 ± 0.3	0.58	88	0.566
	Fast swimming distance (cm)	57 ± 7	64 ± 7	0.74	92	0.461
	Freezing occurrence	24 ± 13	33 ± 16	0.43	91	0.669

Table 3.10. **Statistical analysis of the effect of varying concentrations of alarm pheromone (protocol 1) on 4 dpf larval swimming behaviour.** The results of one-way ANOVA for the effect of treatment carried out on various behavioural parameters are shown. SEM; standard error of the mean. d.f; degrees of freedom. The alarm 2 extract is double the concentration of alarm 1. N = 40 per group, 1 technical replicate.

Behavioural parameter	Control mean ± SEM	Alarm 1 mean ± SEM	Alarm 2 mean ± SEM	F value	d.f	p value
Distance swam (cm)	163 ± 16	133 ± 13	172 ± 23	2.18	3, 181	0.092
Swimming duration (mins)	8.0 ± 0.5	7.1 ± 0.6	8.3 ± 0.5	3.05	3, 181	0.062
Fast swimming distance (cm)	57 ± 9	43 ± 7	51 ± 13	2.11	3, 181	0.100
Freezing occurrence	77± 34	178 ± 43	103 ± 35	2.53	3, 181	0.116

Table 3.11. Statistical analysis of the effect of alarm pheromone (protocol 1) on *disc1* Y472 4 dpf larval swimming behaviour. The results of two-way ANOVA carried out on various behavioural parameters are shown. d.f; degrees of freedom; WT, wild type in-cross larvae; Mut, homozygous mutant in-cross larvae. N=32 per group, 1 technical replicate.

Behavioural parameter	Factor	F value	d.f	p value
Distance swam	Genotype	0	1, 124	0.984
	Treatment	2.43	1, 124	0.122
	Genotype: treatment	2.00	1, 124	0.160
Swimming duration	Genotype	0.17	1, 124	0.679
	Treatment	0.63	1, 124	0.428
	Genotype: treatment	0.39	1, 124	0.532
Fast swimming distance	Genotype	0.05	1, 124	0.829
	Treatment	2.34	1, 124	0.129
	Genotype: treatment	1.86	1, 124	0.175

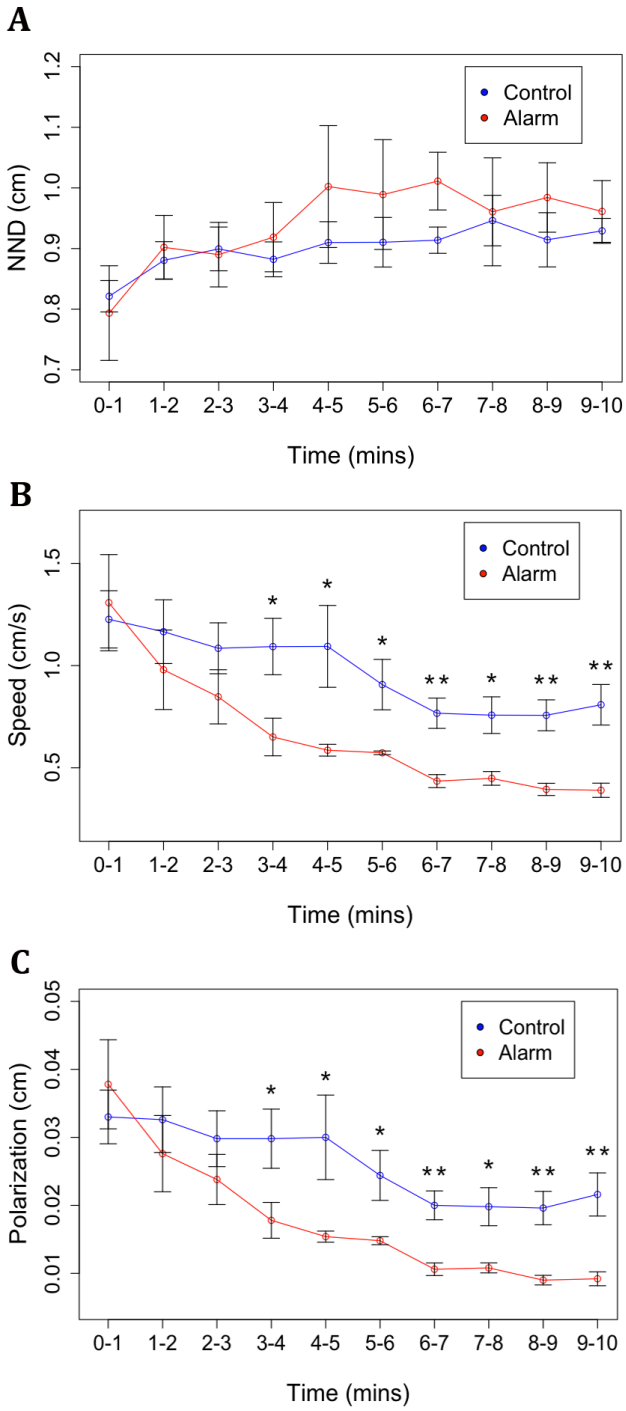

Figure 3.8. The effect of alarm pheromone (protocol 1) on the shoaling behaviour of 5 dpf wild type zebrafish larvae during a 10 minute test. Points and bars represent mean ± standard error. NND, nearest neighbour distance; Time, time interval in the experiment. N = 5 shoals per group, 1 technical replicate. See Table 3.12 for statistics.

(A) Mean nearest neighbour distance of groups of wild type zebrafish larvae exposed to alarm pheromone or control solution.

(B) Mean speed of groups of wild type zebrafish larvae exposed to alarm pheromone or control solution.

(C) Mean polarization of groups of wild type zebrafish larvae exposed to alarm pheromone or control solution.

Table 3.12. Statistical analysis of the effect of alarm pheromone (protocol 1) on the shoaling behaviour of wild type zebrafish at 5 dpf. The results of two-way ANOVA tests, with reapeated masures for factor time, carried out on various behavioural parameters are shown. d.f; degrees of freedom. Time, time interval in the experiment. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 4 shoals per group, 1 technical replicate. See Figure 3.8 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Treatment	0.36	1, 8	0.568	
	Time	30.49	1, 88	<0.0001	***
	Treatment: time	2.30	1, 88	0.133	
Swimming speed	Treatment	6.08	1, 8	0.039	*
	Time	113.09	1, 88	<0.0001	***
	Treatment: time	5.91	1, 88	0.017	*
Polarization	Treatment	5.34	1, 8	0.049	*
	Time	117.29	1, 88	<0.0001	***
	Treatment: time	7.96	1, 88	0.006	**

Y472 + alarm: 8 dpf

In a 10 minute test, there was no effect of alarm treatment (protocol 1) on NND, swimming speed or polarization in either genotype. There was no effect of genotype or time on nearest neighbour distance and no interactions (Figure 3.10 A-B, Table 3.14). Y472 mutants swam significantly faster and were more polarized than wild type larvae, regardless of treatment (Figure 3.10 C-F, Table 3.14). Speed and polarization decreased throughout the test, regardless of genotype.

3.2.6.2 Alarm pheromone extraction protocol 2

L115 + alarm: 5 dpf

In a 10 minute test, exposure to alarm pheromone (protocol 2) resulted in a significant increase in NND of wild types, but had no significant effect on mutants (Figure 3.11 A-B, Table 3.15). Exposure resulted in a significant decrease in swimming speed and polarization in both wild types and mutants. There was no effect of genotype on NND, swimming speed or polarization. NND increased throughout the test, regardless of genotype, whilst speed and polarization decreased throughout the test for mutant larvae only (Figure 3.11 C-F, Table 3.15).

Y472 + alarm: 5 dpf

In a 10 minute test, treatment with alarm pheromone (protocol 2) resulted in a significant increase in NND in wild types, but had no significant effect on mutants (Figure 3.12 A-B, Table 3.16). There was a significant treatment: time interaction, with exposure resulting in a stronger increase in NND over the course of the test, in comparison to that of controls.

Exposure resulted in a significant reduction in swimming speed and polarization in both genotypes (Figure 3.12 C-F, Table 3.16). Y472 mutants had a significantly larger NND and slower swimming speed and polarization than wild types. Regardless of genotype, NND increased throughout, whilst speed and polarization decreased.

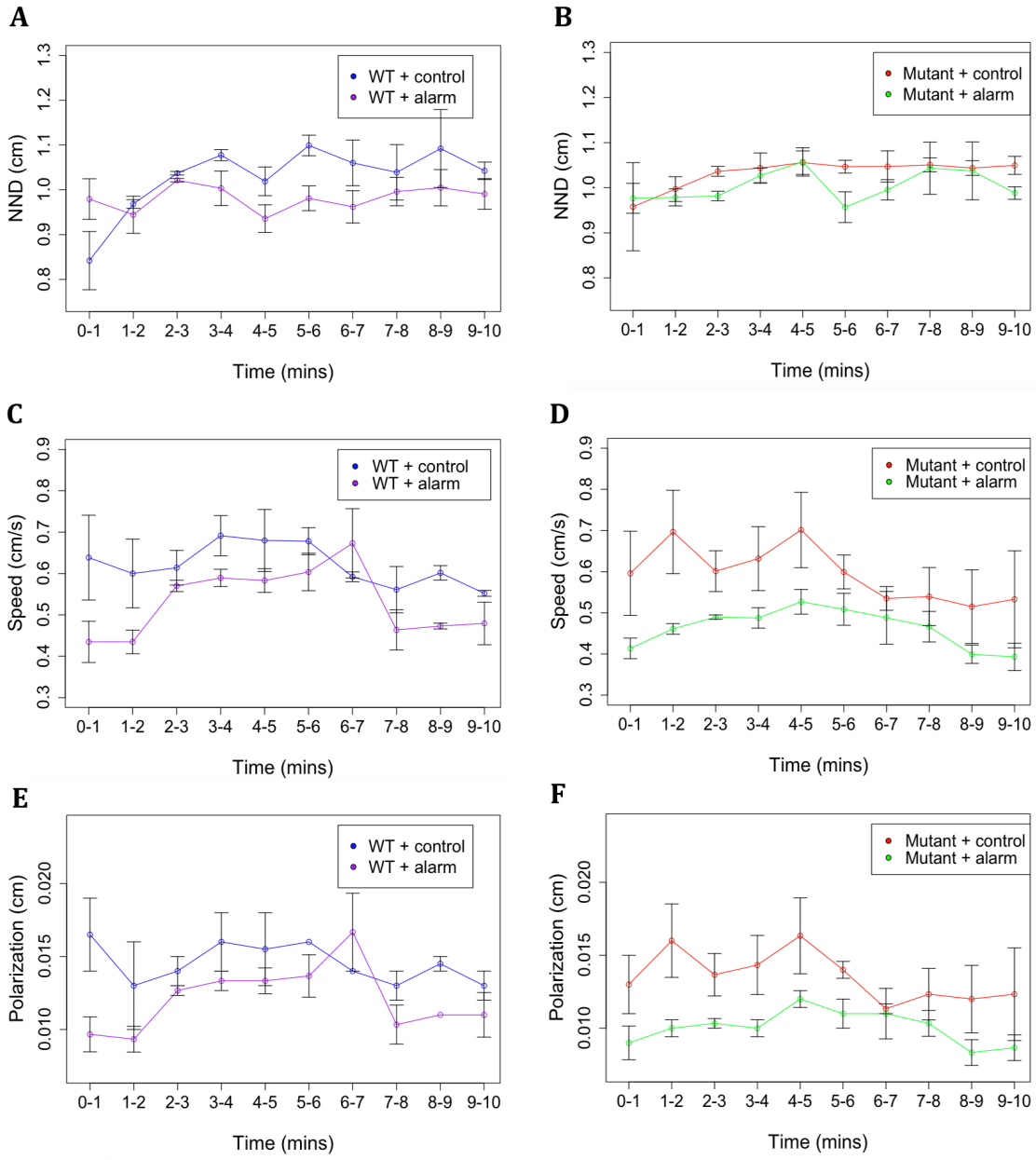

Figure 3.9. The effect of alarm pheromone (protocol 1) on the shoaling behaviour of *disc1* L115 4 dpf zebrafish larvae during a 10 minute test. Points and bars represent mean ± standard error. N=21 larvae per shoal. NND, nearest neighbour distance; WT; wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; Time, time elapsed in the experiment. N= 3 shoals per treatment group, 1 technical replicate. See Table 3.13 for statistics.

(A) Mean NND of wild type larvae exposed to alarm pheromone or control solution.
(B) Mean NND of mutant larvae exposed to alarm pheromone or control solution.
(C) Mean speed of wild type larvae exposed to alarm pheromone or control solution.
(D) Mean speed of mutant larvae exposed to alarm pheromone or control solution.
(E) Mean polarization of wild type larvae exposed to alarm pheromone or control solution.
(F) Mean polarization of mutant larvae exposed to alarm pheromone or control solution.

Table 3.13. Statistical analysis of the effect of alarm pheromone (protocol 1) on shoaling behaviour in *disc1* L115 zebrafish at 4 dpf. The results of two-way ANOVA, with repeated measures carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 3 shoals per group, 1 technical replicate. See Figure 3.9 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	1.36	1, 7	0.283	
	Treatment	5.19	1, 7	0.057	
	Time	10.84	1, 95	0.001	**
	Genotype: treatment	0.29	1, 7	0.606	
	Genotype: time	0.28	1, 95	0.600	
	Treatment: time	4.39	1, 95	0.039	*
	Genotype: treatment: time	1.79	1, 95	0.184	
Swimming speed	Genotype	1.10	1, 7	0.330	
	Treatment	9.91	1, 7	0.016	*
	Time	4.57	1, 95	0.035	*
	Genotype: treatment	0.32	1, 7	0.589	
	Genotype: time	1.98	1, 95	0.163	
	Treatment: time	3.32	1, 95	0.072	
	Genotype: treatment: time	0.00	1, 95	0.982	
Polarization	Genotype	3.04	1, 7	0.125	
	Treatment	16.68	1, 7	0.005	**
	Time	1.56	1, 95	0.215	
	Genotype: treatment	0.47	1, 7	0.516	
	Genotype: time	1.42	1, 95	0.237	
	Treatment: time	2.68	1, 95	0.105	
	Genotype: treatment: time	0.07	1, 95	0.797	

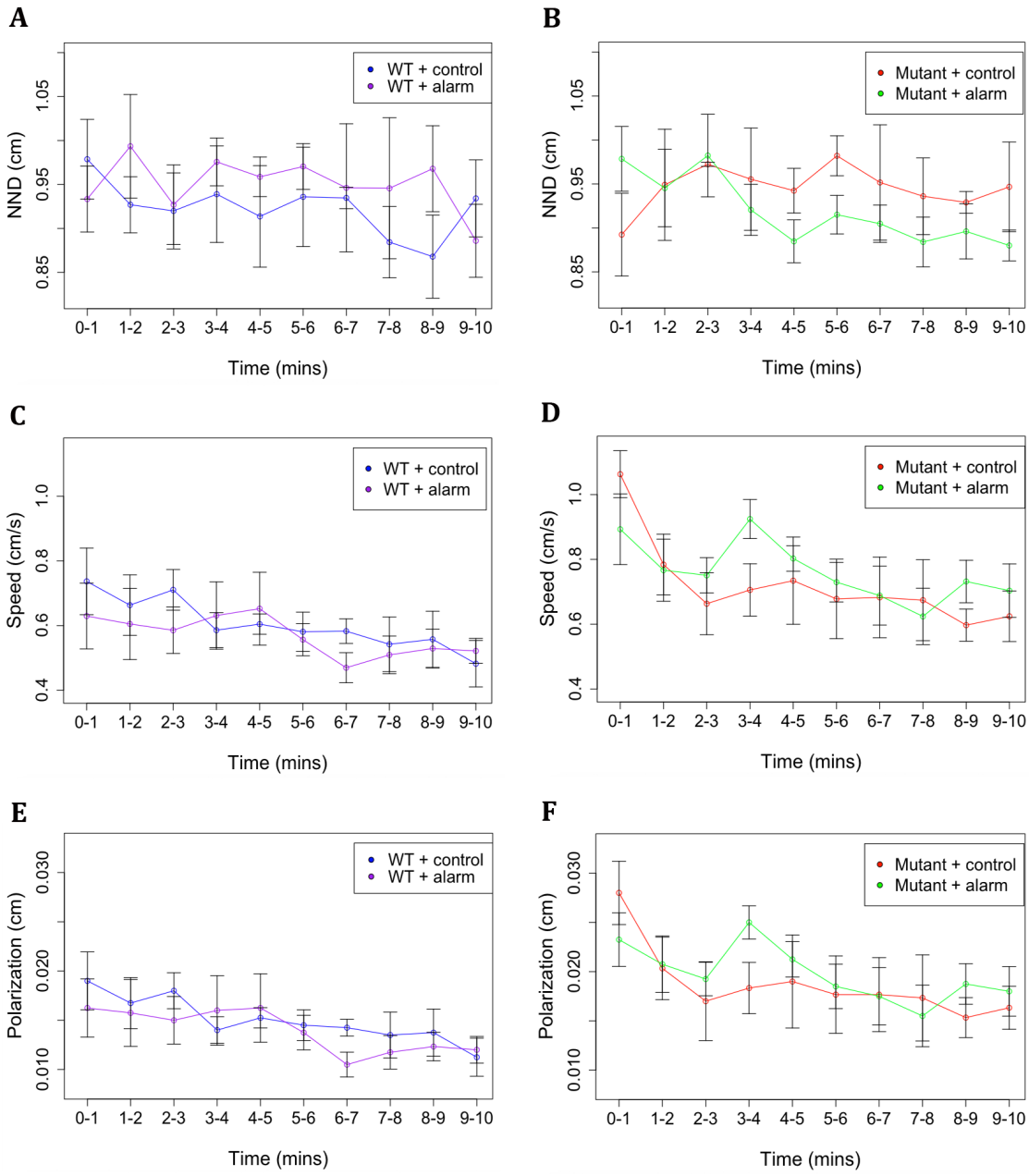

Figure 3.10. The effect of alarm pheromone (protocol 1) on the shoaling behaviour of *disc1* Y472 8 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. NND, nearest neighbour distance; WT, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; Time, time interval in the experiment. N = 3 shoals per group, 2 biological replicates. See Table 3.14 for statistics.

(A) Mean NND of wild type larvae exposed to alarm pheromone or control solution.
(B) Mean NND of mutant larvae exposed to alarm pheromone or control solution.
(C) Mean speed of wild type larvae exposed to alarm pheromone or control solution.
(D) Mean speed of mutant larvae exposed to alarm pheromone or control solution.
(E) Mean polarization of wild type larvae exposed to alarm pheromone or control solution.
(F) Mean polarization of mutant larvae exposed to alarm pheromone or control solution.

Table 3.14. Statistical analysis of the effect of alarm pheromone (protocol 1) on the shoaling behaviour of *disc1* Y472 8 dpf zebrafish. The result of two-way ANOVA with repeated measures are shown. WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae. Time, time interval in the experiment. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$.N= 3 shoals per group, 2 technical replicates. See Figure 3.10 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	0.04	1, 10	0.843	
	Treatment	0.00	1, 10	0.954	
	Time	9.56	1, 130	0.002	**
	Genotype: treatment	1.14	1, 10	0.311	
	Genotype: time	0.01	1, 130	0.931	
	Treatment: time	1.80	1, 130	0.182	
	Genotype: treatment: time	3.60	1, 130	0.060	
Swimming speed	Genotype	5.62	1, 10	0.039	*
	Treatment	0.00	1, 10	0.985	
	Time	51.27	1, 130	<0.0001	***
	Genotype: treatment	0.47	1, 10	0.511	
	Genotype: time	1.04	1, 130	0.309	
	Treatment: time	2.84	1, 130	0.095	
	Genotype: treatment: time	0.05	1, 130	0.831	
Polarization	Genotype	5.48	1, 10	0.041	*
	Treatment	0.00	1, 10	0.981	
	Time	51.60	1, 130	<0.0001	***
	Genotype: treatment	0.47	1, 10	0.507	
	Genotype: time	0.31	1, 130	0.580	
	Treatment: time	0.78	1, 130	0.380	
	Genotype: treatment: time	0.07	1, 130	0.791	

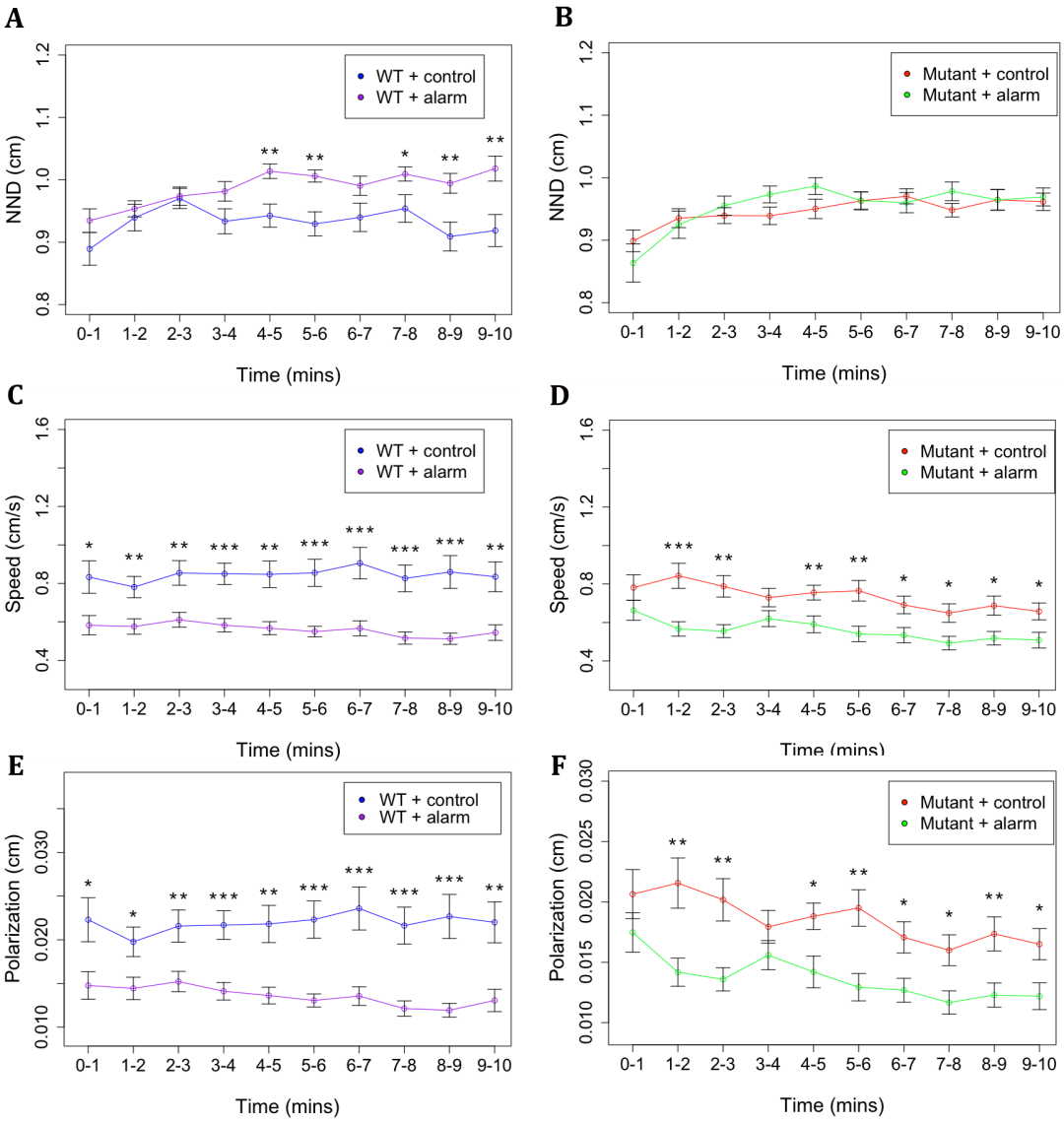

Figure 3.11. The effect of alarm pheromone (protocol 2) on the shoaling behaviour of *disc1* L115 5 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. WT, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; NND, nearest neighbour distance; Time, time interval in the experiment. N = 3 shoals per group, 3 technical replicates. See Table 3.15 for statistics.

(A) Mean NND of wild type larvae exposed to alarm pheromone or control solution.
(B) Mean NND of mutant larvae exposed to alarm pheromone or control solution.
(C) Mean speed of wild type larvae exposed to alarm pheromone or control solution.
(D) Mean speed of mutant larvae exposed to alarm pheromone or control solution.
(E) Mean polarization of wild type larvae exposed to alarm pheromone or control solution.
(F) Mean polarization of mutant larvae exposed to alarm pheromone or control solution.

Table 3.15. Statistical analysis of the effect of alarm pheromone (protocol 2) on shoaling behaviour in 5 dpf *disc1* L115 zebrafish. The results of ANOVA with repeated measures and pairwise comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type in-cross; mut, homozygous mutant in-cross; Time, time interval in the experiment. * indicates a significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N= 3 shoals per group, 3 technical replicates. See Figure 3.11 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	0.72	1, 63	0.398	
	Treatment	6.82	1, 63	0.011	*
	Time	42.81	1, 565	<0.0001	***
	Genotype: treatment	4.07	1, 63	0.048	*
	WT control: WT alarm	8.86	1,34	0.005	**
	Mut control: mut alarm	0.12	1,33	0.735	
	WT control: mut control	0.63	1, 34	0.434	
	WT alarm: mut alarm	7.05	1, 33	0.012	*
	Genotype: time	1.45	1, 565	0.228	
	Treatment: time	10.08	1, 565	0.001	**
	Genotype: treatment: time	1.99	1, 565	0.159	
Swimming speed	Genotype	1.65	1, 63	0.204	
	Treatment	28.98	1, 63	<0.0001	***
	Time	33.26	1, 565	<0.0001	***
	Genotype: treatment	1.27	1, 63	0.265	
	Genotype: time	13.02	1, 565	0.0003	***
	Treatment: time	4.40	1, 565	0.036	*
	Genotype: treatment: time	2.84	1, 565	0.092	
Polarization	Genotype	1.67	1, 63	0.202	
	Treatment	27.95	1, 63	<0.0001	***
	Time	36.34	1, 565	<0.0001	***
	Genotype: treatment	1.58	1, 63	0.214	
	Genotype: time	13.28	1, 565	0.0003	***
	Treatment: time	7.92	1, 565	0.005	**
	Genotype: treatment: time	2.92	1, 565	0.088	

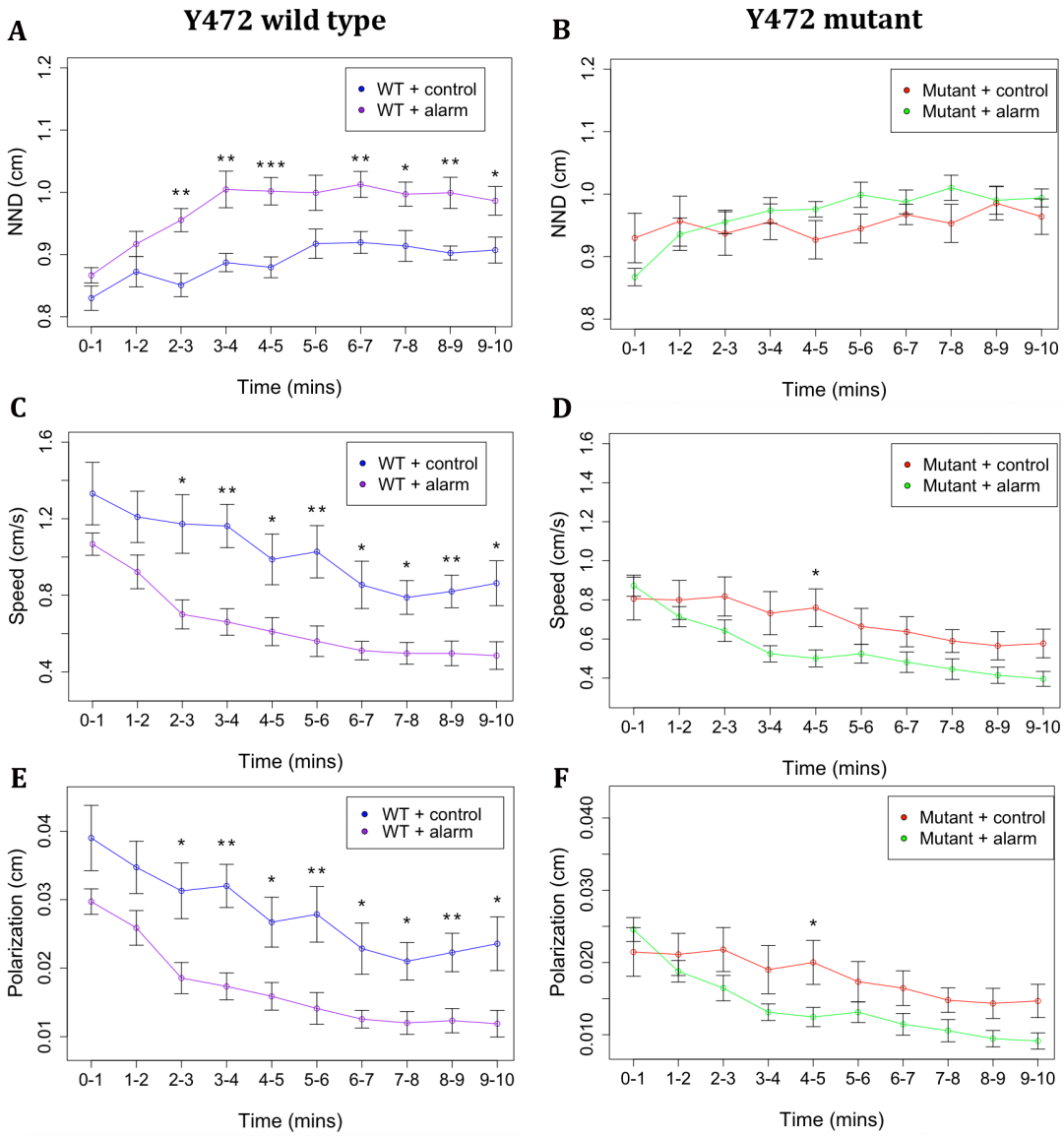

Figure 3.12. The effect of alarm pheromone (protocol 2) on the shoaling behaviour of *disc1* Y472 5 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. NND, nearest neighbour distance; WT, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; Time, time interval in the experiment. N = 3 shoals per group, 3 technical replicates. See Table 3.16 for statistics.

(A) Mean NND of wild type larvae exposed to alarm pheromone or control solution.
(B) Mean NND of mutant larvae exposed to alarm pheromone or control solution.
(C) Mean speed of wild type larvae exposed to alarm pheromone or control solution.
(D) Mean speed of mutant larvae exposed to alarm pheromone or control solution.
(E) Mean polarization of wild type larvae exposed to alarm pheromone or control solution.
(F) Mean polarization of mutant larvae exposed to alarm pheromone or control solution.

Table 3.16. Statistical analysis of the effect of alarm pheromone (protocol 2) on the shoaling behaviour of *disc1* Y472 zebrafish at 5 dpf. The results of ANOVA with repeated measures and pairwise comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type in-cross; mut, homozygous mutant in-cross; Time, time elapsed in the experiment. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N = 3 shoals per group, 3 technical replicates. See Figure 3.12 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	1.86	1, 28	0.183	
	Treatment	8.89	1, 28	0.005	**
	Time	65.88	1, 298	<0.0001	***
	Genotype: treatment	5.06	1, 28	0.033	*
	WT control: WT alarm	22.33	1, 14	0.0003	***
	Mut control: mut alarm	0.39	1, 16	0.541	
	WT control: mut control	5.11	1, 14	0.040	*
	WT alarm: mut alarm	0.20	1, 16	0.658	
	Genotype: time	1.09	1, 298	0.297	
	Treatment: time	6.39	1, 298	0.012	*
	Genotype: treatment: time	1.16	1, 298	0.282	
Swimming speed	Genotype	6.30	1, 28	0.018	*
	Treatment	11.86	1, 28	0.002	**
	Time	309.74	1, 298	<0.0001	***
	Genotype: treatment	2.49	1, 28	0.125	
	Genotype: time	12.89	1, 298	0.0003	***
	Treatment: time	1.55	1, 298	0.215	
	Genotype: treatment: time	2.12	1, 298	0.147	
Polarization	Genotype	7.31	1, 28	0.011	*
	Treatment	12.72	1, 28	0.001	**
	Time	295.38	1, 298	<0.0001	***
	Genotype: treatment	2.75	1, 28	0.108	
	Genotype: time	13.18	1, 298	0.0003	***
	Treatment: time	2.25	1, 298	0.135	
	Genotype: treatment: time	2.69	1, 298	0.102	

3.2.7 Analysis of sodium chloride exposure on shoaling behaviour in larval zebrafish

L115 + NaCl: 4 dpf

In a 10 minute test, exposure to sodium chloride resulted in a significant reduction in NND in wild types and L115 mutant larvae (Figure 3.13 A-B, Table 3.17). Exposure had no effect on swimming speed or polarization of either wild type or mutant larvae (Figure 3.13 C-F, Table 3.17). L115 mutant shoaling behaviour was not different to wild types in any parameters measured here. NND increased throughout the test, whilst speed and polarization decreased, regardless of genotype.

L115 + NaCl: 5 dpf

In a 10 minute test, sodium chloride exposure caused a significant reduction in NND in wild types but had no effect on the L115 mutants (Figure 3.14 A-B, Table 3.18). Exposure resulted in significantly lower swimming speed and polarization in both wild types and mutants (Figure 3.14 C-F, Table 3.18). There was no significant main effect of genotype on NND, swimming speed or polarization. Both genotypes exhibited a significant increase in NND over the course of the test.

Y472 + NaCl: 5 dpf

In a 10 minute test, exposure to sodium chloride resulted in a significant increase in NND in wild types, but had no significant effect on Y472 mutants (Figure 3.15 A-B, Table 3.19). Exposure resulted in a significant reduction in swimming speed and polarization in both wild types and mutants (Figure 3.15 C-F, Table 3.19). There were no significant differences between wild types and mutants in any of the behavioural parameters. Exposure to sodium chloride caused NND to gradually increase, whilst speed and polarization decreased throughout the test.

3.2.8 Body size of *disc1* larvae

There was no significant difference between the head size or body length of the Y472 and L115 homozygous mutant in-cross and wild type in-cross larvae at 5 dpf (Table 3.20).

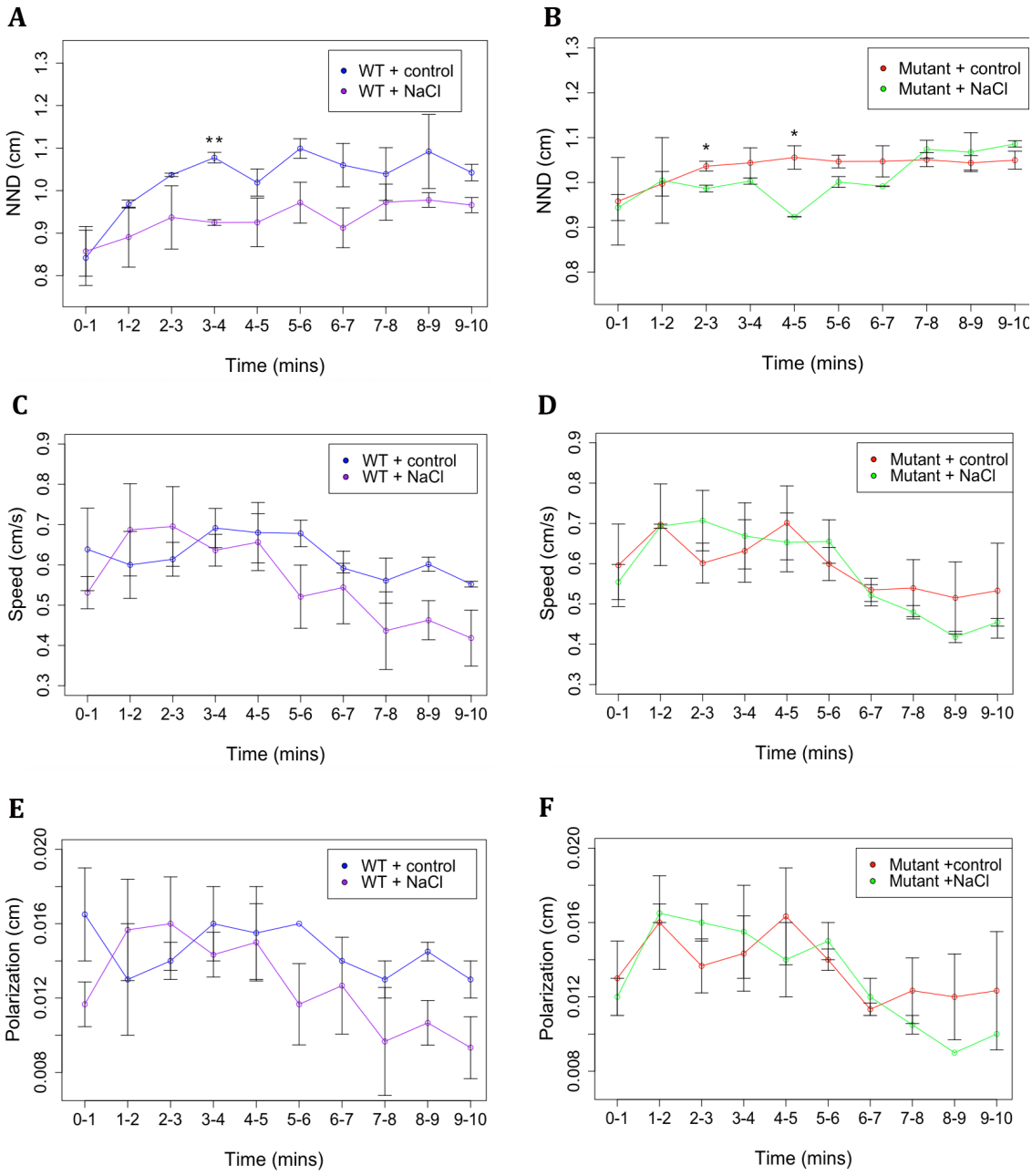

Figure 3.13. The effect of sodium chloride on the shoaling behaviour of *disc1* L115 4 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. NND, nearest neighbour distance; WT, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; Time, time interval in the experiment. N = 3 shoals per group, 1 technical replicate. See Table 3.17 for statistics.

(A) Mean NND of wild type larvae exposed to sodium chloride or control solution.
(B) Mean NND of mutant larvae exposed to sodium chloride or control solution.
(C) Mean speed of wild type larvae exposed to sodium chloride or control solution.
(D) Mean speed of mutant larvae exposed to sodium chloride or control solution.
(E) Mean polarization of wild type larvae exposed to sodium chloride or control solution.
(F) Mean polarization of mutant larvae exposed to sodium chloride or control solution.

Table 3.17. Statistical analysis of the effect of sodium chloride on the shoaling behaviour of *disc1* L115 zebrafish at 4 dpf. The results of two-way ANOVA with repeated measures and pairwise comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type in-cross; mut, homozygous mutant in-cross; Time, time interval in the experiment. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N = 3 shoals per group, 1 technical replicate. See Figure 3.13 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	5.04	1, 6	0.066	
	Treatment	6.37	1, 6	0.045	*
	Time	28.35	1, 86	<0.0001	***
	Genotype: treatment	2.17	1, 6	0.191	
	Genotype: time	0.57	1, 86	0.452	
	Treatment: time	0.001	1, 86	0.975	
	Genotype: treatment: time	1.74	1, 86	0.190	
Swimming speed	Genotype	0.01	1, 6	0.929	
	Treatment	0.41	1, 6	0.547	
	Time	39.30	1, 86	<0.0001	***
	Genotype: treatment	0.16	1, 6	0.704	
	Genotype: time	0.03	1, 86	0.862	
	Treatment: time	5.36	1, 86	0.023	*
	Genotype: treatment: time	0.28	1, 86	0.600	
Polarization	Genotype	0.003	1, 6	0.955	
	Treatment	0.71	1, 6	0.431	
	Time	24.74	1, 86	<0.0001	***
	Genotype: treatment	0.25	1, 6	0.635	
	Genotype: time	0.02	1, 86	0.891	
	Treatment: time	4.10	1, 86	0.046	*
	Genotype: treatment: time	0.02	1, 86	0.880	

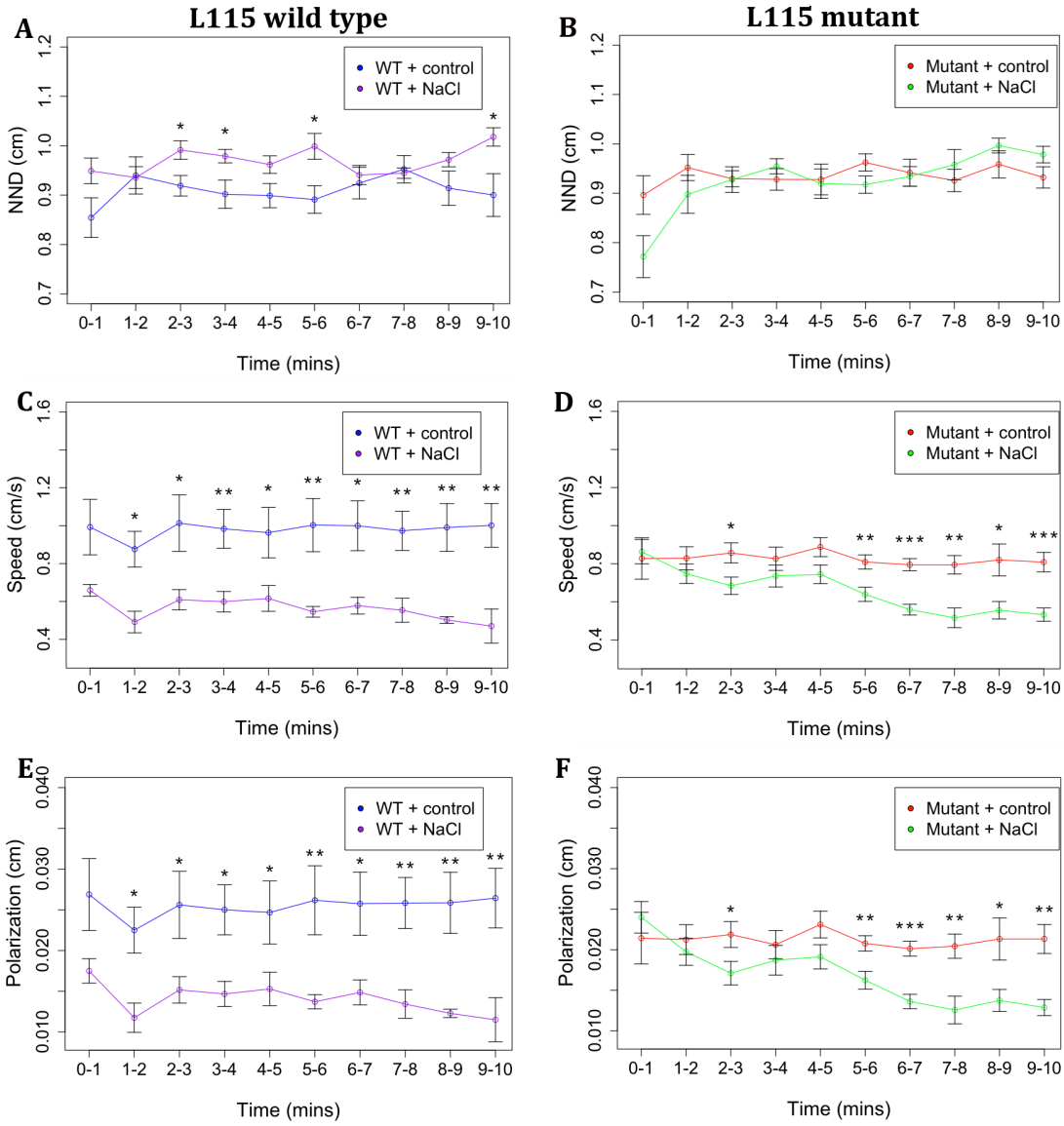

Figure 3.14. The effect of sodium chloride on the shoaling behaviour of *disc1* L115 5 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. NND, nearest neighbour distance; WT, wild type in-cross (non-sibling); mutant, homozygous mutant in-cross; Time, time interval in the experiment. N = 3 shoals per group, 3 technical replicates. See Table 3.18 for statistics.

(A) Mean NND of wild type larvae exposed to sodium chloride or control solution.
(B) Mean NND of mutant larvae exposed to sodium chloride or control solution.
(C) Mean speed of wild type larvae exposed to sodium chloride or control solution.
(D) Mean speed of mutant larvae exposed to sodium chloride or control solution.
(E) Mean polarization of wild type larvae exposed to sodium chloride or control solution.
(F) Mean polarization of mutant larvae exposed to sodium chloride or control solution.

Table 3.18. Statistical analysis of the effect of sodium chloride on the shoaling behaviour of *disc1* L115 zebrafish at 5 dpf. The results of ANOVA with repeated measures and pairwise comparison tests are shown. WT, wild type in-cross; mut, homozygous mutant in-cross; Time, time interval in the experiment. * indicates a significant difference at p< 0.05, ** p<0.01, *** p<0.001. N= 3 shoals per group, 3 technical replicates. See Figure 3.14 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	0.24	1, 28	0.626	
	Treatment	1.33	1, 28	0.259	
	Time	37.59	1, 250	<0.0001	***
	Genotype: treatment	6.56	1, 28	0.016	*
	WT control: WT NaCl	5.73	1, 16	0.029	*
	Mut control: mut NaCl	0.33	1, 16	0.573	
	WT control: mut control	0.83	1, 16	0.376	
	WT NaCl: mut NaCl	7.95	1, 16	0.012	*
	Genotype: time	9.00	1, 250	<0.0001	***
	Treatment: time	10.41	1, 250	0.001	**
	Genotype: treatment: time	5.38	1, 250	0.021	*
Swimming speed	Genotype	0.37	1, 28	0.549	
	Treatment	21.82	1, 28	<0.0001	***
	Time	37.76	1, 250	<0.0001	***
	Genotype: treatment	5.81	1, 28	0.023	*
	WT control: WT NaCl	14.97	1, 16	0.001	**
	Mut control: mut NaCl	11.29	1, 16	0.003	**
	WT control: mut control	2.19	1, 16	0.158	
	WT NaCl: mut NaCl	5.12	1, 16	0.038	*
	Genotype: time	9.67	1, 250	0.002	**
	Treatment: time	27.86	1, 250	<0.0001	***
	Genotype: treatment: time	0.12	1, 250	0.735	
Polarization	Genotype	0.27	1, 28	0.610	
	Treatment	18.98	1, 28	0.0002	***
	Time	34.93	1, 250	<0.0001	***
	Genotype: treatment	5.84	1, 28	0.022	*
	WT control: WT NaCl	13.16	1, 16	0.002	**
	Mut control: mut NaCl	8.54	1, 16	0.009	**
	WT control: mut control	1.92	1, 16	0.185	
	WT NaCl: mut NaCl	4. 76	1, 16	0.044	*
	Genotype: time	9.19	1, 250	0.003	**
	Treatment: time	26.97	1, 250	<0.0001	***
	Genotype: treatment: time	0.76	1, 250	0.385	

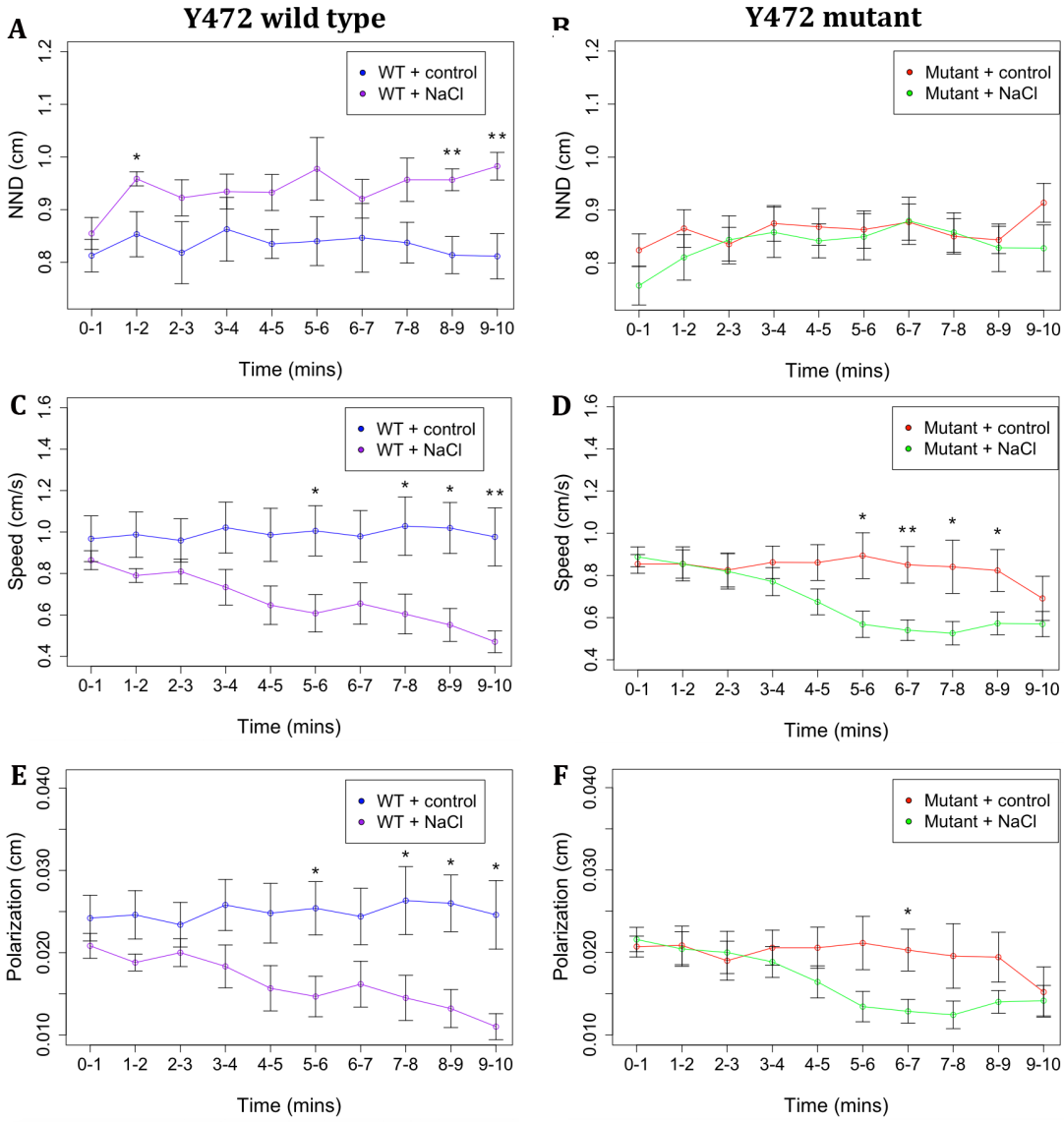

Figure 3.15. The effect of sodium chloride on the shoaling behaviour of *disc1* Y472 5 dpf zebrafish larvae during a 10 minute test. N= 21 per shoal. Points and bars represent mean ± standard error. NND, nearest neighbour distance; WT, wild type in-cross (non-sibling); mutant, homozygous mutant; Time, time interval in the experiment. N = 3 shoals per group, 3 technical replicates. See Table 3.19 for statistics.

(A) Mean NND of wild type larvae exposed to sodium chloride or control solution.
(B) Mean NND of mutant larvae exposed to sodium chloride or control solution.
(C) Mean speed of wild type larvae exposed to sodium chloride or control solution.
(D) Mean speed of mutant larvae exposed to sodium chloride or control solution.
(E) Mean polarization of wild type larvae exposed to sodium chloride or control solution.
(F) Mean polarization of mutant larvae exposed to sodium chloride or control solution.

Table 3.19. Statistical analysis of the effect of sodium chloride on the shoaling behaviour of *disc1* Y472 zebrafish at 5 dpf. The results of ANOVA with repeated measures and pairwise comparison tests are shown. WT, wild type in-cross; mut, homozygous mutant in-cross; Time, time interval in the experiment. * indicates a significant difference at p <0.05, ** p<0.01, *** p<0.001.N= 3 shoals per group, 3 technical replicates. See Figure 3.15 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Nearest neighbour distance	Genotype	1.55	1, 19	0228	
	Treatment	0.98	1, 19	0.333	
	Time	10.48	1, 216	0.001	**
	Genotype: treatment	4.44	1, 19	0.048	*
	WT control: WT NaCl	5.29	1, 10	0.044	*
	Mut control: mut NaCl	0.24	1, 12	0.633	
	WT control: mut control	0.31	1, 11	0.588	
	WT NaCl: mut NaCl	4.33	1, 11	0.062	
	Genotype: time	0.13	1, 216	0.724	
	Treatment: time	5.38	1, 216	0.021	*
	Genotype: treatment: time	1.17	1, 216	0.281	
Swimming speed	Genotype	0.92	1, 19	0.349	
	Treatment	10.52	1, 19	0.004	**
	Time	103.89	1, 216	<0.0001	***
	Genotype: treatment	1.32	1, 19	0.265	
	Genotype: time	0.68	1, 216	0.412	
	Treatment: time	70.68	1, 216	<0.0001	***
	Genotype: treatment: time	0.20	1, 216	0.652	
Polarization	Genotype	1.36	1, 19	0.258	
	Treatment	8.10	1, 19	0.010	*
	Time	71.34	1, 216	<0.0001	***
	Genotype: treatment	1.69	1, 19	0.209	
	Genotype: time	0.75	1, 216	0.389	
	Treatment: time	2.24	1, 216	<0.0001	***
	Genotype: treatment: time	0.01	1, 216	0.936	

Table 3.20. Statistical analysis of *disc1* L115 and Y472 larval body size at 5 dpf. The results of two-sample t-tests carried out for body length and head width are shown. SEM; standard error of the mean. d.f; degrees of freedom. N = 18 per group, 3 technical replicates.

	Body measurement	Wild type in-cross mean ± SEM	Homozygous in-cross mean ± SEM	t value	d.f	p value
L115 5 dpf	Body length (mm)	3.97	4.06	0.94	34	0.355
	Head width (mm)	0.95	1.06	1.45	10	0.178
Y472 5 dpf	Body length (mm)	4.06	4.13	0.50	34	0.621
	Head width (mm)	0.93	093	-0.01	10	0.993

3.3 Discussion

3.3.1 The effect of *disc1* on baseline swimming behaviour in larval zebrafish

There was no difference in the basic swimming behaviour of Y472 wild type in-cross or homozygous mutant in-cross larvae in a 10 minute test, but a 1 hour analysis of the L115 larvae revealed hypomotility in the mutants. Further analyses are required to establish if this represents a difference between the two *disc1* lines, or whether hypomotility was not detected in the tests of the Y472 larvae due to the short testing period.

In an analysis of L115 siblings, no difference in motility was detected. This analysis is unfortunately weakened by having a much smaller sample size than that of the non-sibling experiments. The behaviour of heterozygous mutant siblings was inconsistent in these analyses and so it cannot be confirmed if behaviour is affected by one copy of the *disc1* mutation.

Adult *Disc1* mutant mice (L100P) show hyperactivity in the open field [6, 77]. This behaviour is normalised to control levels after treatment with Haloperidol and the authors therefore concluded that this behaviour is associated with Dopamine sensitivity. Zebrafish with a mutation in the glucocorticoid receptor show reduced exploration in the open field and this behaviour is normalised by treatment with an antidepressant [78]. An analysis of the effect of drug treatments on behaviour in the *disc1* zebrafish may help to characterise these impairments further. There was no significant difference in the size of the *disc1* mutant larvae in comparison to wild types that could account for a locomotion defect and their gross morphology was normal, consistent with the idea that there may be a reduced motivation for swimming in the L115 homozygous mutant larvae.

3.3.2 Analysis of place preference in *disc1* zebrafish larvae

In the open field test, larvae showed a preference for the perimeter of the well, which has been previously reported in zebrafish larvae [1]. In the light response experiment, L115 mutants spent less time in the perimeter of the well than wild types. The avoidance of the centre, or thigmotaxis, has been described by some as

an anxiety-like behaviour [1], in which animals avoid the exposed central area, but by others as motivated exploratory behaviour [79, 80], as fish habituated to the open field will increase thigmotaxic behaviour. An avoidance of the walls of the tank has also been reported in the glucocorticoid receptor mutant, which the authors associate with increased anxiety [78]. In my experiment, the motivation for this behaviour was not clear, but suggests that *disc1* mutants display some differences in their exploratory or anxiety behaviour.

3.3.3 Analysis of light responsiveness in *disc1* zebrafish larvae

It was apparent in both of the light experiments, that *disc1* mutant larvae were able to detect a light source and initiate a locomotor response. In contrast to previous reports [81], L115 larvae did not show increased activity in the dark when compared to the light. On the other hand, dark exposure was associated with a reduction in thigmotaxic behaviour. A light-induced change in place preference in this manner has not been previously described, but this result might suggest that zebrafish show a change in anxiety or exploratory behaviours in the dark.

When *disc1* Y472 larvae were exposed to a dark flash, both wild types and mutants exhibited a response of similar magnitude, suggesting no impairment of the mutant optokinetic startle response. As previously reported [81], post-stimulus activity was significantly higher than pre-stimulus activity.

3.3.4 The effect of *disc1* on shoaling behaviour in larval zebrafish

The behaviour of the *disc1* larvae in the shoaling assays was inconsistent between experiments. This could be due to chance effects, or genetic or environmental differences affecting the different offspring cohorts. In the L115 larvae, experiments revealed no difference between the nearest neighbour distance, swimming speed or polarization of wild type and mutant larvae. On the other hand, shoaling behaviour in the Y472 line was very inconsistent, suggestive of chance effects, and in some experiments Y472 mutants again had significantly reduced swimming speed and polarization, further suggesting hypomotility in the mutants. In these experiments, the effect of the L115 mutation on motility seemed to be very weak, despite a more convincing result in the basic locomotion assays.

For the most part, these results suggest that mutant larvae have the same level of social cohesion as wild types at this early stage of development. In the *DISC1* mouse literature, some studies have shown no effect of the mutation on sociability (L100P *Disc1* [6]; *DISC1* [38]), whilst other mutant lines show reduced sociability (Q3L [6]), which is reminiscent of some psychiatric disorders.

It is clear in all of the experiments that there is a strong correlation between swimming speed and polarization. Due to the nature of how polarization is calculated, taking into account both the direction and distance travelled by the fish, when both the speed and polarization are significantly higher or lower in one group compared to another, it is impossible to ascertain whether more or fewer fish are swimming in the same direction, or whether the change in polarization is due to a concurrent change in speed.

3.3.5 The effect of isolation on *disc1* larval swimming behaviour

Rearing in isolation was intended as a potential stressor, as is seen in rodent models [7] and previous studies in zebrafish had shown that it can affect behaviour [1, 82]. Although zebrafish do not exhibit any parental care, as social animals, I postulated that a lack of social interaction might have an effect on behaviour. It has been demonstrated that tactile stimulation can reduce fear-associated behaviours in larval zebrafish [69], and so I hypothesised that a lack of this stimulation might increase anxiety.

The 8 dpf Y472 homozygous mutant in-cross larvae swam significantly less distance and did less fast swimming than the wild type in-cross fish, further supporting the hypothesis that a mutation in the *disc1* gene is responsible for the observed hypomotility. There was no significant effect of the housing conditions on any of the behavioural parameters measured in the wild type or mutant larvae. It is not clear from this experiment, whether rearing in isolation is stressful in zebrafish and has any effect on behaviours not tested here, such as shoaling, or other long-term effects. It has been shown that tactile stimulation is essential for larvae to develop a normal locomotor response to a dark pulse [1], suggesting that a lack of tactile stimulation might have a detrimental effect on sensory development. It has

been demonstrated in adults that group housed fish show increased anxiety behaviour in an isolated testing situation than the isolation-reared fish [82]. It would therefore have been informative to do a similar experiment in which fish from the two housing conditions were analysed in a group situation. It has also been shown that DISC1 mutant mice are hyper-responsive to an isolation stressor during adolescence [7], but as a species that show parental care it is clear that this experience is stressful in mice.

3.3.6 The effect of alarm pheromone on larval swimming behaviour

The effect of alarm pheromone on swimming behaviour of zebrafish larvae has not been reported. My findings show that exposure to the alarm extract (protocol 1) had no effect on the basic swimming behaviour of wild type larvae or Y472 mutants. These experiments cannot confirm whether early zebrafish larvae, wild types or *disc1* mutants, modulate their behaviour upon exposure to alarm pheromone in a way not detected in this assay.

3.3.7 The effect of alarm pheromone on shoaling behaviour in larval zebrafish

The experiments using the alarm pheromone extraction procedure of protocol 1 yielded some conflicting results, likely due to the inconsistency in the exact concentration and content of the extract. In these experiments NND was unaffected by the alarm extract, whilst speed and polarization were significantly reduced by alarm pheromone in some experiments, but unaffected in others. The detection of some behavioural responses suggests that the larvae are able to detect the olfactory cues of the alarm pheromone. At this early developmental stage, it is likely that the adult zebrafish alarm reaction is not fully developed and behavioural responses to the stressor might be unpredictable. The reduction in swimming speed corresponds with the reported response of adult zebrafish to the alarm pheromone [26], suggesting an increase in caution, as a predator is assumed to be nearby. In the course of my thesis, a new extraction protocol (protocol 2) was described, which was reportedly more potent and could be used in multiple experiments. This protocol was used in later studies.

In the experiments utilising the new alarm pheromone extraction method (protocol 2), exposure resulted in a significant reduction in swimming speed and polarization in both L115 and Y472 wild type and mutant larvae, although the reduction appears smaller in the mutant shoals. In both cases, exposure resulted in a significant increase in nearest neighbour distance in the wild type larvae, but had no effect on this parameter in the mutants. The increase in nearest neighbour distance suggests that the shoal is disrupted when the wild types are stressed, possibly indicative of an escape response. The lack of this behavioural response to the stressor by the mutants suggests that the behavioural stress response is modulated by *disc1* in some way.

3.3.8 The effect of sodium chloride on shoaling behaviour in larval zebrafish

The effect of sodium chloride on larval shoaling behaviour was also slightly inconsistent. In the experiments exposing the 4 dpf L115 larvae to sodium chloride, exposure resulted in a reduction in nearest neighbour distance, whilst in 5 dpf Y472 and L115 wild type NND was significantly increased upon exposure. In these cases exposure had no effect on mutant NND, again suggestive of an interaction between *disc1* and the behavioural response to stress. In most of the experiments, exposure to NaCl resulted in a significant reduction in swimming speed and polarization in both wild type and mutant larvae.

3.4 Concluding remarks

In all of the behavioural experiments, I have exerted caution when interpreting p values from statistical tests. When dealing with large data sets, the risk of a chance effect, not linked to the dependent variables, having a statistically significant effect on the independent variable, is much higher. For the most part, baseline shoal cohesion was not effected by the *disc1* mutation, but this parameter was changed significantly upon exposure to a stressor in the wild type larvae, but unaffected in the mutants. This effect was conserved between the two stressors and two *disc1* fish lines, strongly suggesting a link between *disc1* and the HPI axis. These data are consistent with mouse studies in which animals with a mutation in DISC1 also show different behavioural responses to a stressor in comparison to wild type mice [7, 38]. In contrast with the mouse studies in which animals were hyper-

responsive, mutant zebrafish appeared to be less responsive to a chemical stressor. A reduced responsiveness to stress has also been observed in humans and in animal models, where individuals have experienced early life stress and has been linked to epigenetic programming of the HPA axis [83]. Indeed, a reduction in anxiety behaviours has also been observed in zebrafish that were pre-exposed to a stressor [80], again suggestive of some programming of the stress response. The *disc1* protein could act via similar or distinct mechanisms to interact with the HPI axis in fish and modulate the behavioural response to stress.

4 Behavioural analysis of *disc1* adult zebrafish

4.1 Introduction

In the past two decades, zebrafish behavioural phenotypes relevant to the study of stress have been described [23]. The *DISC1* gene has been linked to the stress response in animal models [7] and the development of psychiatric illness in humans [4]. As previously discussed, *DISC1* mutant mice show a variety of different behavioural abnormalities including hyperactivity in an open field test, impaired pre-pulse inhibition, impaired latent inhibition and deficits in a forced swim test, with phenotypes varying between models [6].

Behaviour is developmentally plastic. Early life experiences, as well as varying expression patterns of relevant genes throughout development will have implications for behavioural phenotypes in a given test situation. This plasticity is demonstrated in the *DISC1* mouse field, in which phenotypes differ with developmental stage. The *Disc1* Q31L mouse model showed impairments in PPI at 16 weeks of age but not at 8 weeks [84] and the effect of maternal immune activation on PPI in wild type mice, in the same experiment, was apparent only in the 16 week group. Another GXE study demonstrated that behavioural defects were only apparent under continuous expression of *DISC1* [38], suggesting that phenotypes may vary with varying expression of *DISC1* throughout development. These findings highlight how developmental timing can affect behavioural phenotypes and responses to stressors. The zebrafish develops quickly and so is a useful model to study the ontogeny of behaviour.

A common behavioural test for anxiety is the open field test [1] (Figure 2.3 A). This test is assumed to evoke anxious behaviour, as a single zebrafish is placed in a large novel environment. Champagne reported an avoidance of the centre of the tank, where fish spent more than 90% of their time in the outer field [80]. This behaviour, known as thigmotaxis, is also observed in rodents and humans in aversive and novel environments [85]. Thigmotaxis may resemble an anxiety response, in which subjects avoid the centre of the environment because of its increased exposure to a predator. In contrast, others have suggested that this

behaviour is indicative of motivated exploration in zebrafish, rather than anxiety [79, 80].

The tank diving test induces an anxiety response that is fish specific [1] (Figure 2.3 C). When fish are first transferred into an open novel tank they typically sink to the bottom of the tank for the first minute, and then are fully habituated by the end of a 10 minute test [25, 28]. Tanks used in this test are designed to be narrow to restrict horizontal movement, and deep to exaggerate the difference between the test tank and the zebrafish's natural shallow water habitat [24]. The duration spent at the bottom of the tank and latency to enter the upper compartment are commonly used as measures of anxiety [23]. This bottom dwell behaviour has been shown to be sensitive to alarm pheromone as well as anxiogenic and anxiolytic drugs [25], and so is a valid measure of anxiety in zebrafish.

Another interesting paradigm is the scototaxis test, which involves measuring behaviour in a tank that is divided into light and dark compartments (Figure 2.3 B). Blaser reported a light avoidance response, which might indicate that the fish were hiding in the dark compartment [79]. In contrast, Champagne reported that fish spent 70% of their time in the light compartment during their study [80]. This might reflect the zebrafish's diurnal habit, as fish will search in the light for food and mates. It is important to note that the scototaxis test has not been pharmacologically validated, although there is some evidence in larvae that Diazepam might impact behaviour in this test, which would validate this test as a measure of anxiety [1].

The experiments described in this chapter utilise the above-described behavioural tests to investigate the effect of *disc1* mutation on behaviour in the adult zebrafish. Previous work in *DISC1* mouse models has demonstrated that the mutation might affect baseline behaviour [6], the behavioural response to stress [7] and that these effects could vary with developmental stage [84]. This highlights the importance of studying the effect of *disc1* on behaviour in the adult, as well as larval zebrafish, allowing for investigation of more complex and validated anxiety-related behaviours. The two lines of *disc1* zebrafish, L115 and Y472, were analysed in

terms of their baseline swimming behaviour and their behavioural response to the alarm pheromone stressor.

4.2 Results

4.2.1 Analysis of the baseline swimming behaviour of adult *disc1* zebrafish

4.2.1.1 The open field test: L115

There was no significant effect of genotype or sex on time spent in the perimeter of the tank; total distance swam or freezing occurrence and no significant genotype: sex interactions (Figure 4.1 A, B, D, Table 4.1). Male fish did significantly less fast swimming than females (Figure 4.1 C, Table 4.1), but there was no effect of genotype and no genotype: sex interaction.

4.2.1.2 The scototaxis test: L115

There was no significant effect of genotype or sex on time spent in the dark compartment, distance swam, fast swimming distance or freezing occurrence and no significant genotype: sex interactions (Figure 4.2, Table 4.2).

4.2.1.3 The tank diving test: Y472

In a ten minute tank diving test, there was no effect of genotype on the behaviours measured (Figure 4.3, Table 4.3). Genotypes differed more strongly in distance swam, fast swimming and freezing occurrences during the first half of the test, after which, differences were not detectable (Figure 4.3 C-H, Table 4.3). For all genotypes and sexes, bottom dwell, distance swam and fast swimming exhibited a decrease throughout the ten minute test (Figure 4.3 A-F, Table 3.4). Males of all genotypes swam further and faster than females (Figure 4.3 C-F, Table 4.3).

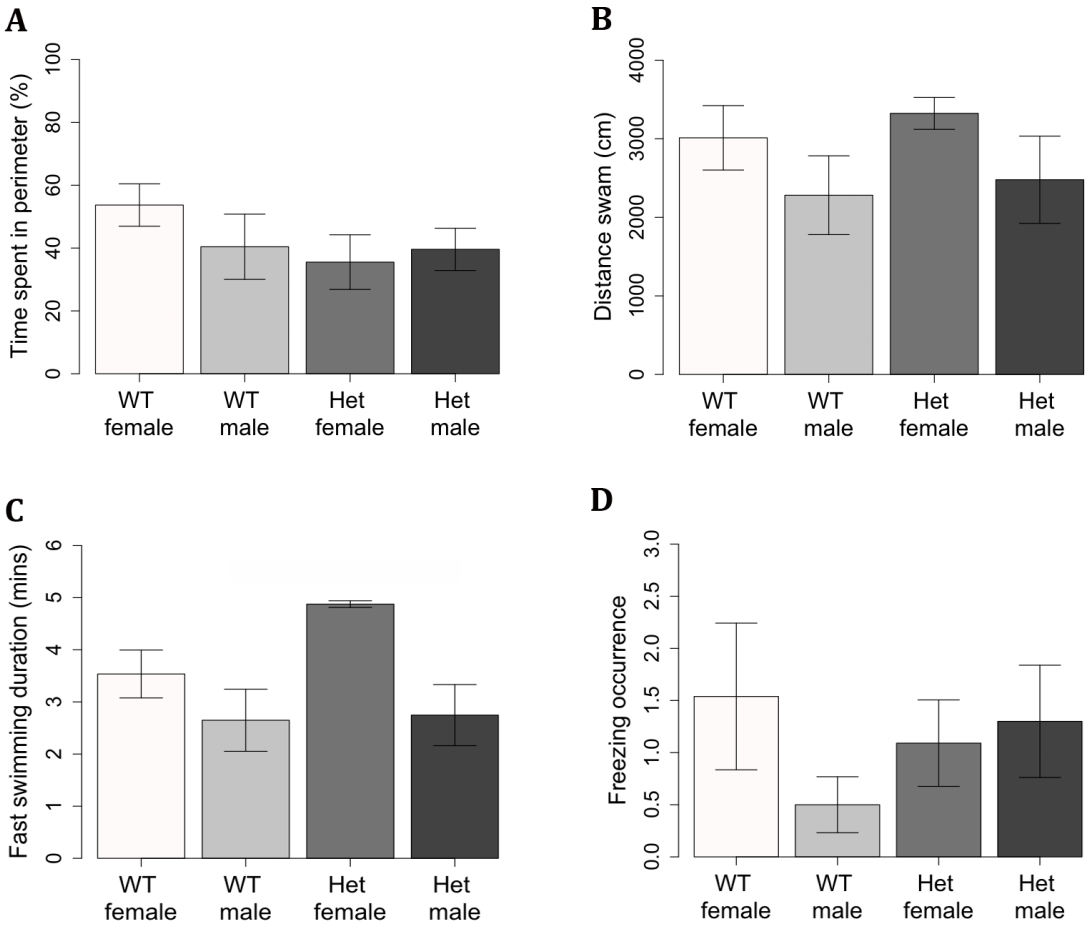

Figure 4.1. Behaviour of *disc1* L115 adult zebrafish in the 5 minute open field test. Points and bars represent mean ± standard error. WT, wild type siblings; Het, heterozygous mutant. * indicates statistically significant difference at $p<0.05$. N= 10 per group, 1 technical replicate. See Table 4.1 for statistics.

(A) Mean percentage of time spent in the perimeter region of the tank by wild type and heterozygous mutant L115 zebrafish.
(B) Mean total distance swam by wild type and heterozygous mutant L115 zebrafish.
(C) Mean fast swimming duration by wild type and heterozygous mutant L115 zebrafish.
(D) Mean number of freezing occurrences by wild type and heterozygous mutant L115 zebrafish.

Table 4.1. Statistical analysis of the behaviour of *disc1* L115 adult zebrafish in the 5 minute open field test. The results of two-way ANOVA and Tukey Multiple Comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type siblings; Het, heterozygous mutant. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 10 per group, 1 technical replicate. See Figure 4.1 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Time in perimeter	Genotype	1.65	1, 40	0.207	
	Sex	0.36	1, 40	0.550	
	Genotype: sex	1.12	1, 40	0.296	
Distance swam	Genotype	0.28	1, 40	0.601	
	Sex	3.31	1, 40	0.076	
	Genotype: sex	0.02	1, 40	0.896	
Fast swimming duration	Genotype	2.31	1, 40	0.136	
	Sex	9.97	1, 40	0.003	**
	Genotype: sex	1.74	1, 40	0.194	
Freezing occurrence	Genotype	0.04	1, 40	0.849	
	Sex	0.65	1, 40	0.425	
	Genotype: sex	1.32	1, 40	0.258	

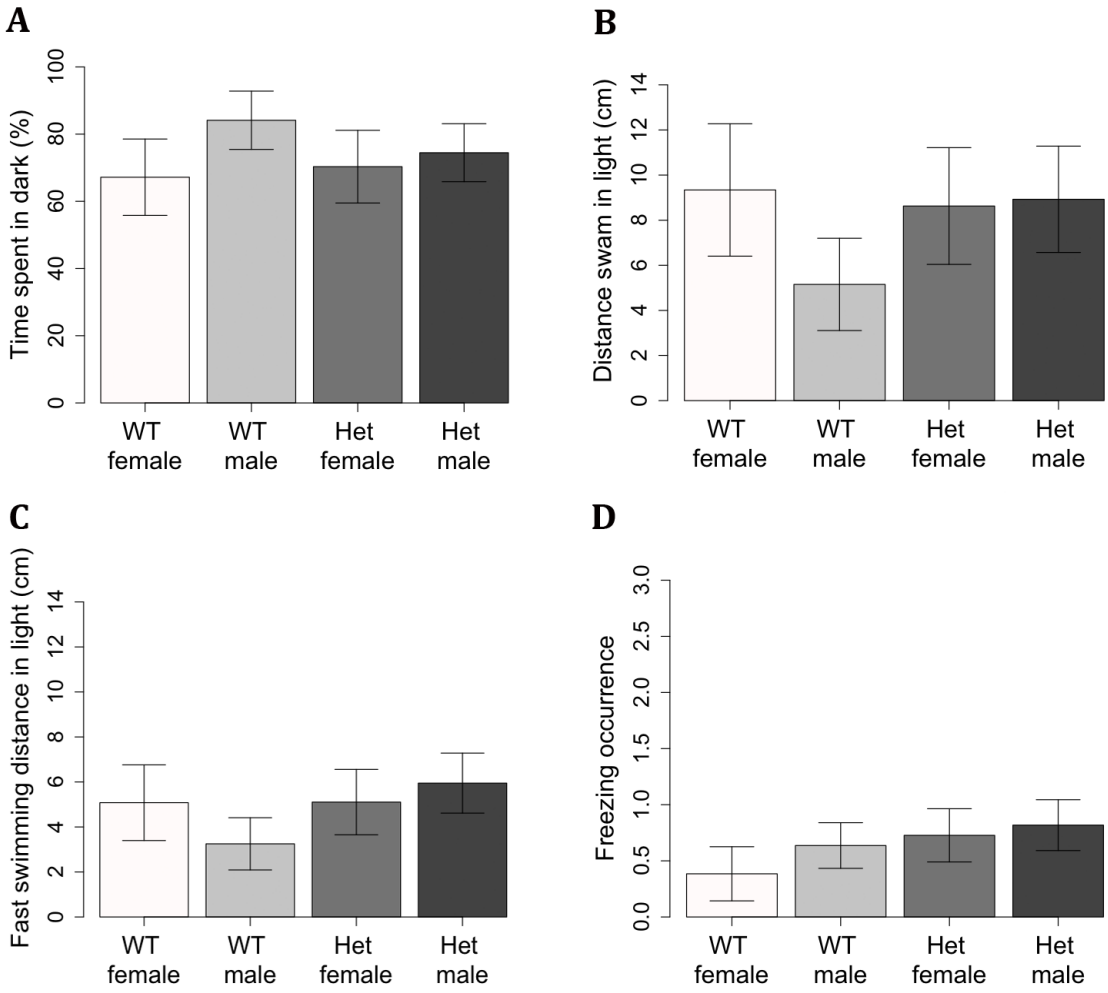

Figure 4.2. Behaviour of *disc1* L115 adult zebrafish in the 5 minute scototaxis test. Points and bars represent mean ± standard error. WT, wild type siblings; Het, heterozygous mutant. * indicates statistically significant difference at $p<0.05$. N= 10 per group, 1 technical replicate. See Table 4.2 for statistics.

(A) Mean percentage of time spent in the dark compartment of the tank by wild type and heterozygous mutant L115 zebrafish.

(B) Mean total distance swam in the light compartment of the tank by wild type and heterozygous mutant L115 zebrafish.

(C) Mean fast swimming distance in the light compartment by wild type and heterozygous mutant L115 zebrafish.

(D) Mean number of freezing occurrences in the light compartment by wild type and heterozygous mutant L115 zebrafish.

Table 4.2. Statistical analysis of the behaviour of *disc1* L115 adult zebrafish in the 5 minute scototaxis test. The results of two-way ANOVA and Tukey Multiple Comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type siblings; Het, heterozygous mutant. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 10 per group, 1 technical replicate. See Figure 4.2 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Time in dark compartment	Genotype	0.06	1, 42	0.802	
	Sex	1.14	1, 42	0.293	
	Genotype: sex	0.40	1, 42	0.532	
Distance swam	Genotype	0.28	1, 42	0.598	
	Sex	0.63	1, 42	0.431	
	Genotype: sex	0.77	1, 42	0.386	
Fast swimming distance	Genotype	0.78	1, 42	0.381	
	Sex	0.14	1, 42	0.710	
	Genotype: sex	0.84	1, 42	0.364	
Freezing occurrence	Genotype	1.41	1, 42	0.242	
	Sex	0.58	1, 42	0.452	
	Genotype: sex	0.12	1, 42	0.728	

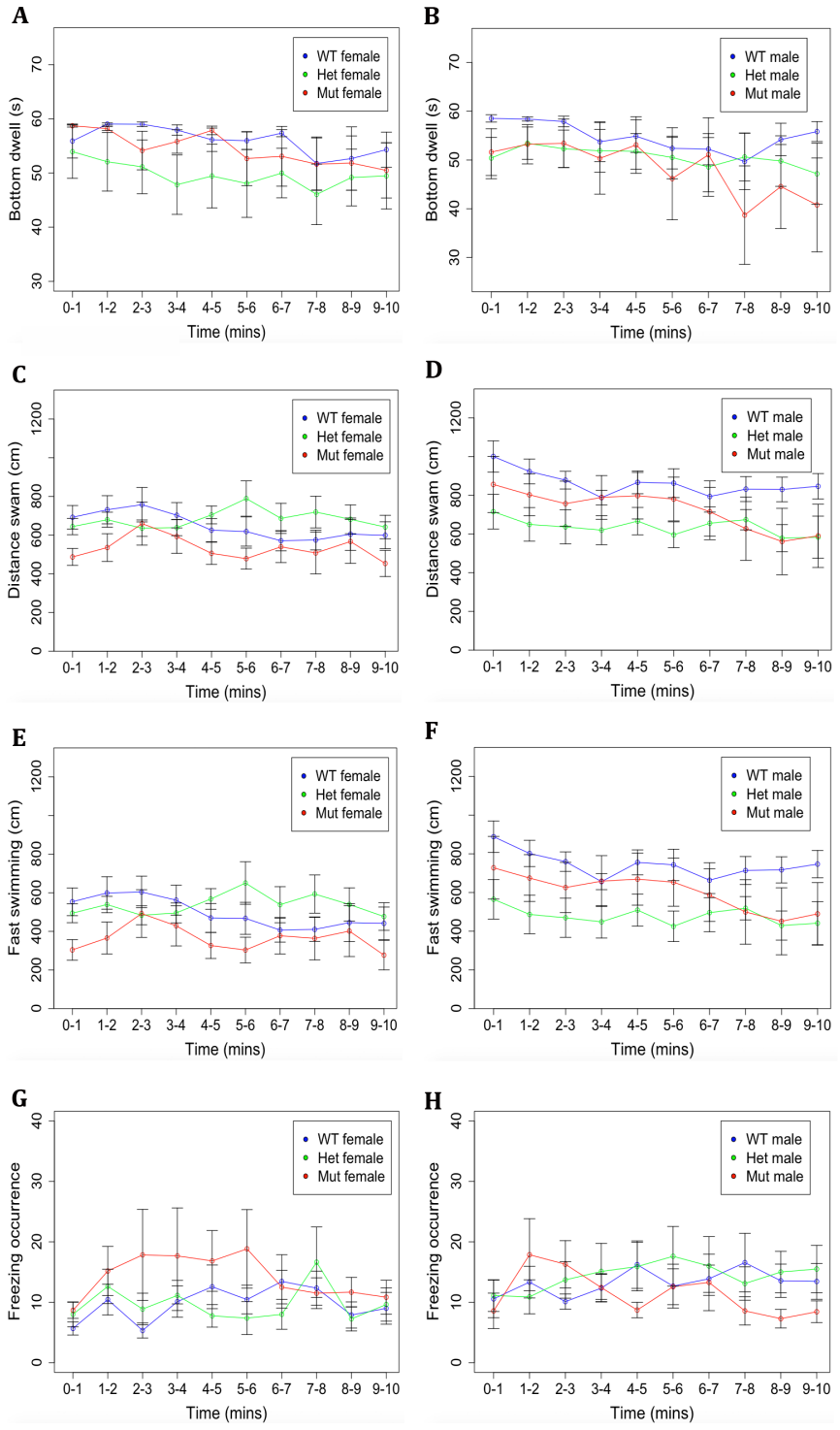

Figure 4.3. Behaviour of *disc1* Y472 adult zebrafish in the 10 minute tank diving test. Points and bars represent mean ± standard error. WT, wild type siblings; Het, heterozygous mutant; Mut, homozygous mutant. N= 10 per group, 1 technical replicate. See Table 4.3 for statistics.

(A-B) Mean duration spent in the bottom compartment of the tank by Y472 females (A) and males (B).

(C-D) Mean distance swam in all compartments of the tank by Y472 females (C) and males (D).

(E-F) Mean distance of fast swimming in all compartments of the tank by Y472 females (E) and males (F).

(G-H) Mean number of freezing occurrences in all compartments of the tank by Y472 females (G) and males (H).

Table 4.3. Statistical analysis of the behaviour of *disc1* Y472 adult zebrafish in the 10 minute tank diving test. The results of two-way ANOVA with repeated measures and pairwise comparison tests carried out on various behavioural parameters are shown. d.f, degrees of freedom; WT, wild type siblings; Het, heterozygous mutant; Mut, homozygous mutant; Time, time interval in experiment. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 9 per group, 1 technical replicate. See Figure 4.3 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Time in lower compartment	Genotype	1.11	2, 43	0.341	
	Sex	0.31	1, 43	0.582	
	Time	30.99	1, 435	<0.0001	***
	Genotype: time	2.60	2, 435	0.075	
	Genotype: sex	0.40	2, 43	0.675	
	Sex: time	0.32	1, 435	0.573	
	Genotype: sex: time	0.49	2, 435	0.616	
Distance swam	Genotype	1.87	2, 43	0.167	
	Sex	1, 43	1, 43	0.038	*
	Time	25.42	1, 435	<0.0001	***
	Genotype: time	4.12	2, 435	0.017	*
	Genotype: sex	2.55	2, 43	0.090	
	Sex: time	3.65	1, 435	0.057	
	Genotype: sex: time	2.86	2, 435	0.058	
Fast swimming distance	Genotype	1.87	2, 43	0.166	
	Sex	4.95	1, 43	0.032	*
	Time	19.93	1, 435	<0.0001	***
	Genotype: time	3.62	2, 435	0.028	*
	Genotype: sex	2.95	2, 43	0.063	
	Sex: time	1.70	1, 435	0.193	
	Genotype: sex: time	2.95	2, 435	0.054	
Freezing occurrence	Genotype	0.12	2, 43	0.891	
	Sex	1.26	1, 43	0.269	
	Time	0.63	1, 435	0.427	
	Genotype: time	4.75	2, 435	0.009	**
	Genotype: sex	1.13	2, 43	0.332	
	Sex: time	0.07	1, 435	0.787	
	Genotype: sex: time	0.79	2, 435	0.455	

4.2.2 The effect of alarm pheromone on the swimming behaviour of adult *disc1* zebrafish

4.2.2.1 The effect of alarm pheromone (protocol 1, cohort 1) on behaviour of *disc1* L115 fish in the tank diving test

A ten minute exposure to alarm pheromone caused a significant increase in bottom dwell time, particularly in wild types and heterozygous mutants, at specific time points, but had no effect homozygous mutants (Figure 4.4 A-C, Table 4.4). Exposure had no effect on swimming distance, fast swimming or slow swim episodes (Figure 4.4 D-L). There was no significant main effect of genotype or sex on any of the behaviours measured (Table 4.4).

4.2.2.2 The effect of alarm pheromone (protocol 1) on behaviour of *disc1* Y472 fish in the tank diving test

In this experiment, a ten minute exposure to alarm pheromone had no significant effect on swimming behaviours (Figure 4.5, Table 4.5). There was no significant main effect of genotype, sex or treatment on bottom dwell, distance swam, fast swimming distance and slow swim episodes and no genotype: treatment interactions (Figure 4.5 C-H, Table 4.5).

4.2.2.3 The effect of alarm pheromone (protocol 2, cohort 2) on behaviour of *disc1* L115 fish in the tank diving test

A ten minute exposure to alarm pheromone resulted in a significant increase in bottom dwell time in wild type female fish, but not heterozygous or homozygous mutants (Figure 4.6 A-C). There was no significant effect of treatment on distance swam, fast swimming or slow swim episodes in any group

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A number of genotypic differences were detected in this test. Homozygous mutants swam significantly less distance and did more slow swim episodes than heterozygous mutants (Figure 4.6 & 4.7 D-L, Table 4.6). Behaviours were also effected by sex and genotype: sex interactions in this test. Females generally exhibited a significantly longer bottom dwell time than males (Figure 4.6 & 4.7 A-C, Table 4.6). Wild type females swam further than wild type males but no sex

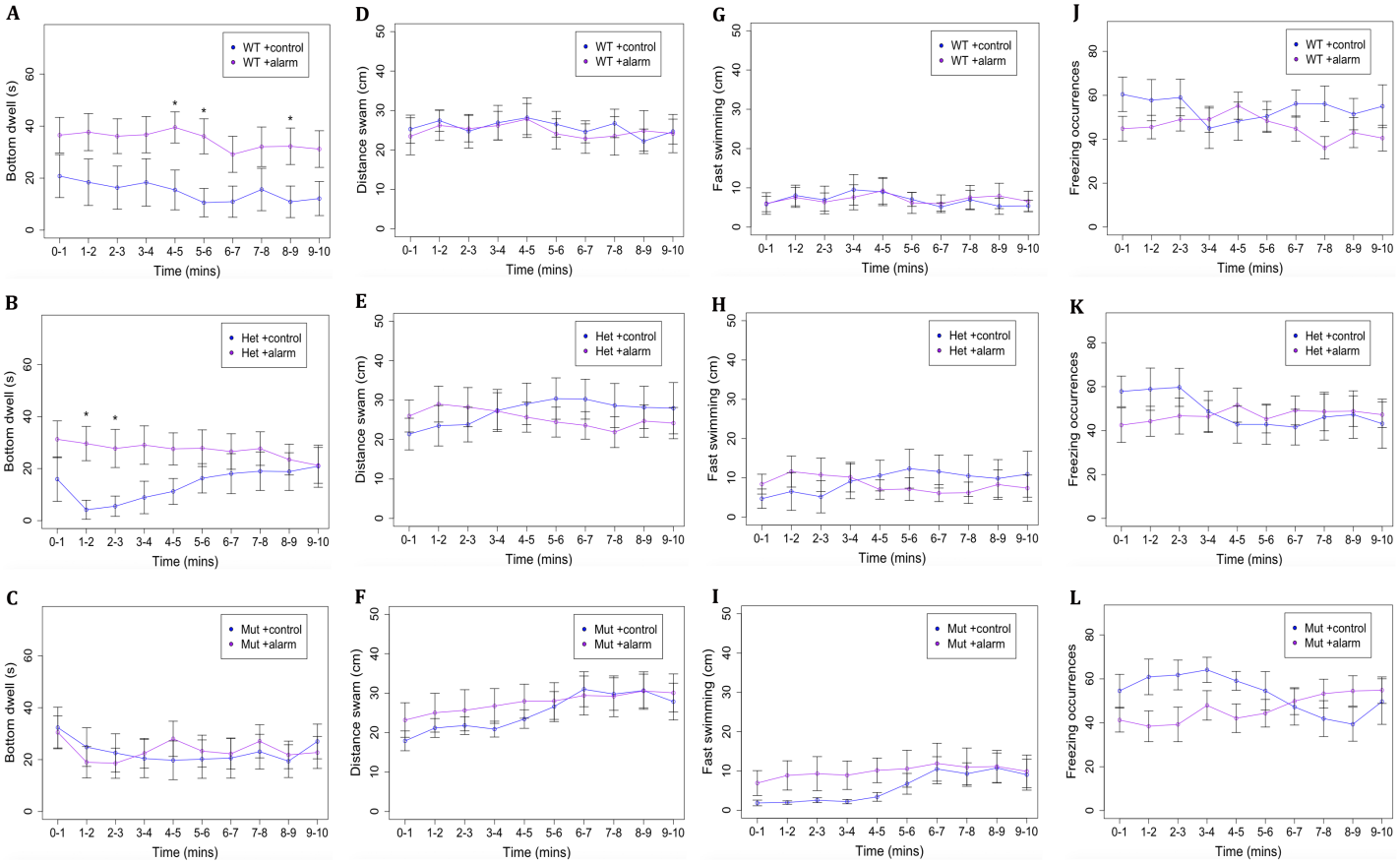

Figure 4.4. The effect of alarm pheromone (protocol 1, cohort 1) on behaviour of *disc1* L115 adult zebrafish in the tank diving test. Points and bars are mean ± standard error. WT, wild type; Het, heterozygote; Mut, homozygous mutant; Slow swimming, <2 cm/s; fast swimming >9 cm/s. N= 4 per group, 1 technical replicate. *, significant difference from control at $p<0.05$. Statistics in Table 4.4.
(A-C) Mean duration spent in the bottom compartment by L115 fish exposed to alarm pheromone or a control solution.
(D-F) Mean distance swam in all compartments by fish when exposed to alarm pheromone or a control solution.
(G-I) Mean distance of fast swimming by fish when exposed to alarm pheromone or a control solution.
(J-L) Mean number of slow swim episodes byfish when exposed to alarm pheromone or a control solution.

Table 4.4. Statistical analysis of the effect of alarm pheromone (protocol 1, cohort 1) on behaviour of *disc1* L115 adult zebrafish in the 10 minute tank diving test. The results of ANOVA with repeated measures carried out on various behavioural parameters are shown. d.f, degrees of freedom; Time, time interval in experiment. * indicates a significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 4 per group, 1 technical replicate. See Figure 4.4 for plot.

Behavioural parameter	Factor	F /t value	d.f	p value	
Time in lower compartment	Genotype	0.16	2, 48	0.850	
	Treatment	4.42	1, 48	0.041	*
	Sex	0.26	1, 48	0.612	
	Time	6.29	1, 528	0.012	*
	Genotype: treatment	1.27	2, 528	0.290	
	Genotype: time	2.99	2, 528	0.051	
	Genotype: sex	0.71	2, 48	0.496	
	Treatment: sex	0.43	1, 48	0.515	
	Treatment: time	2.72	1, 528	0.099	
	Genotype: treatment: time	7.65	2, 528	0.0005	***
Distance swam	Genotype	0.07	2, 48	0.935	
	Treatment	0.00	1, 48	0.972	
	Sex	1.92	1, 48	0.172	
	Time	6.91	1, 528	0.009	**
	Genotype: treatment	0.16	2, 48	0.853	
	Genotype: time	14.22	2, 528	<0.0001	***
	Genotype: sex	3.42	2, 48	0.041	*
	Het female: het male	8.94	188	<0.0001	***
	Treatment: sex	0.53	1, 48	0.471	
	Treatment: time	7.63	2, 528	0.006	**
	Genotype: treatment: time	4.07	2, 528	0.018	*
Fast swimming distance	Genotype	0.16	2, 48	0.850	
	Treatment	0.27	1, 48	0.603	
	Sex	0.62	1, 48	0.434	
	Time	6.24	1, 528	0.0128	*
	Genotype: treatment	0.38	2, 48	0.688	
	Genotype: time	8.51	2, 528	0.0002	***
	Genotype: sex	2.56	2, 48	0.088	
	Treatment: sex	0.08	1, 48	0.784	
	Treatment: time	8.81	1, 528	0.003	**
	Genotype: treatment: time	6.42	2, 528	0.002	**
Slow swim episodes	Genotype	0.05	2, 48	0.947	
	Treatment	1.42	1, 48	0.239	
	Sex	0.02	1, 48	0.886	
	Time	1.61	1, 528	0.205	
	Genotype: treatment	0.15	2, 48	0.861	
	Genotype: time	1.37	2, 528	0.255	
	Genotype: sex	0.02	2, 48	0.976	
	Treatment: sex	4.06	1, 48	0.049	*
	Treatment: time	21.53	1, 528	<0.0001	***
	Genotype: treatment: time	9.72	2, 528	<0.0001	***

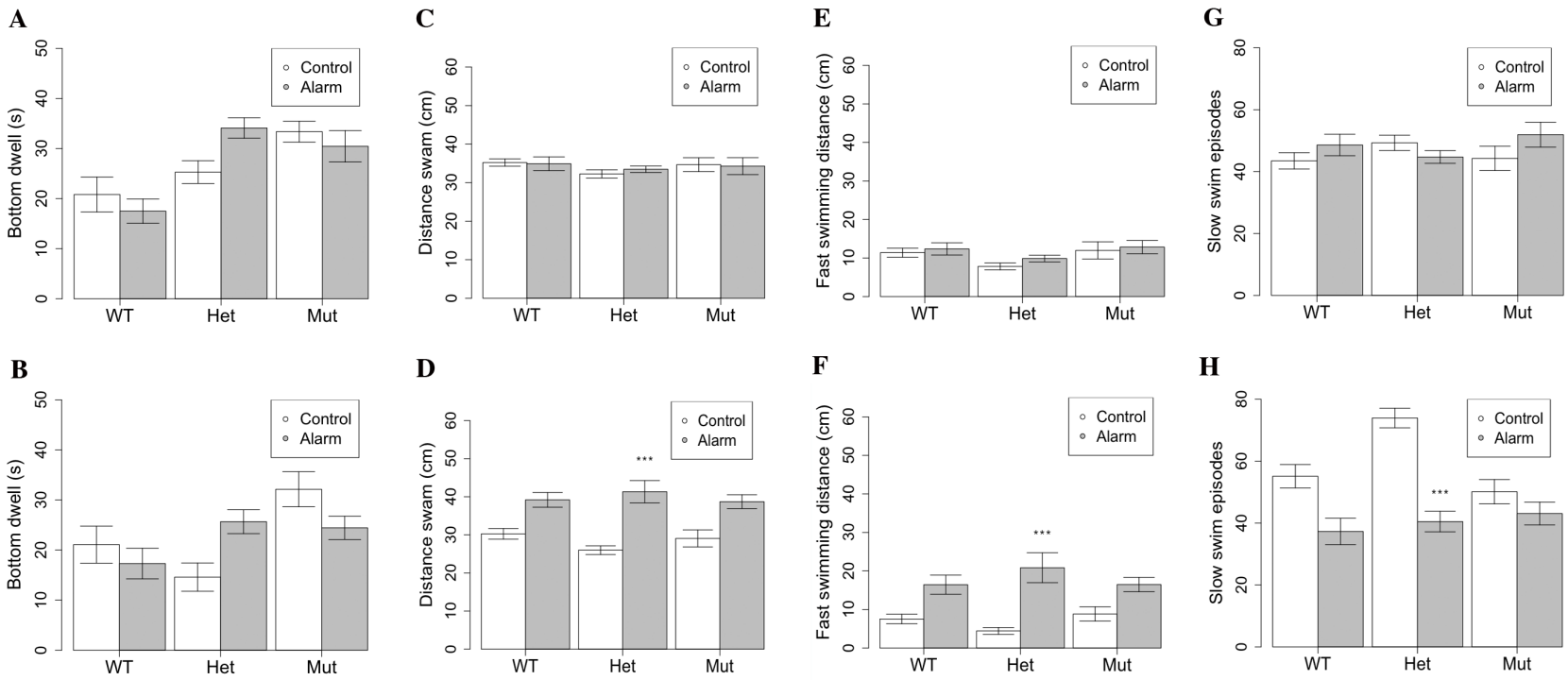

Figure 4.5. The effect of alarm pheromone (protocol 1) on behaviour of *disc1* Y472 adult zebrafish in the 10 minute tank diving test. Points and bars represent mean ± standard error. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant; Slow swimming, <2 cm/s; fast swimming >9 cm/s. N= 4 per group, 1 technical replicate. None of the above behavioural measures exhibited significant alterations throughout the 10 minute experiment and so the mean for the entire experiment is plotted; males and females are plotted separately for clarity. *** indicates statistically significant difference from heterozygous control at $p<0.001$. See Table 4.5 for statistics.

(A-B) Mean duration spent in the lower compartment by Y472 female (A) and male (B) fish when exposed to alarm pheromone or a control solution.

(C-D) Mean distance swam by Y472 female (C) and male (D) fish when exposed to alarm pheromone or a control solution.

(E-F) Mean distance of fast swimming by Y472 female (E) and male (F) fish when exposed to alarm pheromone or a control solution.

(G-H) Mean number of slow swim episodes by Y472 female (G) and male (H) fish when exposed to alarm pheromone or a control solution.

Table 4.5. Statistical analysis of the effect of alarm pheromone (protocol 1) on behaviour of *disc1* Y472 adult zebrafish in a 10 minute tank diving test. The results of ANOVA with repeated measures for various behavioural parameters are shown. d.f, degrees of freedom; Time, time interval in experiment. * indicates a statistically significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N= 5 per group, 1 technical replicate. See Figure 4.5 for plot.

Behavioural parameter	Factor	F value	d.f	p value	
Time in lower compartment	Genotype	1.63	2, 38	0.210	
	Treatment	0.03	1, 38	0.864	
	Sex	1.05	1, 38	0.312	
	Time	6.64	1, 438	0.010	*
	Genotype: treatment	1.09	2, 438	0.348	
	Genotype: time	3.66	2, 438	0.027	*
	Genotype: sex	0.34	2, 38	0.717	
	Treatment: time	0.86	1, 438	0.353	
	Genotype: treatment: sex	0.05	2, 38	0.952	
	Genotype: treatment: time	5.68	2, 438	0.004	**
Distance swam	Genotype	0.06	2, 38	0.947	
	Treatment	2.51	1, 38	0.121	
	Sex	0.04	1, 38	0.841	
	Time	28.76	1, 438	<0.0001	***
	Genotype: treatment	0.15	2, 438	0.865	
	Genotype: time	1.83	2, 438	0.162	
	Genotype: sex	0.01	2, 38	0.992	
	Treatment: time	1.26	1, 438	0.263	
	Genotype: treatment: sex	0.05	2, 38	0.952	
	Genotype: treatment: time	5.87	2, 438	0.003	**
Fast swimming distance	Genotype	0.09	2, 38	0.912	
	Treatment	2.52	1, 38	0.121	
	Sex	0.31	1, 38	0.580	
	Time	16.89	1, 438	<0.0001	***
	Genotype: treatment	0.18	2, 438	0.836	
	Genotype: time	0.72	2, 438	0.486	
	Genotype: sex	0.10	2, 38	0.907	
	Treatment: sex	1.70	1, 38	0.201	
	Treatment: time	0.50	1, 438	0.480	
	Genotype: treatment: sex	0.10	2, 38	0.904	
	Genotype: treatment: time	8.23	2, 438	0.0003	***
Slow swim episodes	Genotype	0.24	2, 38	0.787	
	Treatment	1.86	1, 38	0.181	
	Sex	0.14	1, 38	0.708	
	Time	20.34	1, 438	<0.0001	***
	Genotype: treatment	0.95	2, 438	0.395	
	Genotype: time	3.30	2, 438	0.038	*
	Genotype: sex	0.47	2, 38	0.632	
	Treatment: sex	3.68	1, 38	0.063	
	Treatment: time	4.21	1, 438	0.041	*
	Genotype: treatment: sex	0.13	2, 38	0.877	
	Genotype: treatment: time	2.51	2, 438	0.083	

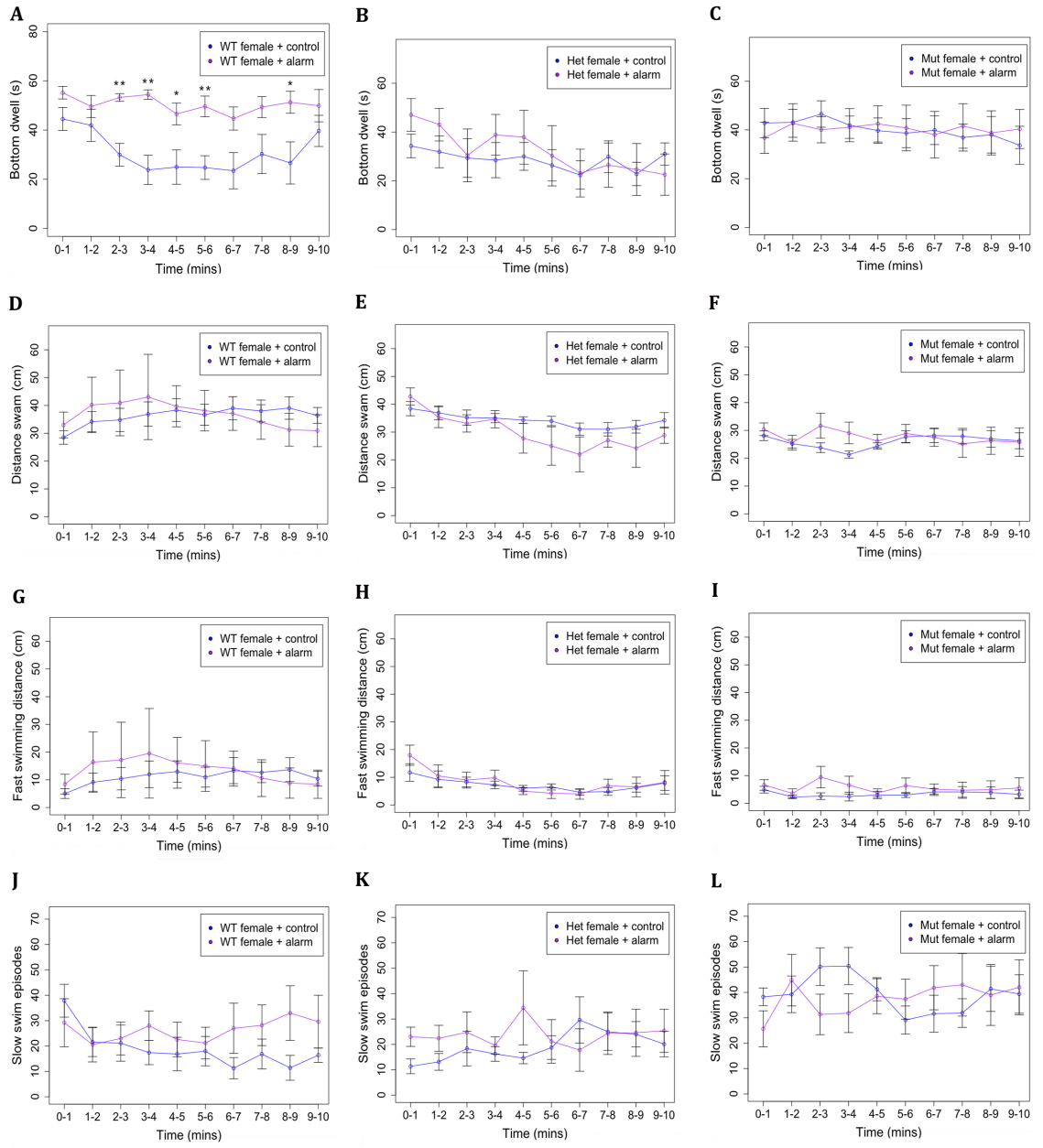

Figure 4.6. The effect of alarm pheromone (protocol 2) on behaviour of *disc1* L115 adult females in the 10 minute tank diving test. Points and bars represent mean ± standard error. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant; Time, time interval in the experiment; slow swimming, <2 cm/s; fast swimming >9 cm/s, * significant difference at $p<0.05$; **, $p<0.01$. N= 6 per group, 1 technical replicate. Genotypes and sexes are plotted separately for clarity. See Table 4.6 for statistics.

(A-C) Mean bottom dwell time by L115 wild type siblings (A), heterozygotes (B) and homozygous mutants (C) when exposed to alarm pheromone or a control solution.

(D-F) Mean distance swam by L115 wild type siblings (D), heterozygous mutants (E) and homozygous mutants (F) when exposed to alarm pheromone or a control solution.

(G-I) Mean distance of fast swimming by L115 wild type siblings (G), heterozygotes (H) and homozygous mutants (I) when exposed to alarm pheromone or a control solution.

(J-L) Mean number of slow swim episodes by L115 wild type siblings (J), heterozygotes (K) and homozygous mutants (L) exposed to alarm pheromone or a control solution.

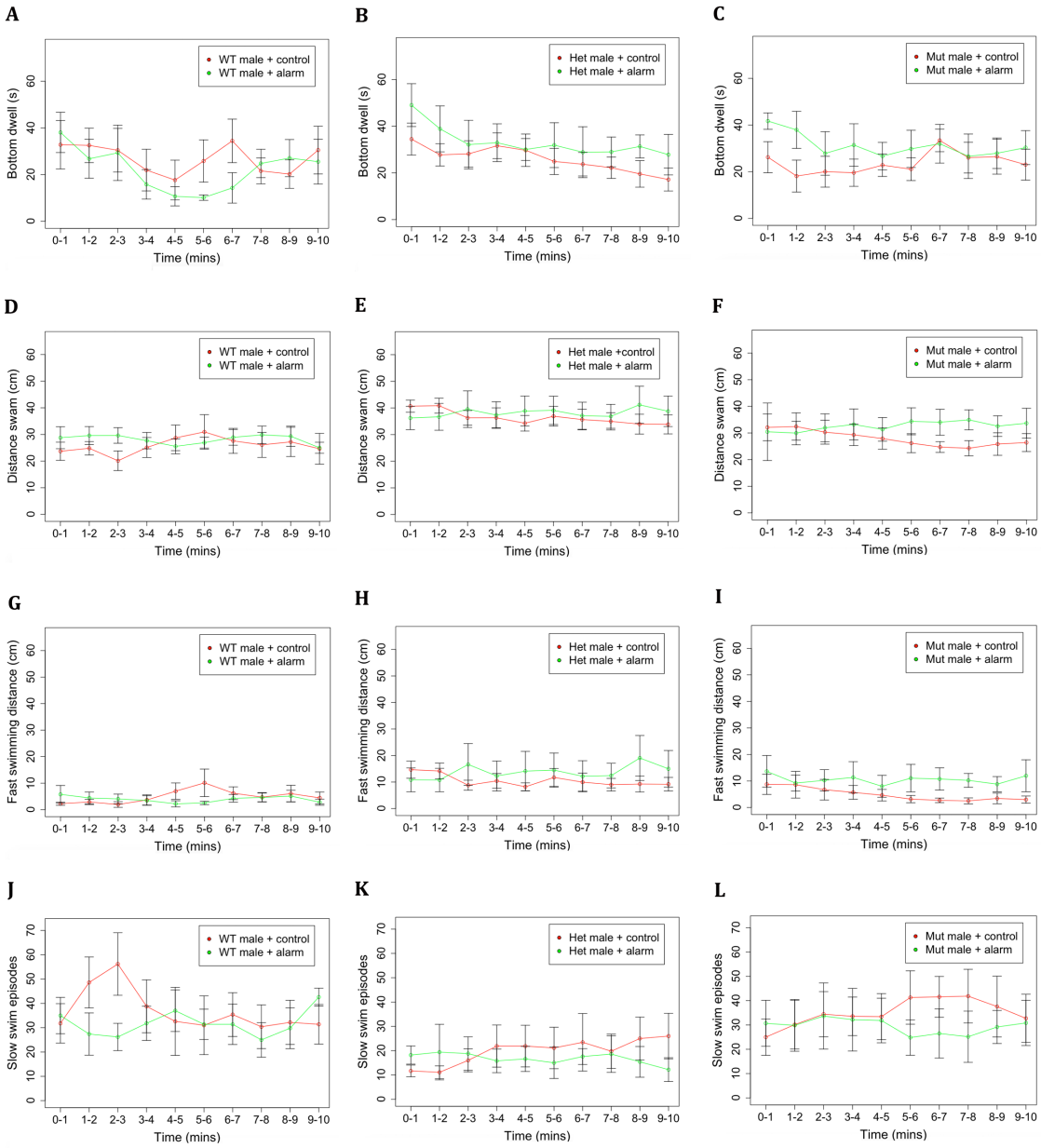

Figure 4.7. The effect of alarm pheromone (protocol 2) on behaviour of *disc1* L115 adult males in the 10 minute tank diving test. Points and bars represent mean ± standard error. WT, wild type siblings; Het, heterozygous mutant; Mut, homozygous mutant; Time, time interval in the experiment; Slow swimming, <2 cm/s; fast swimming >9 cm/s. N= 6 per group, 1 technical replicate. Genotypes and sexes are plotted separately for clarity. See Table 4.6 for statistics.

(A-C) Mean bottom dwell time of L115 wild type siblings (A), heterozygotes (B) and homozygous mutants (C) when exposed to alarm pheromone or a control solution.

(D-F) Mean distance swam by L115 wild type siblings (D), heterozygous mutants (E) and homozygous mutants (F) when exposed to alarm pheromone or a control solution.

(G-I) Mean distance of fast swimming by L115 wild type siblings (G), heterozygotes (H) and homozygous mutants (I) when exposed to alarm pheromone or a control solution.

(J-L) Mean number of slow swim episodes by L115 wild type siblings (J), heterozygotes (K) and homozygous mutants (L) exposed to alarm pheromone or a control solution.

Table 4.6. Statistical analysis of the effect of alarm pheromone (protocol 2) on behaviour of *disc1* L115 adult zebrafish in the 10 minute tank diving test. The results of ANOVA with repeated measures and pairwise comparison tests carried out on various behavioural parameters are shown. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant; Time, time interval in experiment. * indicates a statistically significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N= 6 per group, 1 technical replicate. See Figures 4.6 & 4.7 for plots.

Behavioural parameter	Factor	F value	d.f	p value	
Time in lower compartment	Genotype	0.79	2, 61	0.465	
	Treatment	2.66	1, 61	0.108	
	Sex	9.06	1, 61	0.004	**
	Time	34.88	1, 673	<0.0001	***
	Genotype: treatment	0.15	2, 61	0.864	
	Genotype: time	5.50	2, 673	0.004	**
	Genotype: sex	2.01	2, 61	0.143	
	Treatment: sex	0.28	1, 61	0.599	
	Treatment: time	1.20	1, 673	0.275	
	Sex: time	0.15	1, 673	0.699	
	Genotype: treatment: sex	2.25	2, 61	0.114	
	Genotype: treatment: time	0.81	2, 673	0.447	
	Genotype: treatment: sex: time	4.48	2, 673	0.012	*
Distance swam	Genotype	3.61	2, 62	0.033	*
	WT: het	0.97	1, 45	0.33	
	WT: mut	1.98	1, 47	0.166	
	Het: mut	8.73	1, 50	0.005	**
	Treatment	0.20	1, 62	0.658	
	Sex	0.0	1, 62	0.990	
	Time	12.30	1, 674	0.0005	***
	Genotype: treatment	0.41	2, 62	0.665	
	Genotype: time	9.11	2, 674	0.0001	***
	Genotype: sex	4.31	2, 62	0.018	*
	WT female: WT male	4.74	1, 20	0.042	*
	Treatment: sex	0.73	1, 62	0.395	
	Treatment: time	2.46	1, 674	0.117	
	Sex: time	1.12	1, 674	0.291	
	Genotype: treatment: sex	0.09	2, 62	0.914	
	Genotype: treatment: time	6.88	2, 674	0.001	**

Behavioural parameter	Factor	F value	d.f	p value	
Fast swimming distance	Genotype	1.78	2, 62	0.177	
	Treatment	1.49	1, 62	0.227	
	Sex	0.03	1	0.860	
	Time	6.76	9	0.009	**
	Genotype: treatment	0.37	2	0.690	
	Genotype: time	3.78	18	0.023	*
	Genotype: sex	4.15	2	0.020	*
	Treatment: sex	0.08	1	0.785	
	Treatment: time	0.42	9	0.518	
	Sex: time	0.16	9	0.693	
	Genotype: treatment: sex	0.32	2	0.731	
	Genotype: treatment: time	5.83		0.003	**
Slow swim episodes	Genotype	5.62	2, 62	0.006	**
	WT: het	3.15	1, 45	0.083	
	WT: mut	2.50	1, 47	0.121	
	Het: mut	11.19	1, 50	0.002	**
	Treatment	0.01	1, 62	0.933	
	Sex	0.03	1, 62	0.871	
	Time	1.54	1, 674	0.215	
	Genotype: treatment	0.23	2, 62	0.797	
	Genotype: time	8.72	2, 674	0.0002	***
	Genotype: sex	1.76	2, 62	0.181	
	Treatment: sex	0.90	1, 62	0.346	
	Treatment: time	0.49	1, 674	0.483	
	Sex: time	0.29	1, 674	0.588	
	Genotype: treatment: sex	0.09	2, 62	0.916	
	Genotype: treatment: time	13.67	2, 674	<0.0001	***
	Genotype: treatment: sex: time	2.84	2, 674	0.059	

differences were detected for heterozygous or homozygous mutants (Figure 4.6 & 4.7 D-I, Table 4.6).

4.2.3 Body size

There was no main effect of genotype or sex and no genotype: sex interaction on body mass of L115 cohort 1 fish (Figure 4.8 A, Table 4.7). There was no effect of genotype or genotype: sex interaction on body mass of Y472 fish (Figure 4.8 B, Table 4.7) but females generally had a larger mass than males. L115 homozygous mutants in cohort 2 had a significantly smaller mass than their wild type siblings (Figure 4.8 C, Table 4.7).

4.3 Discussion

4.3.1 Analysis of the baseline swimming behaviour of adult *disc1* zebrafish

4.3.1.1 L115

In the open field test, fish did not exhibit the anxiety-linked thigmotaxic behaviour seen in previous studies [80]. This may be due to differences in tank size, strain or housing conditions, compared to previous studies; factors that have been demonstrated to effect anxiety behaviours [82, 86]. In the scototaxis test, all fish showed a preference for the dark compartment, which is likely an adaptive response to avoid detection [1]. Stressed zebrafish have been shown to spend longer in the dark compartment than control fish [80], but no effect of genotype was seen on compartmental preference in this test, suggesting that heterozygous mutants do not exhibit altered baseline stress levels in this context. There was no difference between heterozygous mutants and their wild type siblings in any of the behaviours measured in these tests, suggesting that adult L115 heterozygous mutants had no major locomotor deficits and did not differ in the aspects of anxiety measured in these tests.

There was also no effect of genotype on any of the behavioural parameters measured in the first L115 tank diving experiment, whereas in the second tank diving experiment homozygous mutants swam less total distance and did more

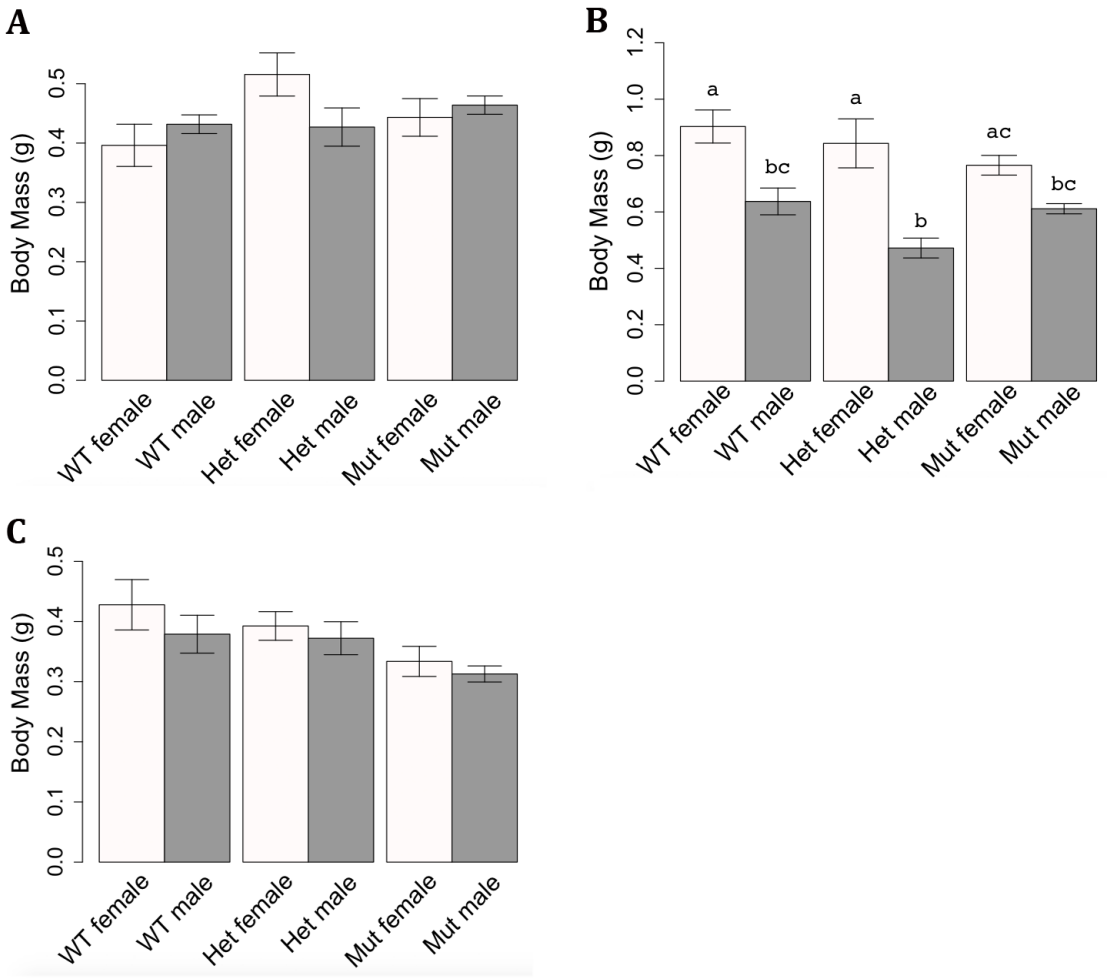

Figure 4.8. Body mass of adult *disc1* L115 and Y472 zebrafish. Points and bars represent mean ± standard error. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant. N= 6 per group, 1 technical replicate. Letters above bars indicate statistically significant differences. Groups that do not share letters in common are significantly different at $p<0.05$. See Table 4.7 for statistics.

(A) Mean body mass of L115 zebrafish (cohort 1, aged 14 months).
(B) Mean body mass of Y472 zebrafish (cohort 1, aged 23 months).
(C) Mean body mass of L115 zebrafish (cohort 2, aged 15 months).

Table 4.7. **Statistical analysis of body mass of *disc1* L115 and Y472 adult zebrafish.** The results of two-way ANOVA and Tukey's Multiple Comparison test for genotype and significant genotype: sex interactions are shown. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant; d.f, degrees of freedom. N= 6 per group, 1 technical replicate. See Figure 4.8 for plot.

	Factor	F value	d.f	p value	
L115 cohort	Genotype	1.82	2, 18	0.191	
	Sex	0.05	1, 18	0.830	
	Genotype: sex	2.40	2, 18	0.119	
Y472 cohort	Genotype	1.94	2, 29	0.162	
	Sex	36.64	1, 29	<0.0001	***
	Genotype: sex	2.08	2, 29	0.143	
L115 cohort 2	Genotype	4.02	2, 37	0.026	*
	WT: het	-	-	0.706	
	WT: mut	-	-	0.026	*
	Het: mut	-	-	0.114	
	Sex	1.54	1, 37	0.222	
	Genotype: sex	0.15	2, 37	0.859	

slow swimming in comparison to heterozygous mutants, possibly suggestive on increased caution or anxiety. This hypomotility was also seen in the homozygous mutant larvae and so is likely to be a real effect of the mutation. In the second experiment, the acclimation tanks were slightly wider than in the first experiment, meaning that there was a bigger difference between the size of the acclimation tank and the experimental tank in the second experiment. This could be a cause of the increased anxiety response detected in the second tank diving test.

4.3.1.2 Y472

In the tank diving tests, there was no significant effect of genotype, and no genotype: sex interactions, on any of the behaviours measured. There was no significant effect of genotype on freezing and, in line with some previous studies, freezing occurred very infrequently [87], suggesting that it is not a consistent measure of anxiety. Despite detection of hypomotility in the Y472 homozygous mutant larvae, this phenotype was not detected in the adult experiments. Unfortunately it is not clear from these experiments whether this is a result of small sample size, or whether the phenotype is not maintained into adulthood.

4.3.2 The effect of alarm pheromone on the swimming behaviour of adult *disc1* zebrafish

4.3.2.1 L115 alarm response

The two alarm pheromone experiments utilised different alarm pheromone extracts and concentrations and this may have contributed to the varying behavioural responses, as well as cohort differences. Alarm pheromone exposure resulted in a significant increase in bottom dwell time in wild types and heterozygotes or wild type females only in the two experiments, but had no significant effect on homozygous mutants in either case. This response corresponds with previous reports of alarm pheromone exposure [28, 88]. A differential effect of alarm pheromone on the sexes has not previously been reported, but zebrafish are known to exhibit sex differences in some aspects of behaviour, such as aggression [89] and social behaviour [90]. The failure to show this typical behavioural response to the stressor in the L115 mutants is interesting

and further supports my larval work in which 5 dpf *disc1* homozygous mutants respond differently than wild types to a variety of stressors.

4.3.2.2 Y472 alarm response

In this experiment, exposure to the alarm pheromone had no significant effect on the behaviours tested. Exposure caused an increase in bottom dwell time, an increase in distance swam and fast swimming distance and a reduction in the number of slow swim episodes. One explanation is that the alarm extract used in this experiment was less effective, and so wild type fish did not show the typical response seen in other experiments. In line with this hypothesis, heterozygous mutants may have been more sensitive to the extract, and so showed a stronger behavioural response.

4.3.3 Body mass

The smaller size of the L115 homozygous mutants in one cohort is interesting, and could be caused by a number of different factors. A couple of studies measured the mass of *DISC1* mice in early life [91] and as adults [92] and found no effect of the mutation. But one study reported a significant increase in time to find food by *DISC1* mutants [92], which could be caused by an impairment in olfaction or feeding motivation. The size difference in the *disc1* fish could be caused by an impairment in muscular-skeletal growth, metabolism or reduced feeding abilities. Factors that might impede feeding in the mutants include monopolisation of food resources by a dominant fish; reduced swimming ability; impaired visual and/or olfactory systems; reduced feeding motivation or appetite. The relationship between stress and food has recently been investigated in many vertebrates. Exposure to a stressor has been demonstrated to supress feeding in adult and larval zebrafish [76, 93] and in the larva feeding did not resume until basal cortisol levels were re-established [76]. Although the *disc1* mutant zebrafish do not appear to have increased baseline cortisol levels, it is possible that an impairment in the HPI axis and dysregulated cortisol synthesis might be linked to a feeding impairment.

4.4 Concluding remarks

In the *DISC1* mice models, some mutants have demonstrated behavioural abnormalities that have been described as reminiscent of depression or schizophrenia [6], whilst others demonstrate normal baseline behaviour [7]. In some experiments baseline behaviour of the *disc1* zebrafish was normal, whilst others suggested increased anxiety. These inconsistencies likely reflect cohort differences, via interactions with other genetic factors. In most of the experiments, control behaviour and response to the stressor did not differ significantly between homozygous and heterozygous mutants, suggesting that the mutation is expressed in a dominant manner. This corresponds with finding in the larval study of sibling behaviour, in which both heterozygous and homozygous mutants displayed hypomotility in comparison to their wild type siblings. The hypomotility phenotype is also relevant to schizophrenia, as a common symptom of the disease is lethargy [94]. Tests to investigate whether the hypomotility phenotype detected in the *disc1* zebrafish is linked to reduced motivation, for example through novel object testing and feeding and reward motivation tasks would be beneficial.

Importantly, within any one experiment, mutants often differed in their response to the stressor when compared with their wild type siblings. In line with the larval behavioural data, this strongly supports a role for *disc1* in modulating the behavioural response to stress. Although *disc1* zebrafish mutants were not obviously hyper- or hypo-responsive to a stressor, as seen in other animal models, it is clear that they do not respond in a 'normal' wild type manner. It is likely that mutants harbour an impairment in the HPI axis and/or other circuits coordinating these responses, and this may have consequences for their fitness.

5 Endocrine analysis of *disc1* zebrafish

5.1 Introduction

The neuroendocrine regulation of the stress response is well conserved in fish and mammals, and zebrafish is increasingly being used as a good model for stress physiology. The HPA axis in mammals is remarkably similar to its fish homolog, the HPI axis, in terms of anatomy, connectivity and molecular components [1]. When homeostasis is threatened, the axis functions to bring about biochemical and physiological changes that will restore homeostasis. In zebrafish, cortisol is produced and secreted by steroidogenic cells in the interrenal tissue, the analogue of the mammalian adrenal gland. As in mammals, there are two corticosteroid receptors, MR (mineralocorticoid receptor) and GR (glucocorticoid receptor), which regulate the action of cortisol via a negative feedback system. GR and MR act as transcription factors by binding glucocorticoid response elements in DNA, upstream of target genes [44]. Cortisol regulates a variety of systems, including glucose metabolism, ionoregulation, immune function, reproduction and behaviour [44].

There are now a number of established methods for extracting and quantifying whole body cortisol in adult and larval zebrafish, including ELISA utilizing commercially available kits [25, 73], a custom-made ELISA kit [68], radioimmunoassay [95, 96] and luminescence immunoassays [97]. Non-invasive extraction and quantification of steroids has also been performed in zebrafish, by assaying fish tank water samples, which has the benefit of allowing for sequential assaying of individuals [98]. More recently, cortisol has been extracted and quantified from blood plasma in zebrafish [78]. Whole body cortisol levels have been demonstrated to increase in adult zebrafish upon acute exposure to a variety of stressors, including alarm pheromone [69], crowding [95], net handling [99], a predator [100], air [96, 101], lysergic acid diethylamide (LSD) [102], caffeine [103], convulsants [103], as well as housing conditions [82, 104] and unpredictable chronic stress [105]. These data support the hypothesis that an increase in whole body cortisol level is a strong and reliable indicator of stress in zebrafish.

The physiological development of the HPI axis in the early zebrafish has also been described [22]. All of the neurons that characterise the HPI axis can be detected by 48 hpf [45, 73](see also chapter 6) and it is around this time, soon after hatching, that de novo synthesis of cortisol begins [44]. After hatching, endogenous cortisol levels steadily increase up to 5 or 6 dpf [44, 45], before dropping again. This point is thought to signify full development of the HPI axis. Prior to this, maternally derived cortisol can be detected in the embryo [73]. The earliest reported increase in cortisol levels in response to a stress was detected in 3 dpf larvae after a 15 minute exposure to seawater [45]. Alsop and Vijayan quantified a significant increase in cortisol at 4 dpf in response to swirling stress, but were not able to detect an increased response to swirling stress earlier [73]. They suggest that the delayed response, despite the presence of the characteristic neuronal components, might be due to a lack of developed neural inputs relaying to the hypothalamus prior to 4 dpf. This is known as the stress hyporesponsive period and its functional relevance is not understood. Interestingly, reproduction of this experiment by Steenbergen and colleagues found no increase in whole-body cortisol in response to either swirling or electric shock stress in zebrafish larvae [1]. This could be due to strain differences or quantification methods. An elevation in whole body cortisol has also been detected in response to sodium chloride, ethanol or heavy metal exposure [68] and light exposure in dark adapted larvae [106] at 5 and 6 dpf respectively. It is possible that prior to 4 dpf, zebrafish only synthesise excess cortisol in response to life-threatening stressors, but more investigation into the development of the HPI axis and development of its neuronal afferents is needed.

Baseline cortisol and the effect of stress on cortisol levels have also been studied in some of the *DISC1* mouse models. Baseline cortisol levels of *DISC1* mutants were not higher than those of controls [7, 38]. In contrast, mutant *DISC1* mice that were subjected to an isolation stress during adolescence had significantly higher corticosterone levels than littermate controls, whilst no such difference was seen in wild types [7]. This suggested that mutant *DISC1* mice are hyper-responsive to the stressor. In another *DISC1* mouse model, acute restraint stress resulted in a similar increase in corticosterone levels in both wild types and mutants, however, in contrast to wild types, cortisol levels in mutants failed to return to baseline

levels after a 60 minute recovery period [38]. In the same experiment, *DISC1* mice infected pre-natally with Poly I:C showed a similar acute response and recovery to an acute restraint stress as the Poly I:C wild types. Both of these studies are suggestive of an impairment in the glucocorticoid negative feedback system in the *DISC1* mice.

Together these data suggest that cortisol is a strong indicator of stress in zebrafish from 4 dpf. The data from *DISC1* mouse studies suggest that *DISC1* interacts with the HPA axis to modulate the endocrine stress response, but the underlying mechanism is unknown. The work presented in this chapter tested the response of wild type larval zebrafish to a number of stressors, to establish when a robust increase in cortisol levels might be detected. These data were then used to inform experiments to test the effect of these stressors on cortisol levels of larval *disc1* zebrafish. Adult *disc1* zebrafish were also exposed to the established stressor, alarm pheromone, and whole body cortisol levels were analysed.

5.2 Results

5.2.1 The effect of stress on cortisol levels in wild type larvae

A linear regression analysis of percentage cortisol bound for cortisol standards ranging from 0.5- 50 ng cortisol ml^{-1} allowed for the creation of a standard curve ($p<0.001$, R-squared= 0.99). In wild type larvae at 4 dpf, a ten minute exposure to alarm pheromone (protocol 2) caused a significant increase in whole body cortisol levels (Figure 5.1 A, Table 5.1), whilst a ten minute exposure to sodium chloride had no significant effect (Figure 5.1 B, Table 5.1). In wild type larvae at 5 dpf, a ten minute exposure to alarm pheromone (protocol 2) or sodium chloride and a 20 minute exposure to Tricaine caused a significant increase in whole body cortisol levels (Figure 5.1 C-E, Table 5.1).

5.2.2 The effect of stress on cortisol levels in *disc1* larvae

In 5 dpf L115 larvae, a ten minute exposure to alarm pheromone (protocol 2) or sodium chloride resulted in a significant increase in whole body cortisol levels in

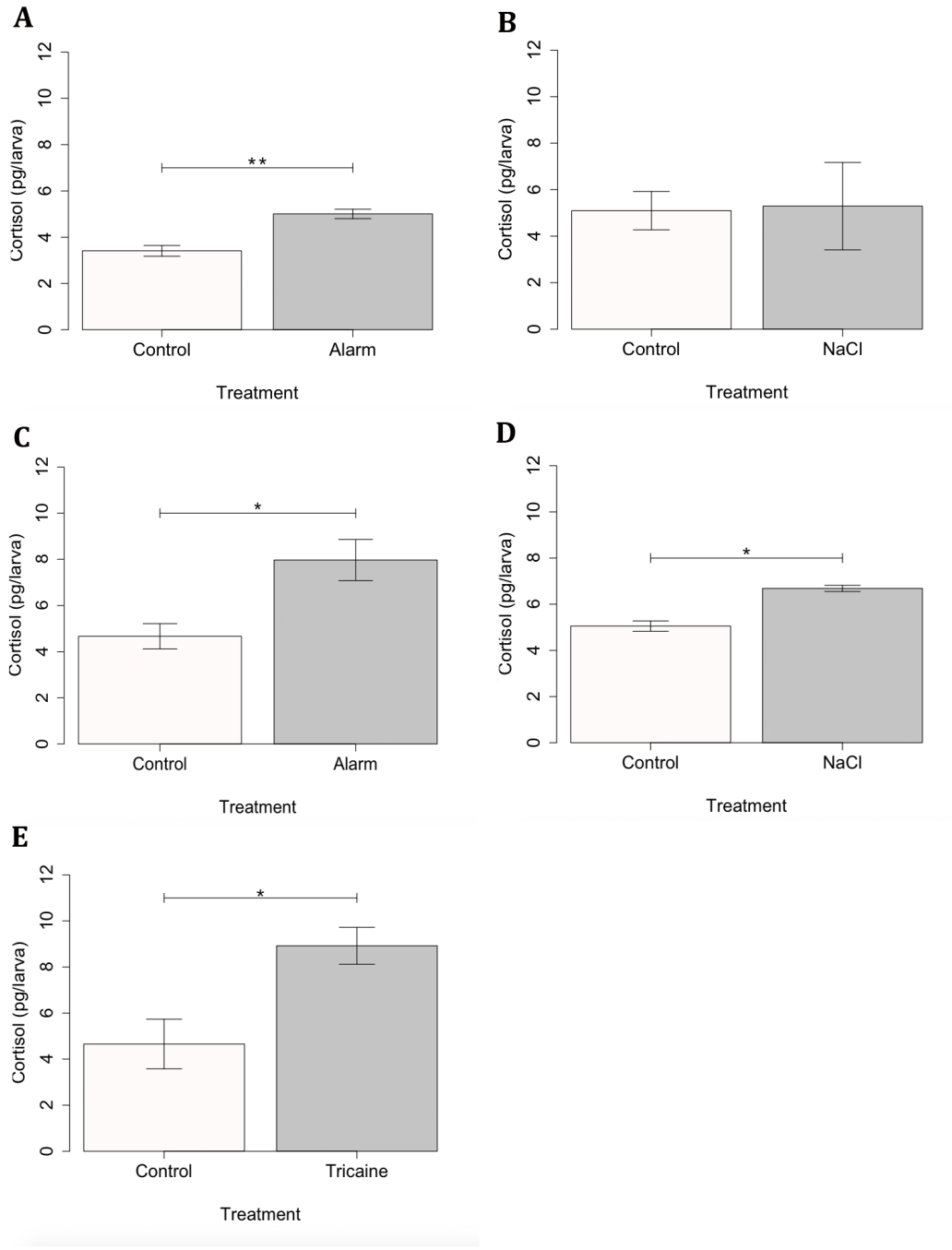

Figure 5.1. Effect of exposure to chemical stressors on whole body cortisol levels of wild type zebrafish larvae. Points and bars represent mean ± standard error. WT, wild type larvae. N= 4 biological replicates, 2 technical replicates. See Table 5.1 for statistics.

(A) Mean whole body cortisol of 4 dpf larvae exposed to alarm pheromone (protocol 2) or control solution.

(B) Mean whole body cortisol of 4 dpf larvae exposed to NaCl or control solution.

(C) Mean whole body cortisol of 5 dpf larvae exposed to alarm pheromone (protocol 2) or control solution.

(D) Mean whole body cortisol of 5 dpf larvae exposed to NaCl or control solution.

(E) Mean whole body cortisol of 5 dpf larvae exposed to Tricaine or control solution.

Table 5.1. Statistical analysis of whole body cortisol levels of larval zebrafish when exposed to chemical stressors. The results of two-sample t-tests comparing mean values for control and stressed larvae at 4 dpf and 5 dpf for various stressors are shown. Alarm pheromone extraction protocol 2 was utilised in these experiments. SEM; standard error of the mean. d.f; degrees of freedom. N = 4 biological replicates, 2 technical replicates. See Figure 5.1 for plots.

	Stressor	Wild type +control mean ± SEM	Wild type +stressor mean ± SEM	t value	d.f	p value	
4 dpf	Alarm pheromone	3.41 ± 0.2	5.01 ± 0.2	-5.14	3.94	0.007	**
	NaCl	5.10 ± 0.8	5.29 ± 1.9	-0.10	2.74	0.931	
5 dpf	Alarm pheromone	4.66 ± 0.5	7.97 ± 0.9	-3.16	5.00	0.025	*
	NaCl	5.05 ± 0.2	6.69 ± 0.1	-6.30	1.63	0.039	*
	Tricaine	4.66 ± 1.1	8.92 ± 0.8	-3.18	5.54	0.021	*

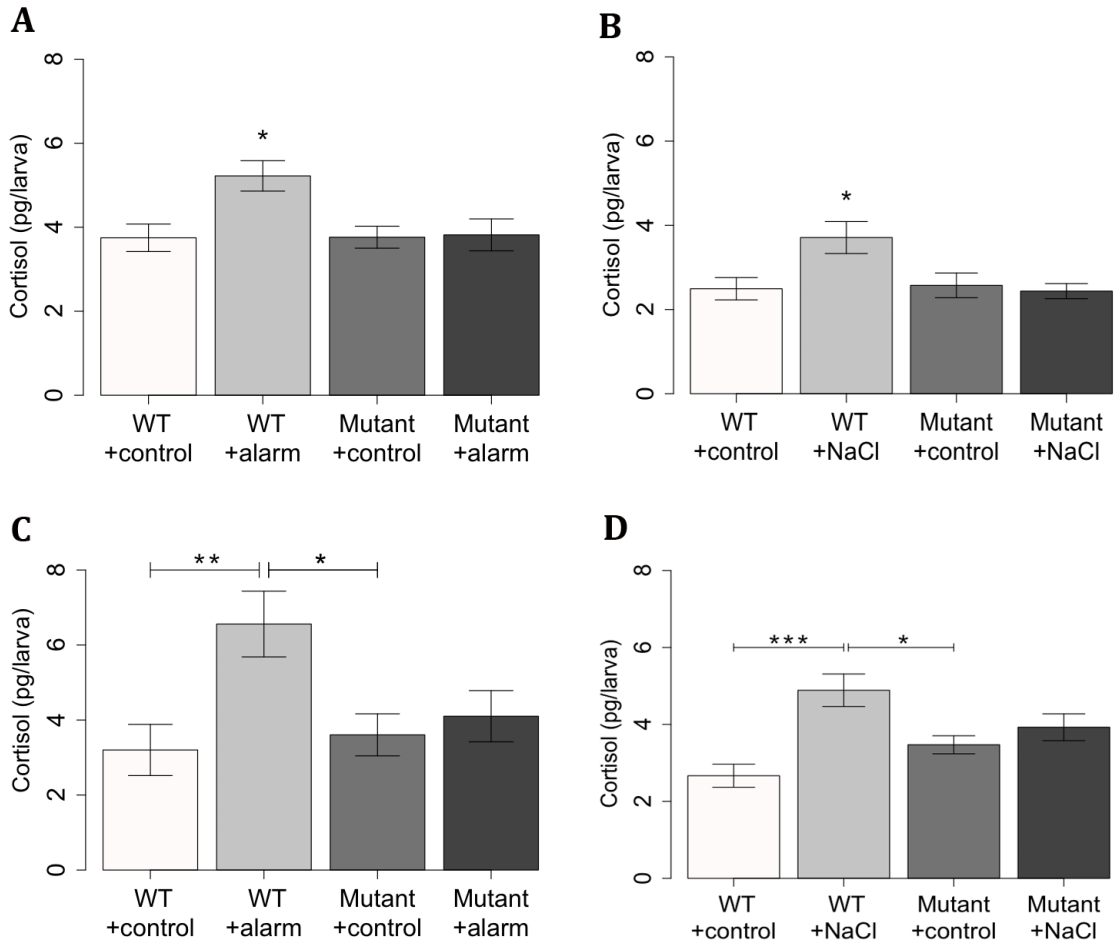

Figure 5.2. Effect of exposure to chemical stressors on whole body cortisol levels of 5 dpf *disc1* L115 and Y472 zebrafish larvae. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; Mut, homozygous mutant in-cross larvae. N= 9 biological replicates, 3 technical replicates. See Table 5.2 for statistics.

(A) Mean whole body cortisol of L115 larvae exposed to alarm pheromone (protocol 2) or control solution.

(B) Mean whole body cortisol of L115 larvae exposed to NaCl or control solution.

(C) Mean whole body cortisol of Y472 larvae exposed to alarm pheromone (protocol 2) or control solution.

(D) Mean whole body cortisol of Y472 larvae exposed to NaCl or control solution.

Table 5.2. Statistical analysis of whole body cortisol levels of *disc1* L115 and Y472 5 dpf larval zebrafish when exposed to chemical stressors. The results of two-way ANOVA and Tukey's multiple comparison test for genotype: treatment interactions are shown. WT, wild type in-cross; Mut, homozygous mutant in-cross; d.f, degrees of freedom. N= 9 biological replicates, 3 technical replicates. See Figure 5.2 for plot.

	Stressor	Factor	F value	d.f	p value	
L115	Alarm pheromone (protocol 2)	Genotype	5.61	1, 100	0.020	*
		Treatment	4.10	1, 100	0.046	*
		Genotype: treatment	4.30	1, 100	0.041	*
		WT control: WT alarm	-	-	0.024	*
		WT control: Mut control	-	-	0.999	
		WT control: Mut alarm	-	-	0.999	
		WT alarm: Mut control	-	-	0.011	*
		WT alarm: Mut alarm	-	-	0.017	*
		Mut control: Mut alarm	-	-	0.999	
	NaCl	Genotype	3.51	1, 32	0.070	
		Treatment	4.28	1, 32	0.047	*
		Genotype: treatment	5.51	1, 32	0.025	*
		WT control: WT NaCl	-	-	0.026	*
		WT control: Mut control	-	-	0.997	
		WT control: Mut NaCl	-	-	0.999	
		WT NaCl: Mut control	-	-	0.042	*
		WT NaCl: Mut NaCl	-	-	0.019	*
		Mut control: Mut NaCl	-	-	0.987	
Y472	Alarm pheromone (protocol 2)	Genotype	2.09	1, 60	0.153	
		Treatment	7.37	1, 60	0.009	**
		Genotype: treatment	4.06	1, 60	0.048	*
		WT control: WT alarm	-	-	0.008	**
		WT control: Mut control	-	-	0.998	
		WT control: Mut alarm	-	-	0.806	
		WT alarm: Mut control	-	-	0.024	*
		WT alarm: Mut alarm	-	-	0.079	
		Mut control: Mut alarm	-	-	0.960	
	NaCl	Genotype	0.06	1, 28	0.816	
		Treatment	16.12	1, 28	0.0004	***
		Genotype: treatment	7.01	1, 28	0.013	*
		WT control: WT NaCl	-	-	0.0003	***
		WT control: Mut control	-	-	0.339	
		WT control: Mut NaCl	-	-	0.057	
		WT NaCl: Mut control	-	-	0.027	*
		WT NaCl: Mut NaCl	-	-	0.199	
		Mut control: Mut NaCl	-	-	0.769	

wild type larvae, but had no significant effect on mutant cortisol levels (Figure 5.2 A-B, Table 5.2). There was no significant difference in cortisol levels of wild type control and mutant control larvae. In 5 dpf Y472 larvae, a ten minute exposure to alarm pheromone (protocol 2) or sodium chloride resulted in a significant increase in whole body cortisol levels in wild type larvae, but had no significant effect on mutant cortisol levels (Figure 5.2 C-D, Table 5.2). There was no significant difference in cortisol levels of wild type control and mutant control larvae.

5.2.3 The effect of alarm pheromone on cortisol levels in *disc1* adult zebrafish

The cortisol response of adult *disc1* zebrafish to alarm pheromone exposure was quantified in three separate experiments. A ten minute exposure to alarm pheromone (protocol 1 or 2) had no significant effect on whole body cortisol levels in wild types or mutants in any of the experiments. (Figure 5.3, Table 5.3). There was also no significant difference in cortisol levels between genotypes or sexes. In the analysis of the Y472 line and L115 cohort 2, there was a significant genotype: sex interaction, however, post-hoc analysis did not detect a significant difference between cortisol levels of males and females of any genotype (Table 5.3), and so the data for both sexes were combined in Figure 5.3.

5.2.4 The effect of circadian rhythms on adult zebrafish cortisol levels

In the alarm pheromone experiment with L115 cohort 1 and the Y472 line, there was no effect of time of day on cortisol levels (Figure 5.4 A-B, Table 5.4). On the other hand, the experiments utilising the L115 cohort 2 fish (Figure 5.4 C, Table 5.4) revealed a significant effect of time of day on cortisol levels.

5.3 Discussion

5.3.1 The effect of stress on cortisol levels in wild type larvae

In 4 dpf larvae, alarm pheromone but not sodium chloride exposure was sufficient to induce an increase in cortisol levels, whereas both were effective at 5 dpf. This suggests that the larvae are still emerging from the stress hyporesponsive period at 4 dpf and that 5 dpf is a more suitable time point to investigate the stress

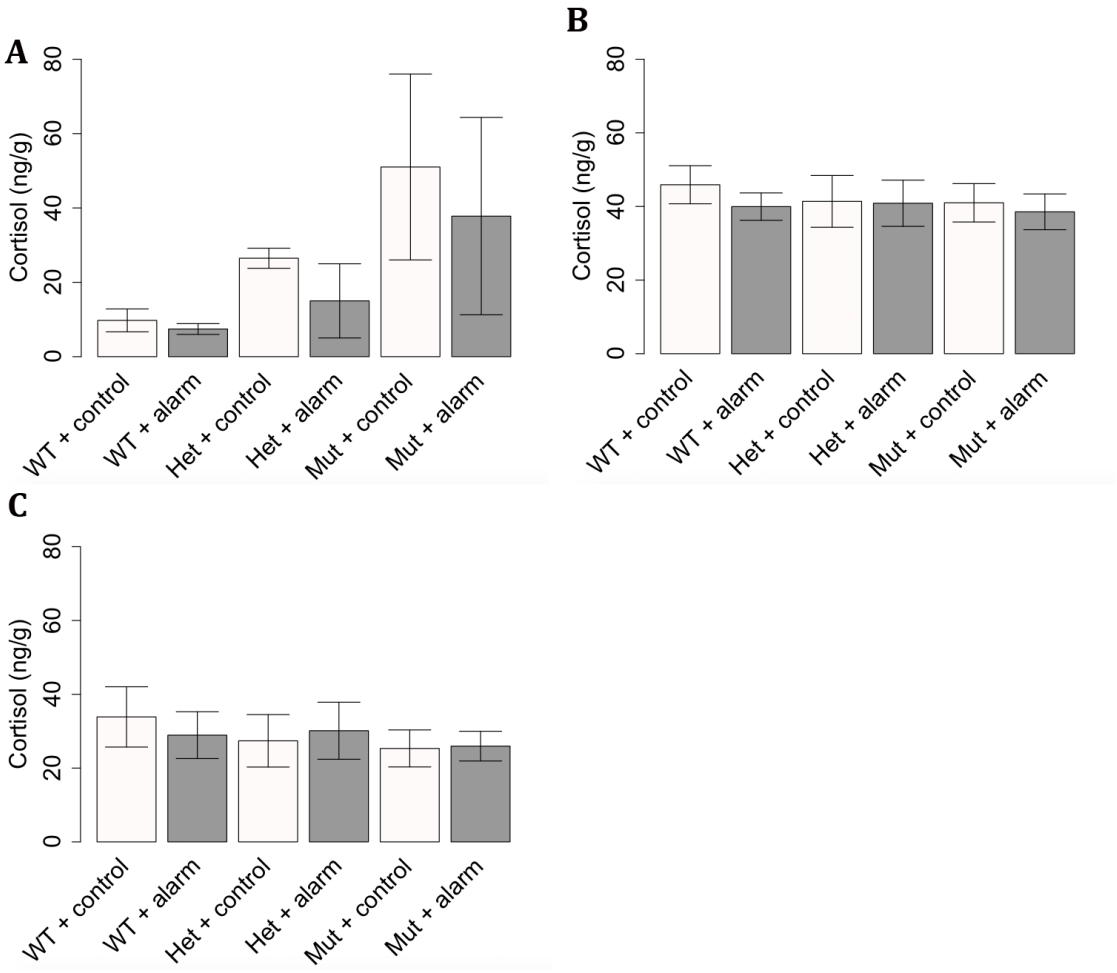

Figure 5.3. Effect of exposure to alarm pheromone on whole body cortisol levels of adult *disc1* L115 and Y472 zebrafish. Points and bars represent mean ± standard error. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant. N= 6 biological replicates, 1 technical replicate. See Table 5.3 for statistics.

(A) Mean whole body cortisol of L115 zebrafish exposed to alarm pheromone (protocol 1, cohort 1) or control solution.

(B) Mean whole body cortisol of Y472 zebrafish exposed to alarm pheromone (protocol 1) or control solution.

(C) Mean whole body cortisol of L115 zebrafish exposed to alarm pheromone (protocol 2, cohort 2) or control solution.

Table 5.3. Statistical analysis of whole body cortisol levels of *disc1* L115 and Y472 adult zebrafish when exposed to alarm pheromone. The results of two-way ANOVA and Tukey's Multiple Comparison test for genotype are shown. WT, wild type sibling; Het, heterozygous mutant; Mut, homozygous mutant; d.f, degrees of freedom. N = 6 biological replicates, 1 technical replicate. See Figure 5.3 for plots.

	Factor	F value	d.f	p value	
L115 cohort 1 (protocol 1)	Genotype	2.35	2, 13	0.135	
	Sex	0.09	1, 13	0.770	
	Treatment	0.34	1, 13	0.571	
	Genotype: sex	0.28	2, 13	0.762	
	Genotype: treatment	0.05	2, 13	0.953	
	Sex: treatment	0.50	1, 13	0.492	
	Genotype: sex: treatment	0.98	1, 13	0.340	
Y472 (protocol 1)	Genotype	0.25	2, 22	0.784	
	Sex	2.51	1, 22	0.127	
	Treatment	0.67	1, 22	0.421	
	Genotype: sex	5.43	2, 22	0.012	*
	Genotype: treatment	0.17	2, 22	0.842	
	Sex: treatment	0.0	1, 22	0.985	
	Genotype: sex: treatment	0.79	2, 22	0.467	
L115 cohort 2 (protocol 2)	Genotype	0.54	2, 30	0.588	
	Sex	0.13	1, 30	0.716	
	Treatment	0.45	1, 30	0.509	
	Genotype: sex	3.50	2, 30	0.043	*
	Genotype: treatment	0.11	2, 30	0.897	
	Sex: treatment	0.68	1, 30	0.418	
	Genotype: sex: treatment	0.34	1, 30	0.714	

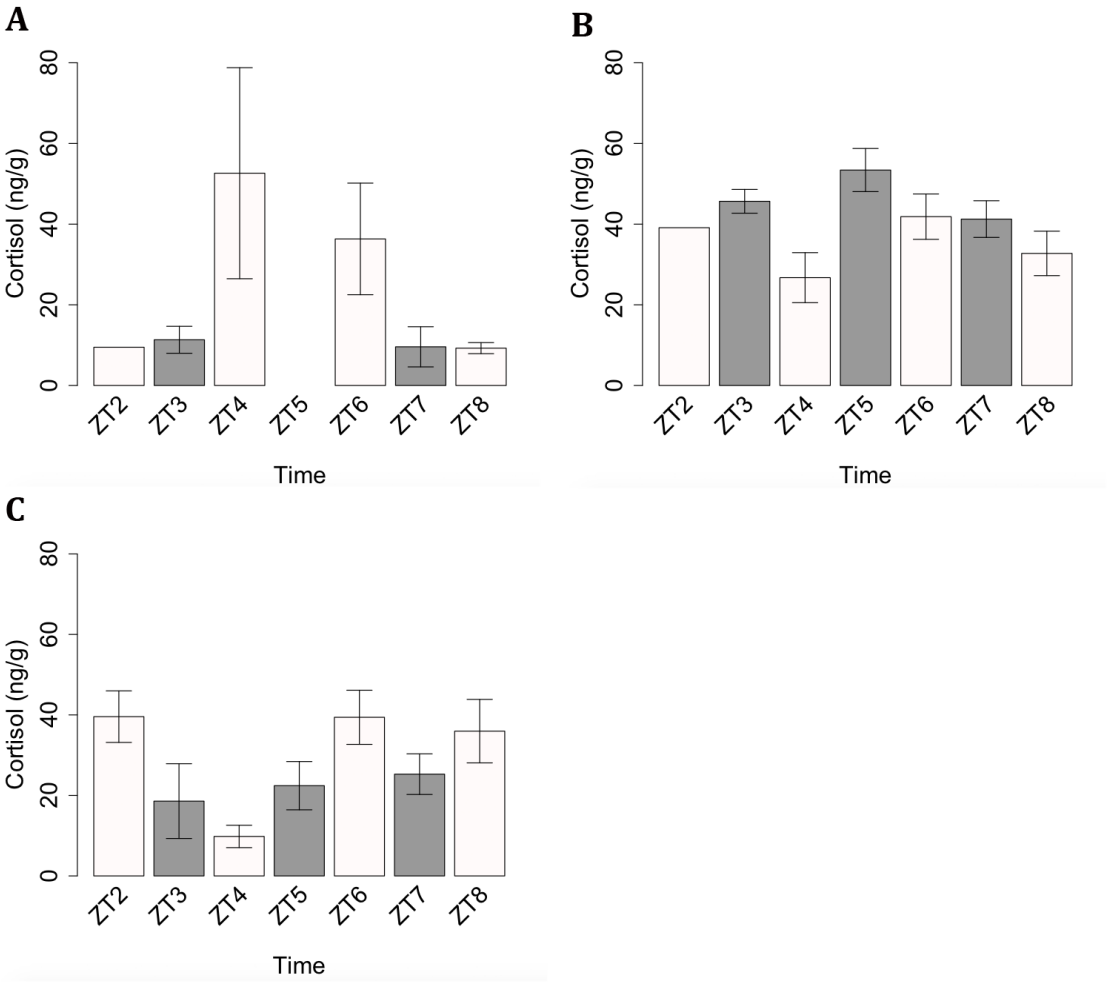

Figure 5.4. Effect of time of day on whole body cortisol levels of adult *disc1* L115 and Y472 zebrafish. Wild type, heterozygous and homozygous mutant disc1 zebrafish were exposed to alarm pheromone or a control solution, plotted here by extraction time, regardless of genotype or treatment. Points and bars represent mean ± standard error. ZT, Zeitgeber time (hours after lights on). N= 1-4 biological replicates per time point, 1 technical replicate. See Table 5.4 for statistics.

(A) Mean whole body cortisol of L115 zebrafish (cohort 1).
(B) Mean whole body cortisol of Y472 zebrafish.
(C) Mean whole body cortisol of L115 zebrafish (cohort 2).

Table 5.4. Statistical analysis of the effect of time of day on whole body cortisol levels of *disc1* L115 and Y472 adult zebrafish. Wild type, heterozygous and homozygous mutant disc1 zebrafish were exposed to alarm pheromone or a control solution, analysed here by extraction time, regardless of genotype or treatment. The results of Kruskal Wallis test for time of day followed by p values for pairwise comparisons using Tukey and Kramer (Nemenyi) test are shown. d.f, degrees of freedom. N = 1-4 per time point, 1 technical replicate. See Figure 5.4 for plots.

	Factor	χ^2	d.f	p value			
L115 (cohort 1)	**Time of day**	3.53	5	0.618			
		ZT2	**ZT3**	**ZT4**	**ZT5**	**ZT6**	**ZT7**
	ZT3	1.0	-	-	-	-	
	ZT4	1.0	0.970	-	-	-	
	ZT5	0.830	0.590	0.890	-	-	
	ZT6	0.990	0.920	1.0	0.950	-	
	ZT7	1.0	1.0	0.840	0.420	0.730	
	ZT8	1.0	1.0	0.990	0.700	0.980	1.0
Y472	**Time of day**	10.22	6	0.116			
		ZT2	**ZT3**	**ZT4**	**ZT5**	**ZT6**	**ZT7**
	ZT3	1.0	-	-	-	-	
	ZT4	0.980	0.160	-	-	-	
	ZT5	0.970	0.980	0.160	-	-	
	ZT6	1.0	0.980	0.580	0.820	-	
	ZT7	1.0	0.990	0.490	0.860	1.0	
	ZT8	1.0	0.760	1.0	0.510	0.960	0.940
L115 (cohort 2)	**Time of day**	15.20	6	0.019		*	
		ZT2	**ZT3**	**ZT4**	**ZT5**	**ZT6**	**ZT7**
	ZT3	0.557	-	-	-	-	-
	ZT4	0.066	0.990	-	-	-	-
	ZT5	0.640	1.0	0.788	-	-	-
	ZT6	1.0	0.596	0.080	0.684	-	-
	ZT7	0.875	0.983	0.447	0.999	0.901	-
	ZT8	1.0	0.703	0.101	0.794	1.0	0.961

response. Cortisol levels in control fish at 5 dpf, were similar to those previously reported in the paper from which the cortisol extraction protocol was taken [68]. This report showed a 100-200% increase in whole body cortisol after exposure to sodium chloride, whilst my experiments saw a 40% increase. This discrepancy could be due to strain differences, or to small differences in experimental set-up. The impact of sodium chloride and alarm pheromone on cortisol levels in 5 dpf larvae confirm that they are effective stressors.

Exposure to Tricaine also increased cortisol levels in 5 dpf fish. This widely used anaesthetic has previously been reported to be aversive to adult zebrafish [107], but its effect on cortisol levels has not previously been reported. The effectiveness of Tricaine exposure as a stressor meant that this exposure was minimised in all of the adult zebrafish experiments in an attempt to eliminate any confounding effect.

5.3.2 The effect of stress on cortisol levels in *disc1* larvae

As seen in the *DISC1* mice [7, 38], baseline cortisol levels in *disc1* zebrafish larvae were not different to those of wild types, but when exposed to a stressor, mutants failed to increase their cortisol levels. The conservation of this effect in both fish lines and by two different stressors strongly suggests that *disc1* is interacting with the HPI axis. This is in contrast to responses seen in the mouse models, in which one *DISC1* mutant was hyper-responsive to a stressor [7], and a second *DISC1* mutant showed a normal acute stress response but abnormal recovery [38]. The different responses to stress in the literature highlight the differences between the models and may be due to the fact that each represents a different *DISC1* mutation. Despite these differences, in all cases the response to stress is dissimilar to that of wild types. The hypo-responsiveness to stress in my model is not suggestive of an impairment of the negative feedback loop as in the mouse models, but an impairment in the upstream circuitry controlling the detection or processing of the stress signals prior to cortisol synthesis. This may suggest that the abnormal development of the HPI axis that I describe in chapter 6 may have functional consequences, i.e. lead to an impairment of the normal stress response.

5.3.3 The effect of alarm pheromone on cortisol levels in *disc1* adult zebrafish

Whole body cortisol levels of adult zebrafish reported in the literature vary greatly, from 0.012 ng/g [102] to 14 ng/g [99] for control fish, whilst readings in my experiments varied from 10 ng/g to 45 ng/g. These differences are likely due to differences in extraction and quantification protocols, experimental temperature [99], fish age and strain. Similarly to the zebrafish larvae, baseline cortisol levels were not different in wild types or *disc1* mutants, further suggesting that under baseline conditions, the HPI axis functions as normal. In contrast to reports in the literature [69], exposure to alarm pheromone did not induce a significant increase in cortisol levels in the wild type fish. Wild type fish did display some of the reported behavioural responses to the stressor (see chapter 3 & 4), and so the lack of endocrine response is likely to be related to the experimental methodology, rather than an ineffectiveness of the stressor.

One explanation is that cortisol levels did not increase upon exposure as a result of the feeding regime. In a study that detected a four-fold increase in whole-body cortisol in crowded, fasted fish in comparison with controls [95], no effect was seen on crowded fish that were subjected to a normal feeding regime. This suggests that food energy ameliorates the response to crowding and suppresses cortisol synthesis. In contrast, other evidence has suggested that if the fish are very hungry, no alarm reaction is observed (Mathuru, personal communication).

Another possibility is that a longer exposure duration is required. Studies have reported a stress-induced increase in zebrafish cortisol levels that was statistically significant, from 3 minutes [99] to 4 hours [101] after the onset of acute exposure. This variation is likely due to differences in stressor severity, age of fish and testing protocol. The previous report of the effectiveness of alarm pheromone to increase cortisol levels in zebrafish had collected samples approximately 12 minutes post exposure onset [69]. The ten minute exposure to alarm pheromone was sufficient to increase cortisol levels in zebrafish larvae in my experiments, and a previous study had demonstrated that acute (6 minute) but not prolonged (30 minute) exposure to alarm pheromone would induce behavioural changes in zebrafish [25]. For these reasons the ten minute exposure to alarm pheromone

was utilised in the adult experiments; however, a time course experiment would have been useful to determine the optimal exposure duration.

The experiment would have also benefitted from larger sample sizes. The previous report describing an increase in cortisol levels of adult zebrafish in response to alarm pheromone had utilized 10 fish per treatment group [69], whilst my experiment only had 6. Combined with high within-group variation, this meant that power of statistical tests was reduced.

5.3.4 The effect of circadian rhythms on adult zebrafish cortisol levels

Dickmeis and colleagues claim that cortisol levels in zebrafish follow a circadian rhythm, as seen in other animals [97]. They report a peak in larval zebrafish cortisol levels at ZT7 and a trough at ZT21. In the experiments described here, cortisol levels did not peak at ZT7 (ZT21 was not analysed), but morning cortisol levels were relatively high and then reduced over the next couple of hours, as previously reported [97]. It is not clear why time of day was a factor in this experiment alone, however unequal sampling throughout the day and small sample sizes meant that statistical analysis of this data was difficult. These preliminary data suggest that future collection of cortisol samples would benefit from a shorter collection time frame, perhaps avoiding the morning in which a large amount of variability was seen.

5.4 Concluding remarks

The experiments with larval *disc1* zebrafish strongly suggest that *disc1* interacts with the HPI axis. The failure to elevate cortisol levels in mutant fish when exposed to a stressor suggests an impairment in some upstream circuitry, either in the detection of the stressor or the processing of the signal upstream of cortisol synthesis. It is not yet clear whether *disc1* is required only during the early development of the HPI axis, up to 5-6 dpf [44, 45], or throughout the life course. A blunted cortisol response to a stressor has also been observed in human patients with schizophrenia and is not understood [8]. In this study patients showed a normal arousal of the autonomic nervous system in response to a psychosocial stressor but cortisol levels did not elevate as seen in the control group. It would be

interesting to measure heart rate in the *disc1* zebrafish upon exposure to a stressor to see if acute autonomic arousal does occur. Reduced cortisol responses to stress have also been observed in animals that were pre-exposed to a stressor, suggestive of adaptation or resilience [108]. Any fitness costs or benefits of this effect in the *disc1* zebrafish are not clear. On the one hand the lack of endocrine response might conserve energy, but equally it could put many aspects of health at risk.

6 Gene expression in the hypothalamus of *disc1* zebrafish

6.1 Introduction

DISC1 has been studied in a number of vertebrates and has been shown to be expressed widely throughout the body [109]. In the adult mouse, Disc1 protein is detected in many neurons including those in the olfactory bulb, cortex, hippocampus, cerebellum and brain stem [110, 111]. Expression studies in primates [40] and rats suggest that *DISC1* expression is fairly well conserved in the vertebrate. Analysis in the human brain has been restricted to the hippocampus but expression here corresponds well to that in primates and rodents [41]. *Disc1* is also expressed developmentally in the mouse and two major peaks in expression have been observed, corresponding to the period of neurogenesis in the developing brain and puberty [110]. Expression of *Disc1* in regions such as the olfactory bulb and dentate gyrus also suggest a possible role of *Disc1* in adult neurogenesis [111]. In addition to these sites of expression, *Disc1* appears to be highly expressed through the entire hypothalamus in many different animals, both in prenatal and postnatal life [40, 110, 111]. In mice, moreover, mutations in *Disc1* have been linked to aberrant HPI axis activity [7, 38]. The role of *DISC1* in the hypothalamus is not clear but its expression here opens an avenue to study its potential role in hypothalamic development and function.

The transparent nature of the early zebrafish brain lends itself to the study of gene expression in early development. The zebrafish develops rapidly and within 24 hpf a functional brain is established. Throughout the few days of development, genes display dynamic temporal and spatial patterns of expression, and interact in complex networks, which are crucial for normal development of the body systems. During this period, normal development of the nervous system is sensitive to both internal and external stimuli and this can have consequences for its functionality throughout the life course [112].

The experiments described here utilise mRNA *in situ* hybridization to explore gene expression in the *disc1* L115 and Y472 zebrafish hypothalamus. Expression of genes linked to hypothalamus development, in particular development of the

tuberal region/mid hypothalamus, which forms a key part of the HPI axis, (see section 1.6 Introduction) were analysed in zebrafish from 24 hpf up to 5 dpf. These stages are easy to work with and represent time points concurrent with formation of a functional brain, including a functional hypothalamus and activation of the HPI axis. The effect of a number of stressors (alarm pheromone and sodium chloride) on expression of key genes was also analysed. I hypothesised that *disc1* mutant fish may show altered patterns of gene expression in the developing tuberal (mid) hypothalamus.

6.2 Results

6.2.1 Expression of *disc1* in wild type larvae

In ventral whole-mount views, expression of *disc1* can be detected in the lower jaw cartilages and ventral midbrain (Figure 6.1). Transverse sections through the hypothalamus reveal expression throughout the hypothalamus, particularly surrounding the 3rd ventricle and lateral recesses of the hypothalamus (Figure 6.1).

6.2.2 Expression of *rx3* in *disc1* larvae

In situ hybridisation of *rx3* in 24 hpf embryos revealed expression in a broad band of cells in the forming diencephalon, as seen in previous studies [43] (Figure 6.2). Expression at 24 hpf appeared weaker in the Y472 mutant, in comparison to controls (Figure 6.2 A-B). From 52 hpf, expression of *rx3* becomes restricted to the inner nuclear layer of the retina and to cells around the 3rd ventricle and lateral recess of the mid hypothalamus (Figure 6.3-6.6). Previous study in the lab has indicated that these are proliferating progenitor cells [43]. At 52 hpf, expression of *rx3* in the Y472 mutants appeared reduced in the anterior and mid regions of the hypothalamus in comparison to wild types; specifically expression is reduced in the ventral most cells surrounding the 3rd ventricle and from the lateral recesses (Figure 6.3 C, G). At 3-5 dpf there was a subtle reduction in expression of *rx3* in the Y472 mutant in the mid hypothalamus (3dpf and 5 dpf) or posterior hypothalamus (4 dpf), when compared to controls (Figure 6.4, 6.5, 6.6).

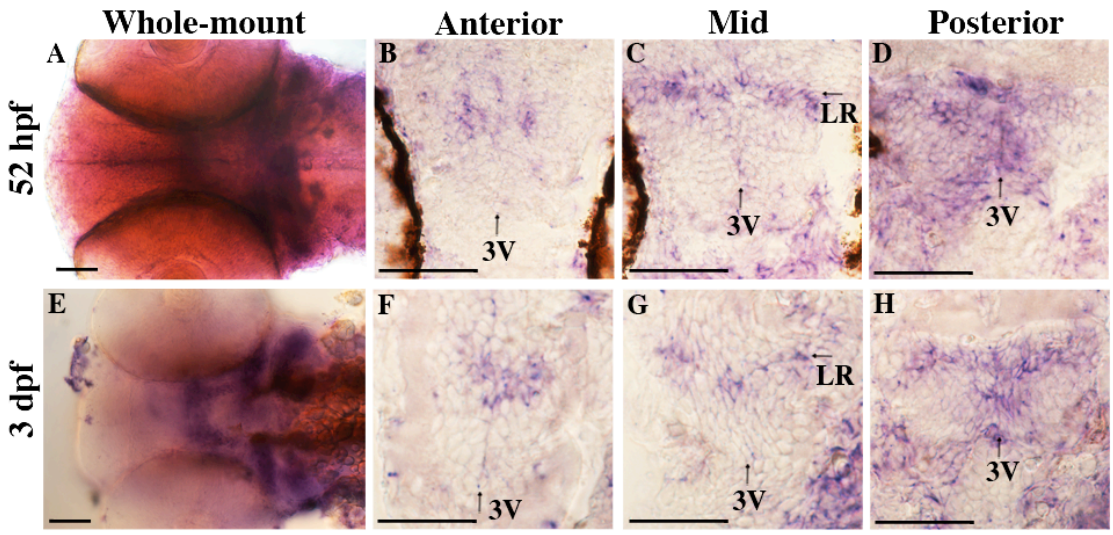

Figure 6.1 . Expression of *disc1* in the larval zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *disc1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. 3V, 3rd ventricle; LR, lateral recess. N= 6 per group, 2 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *disc1* in the 52 hpf wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *disc1* in the 3 dpf wild type brain in a whole-mount view and sections through the hypothalamus.

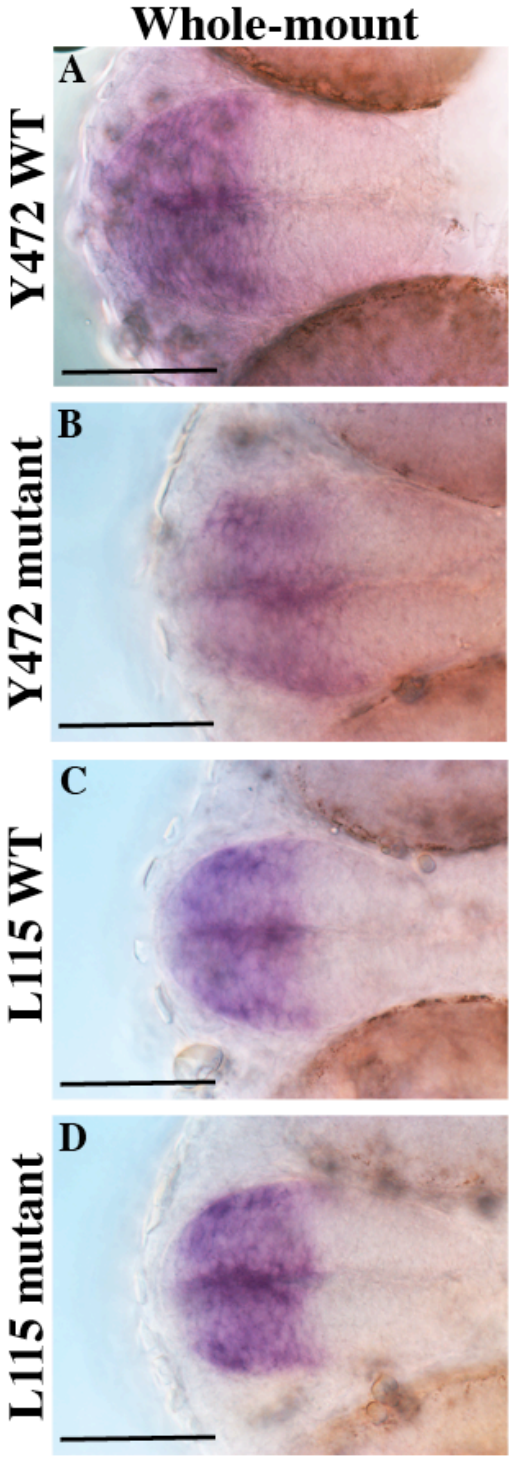

Figure 6.2. Expression of *rx3* in the 24 hpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *rx3* in the zebrafish brain. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 1 technical replicate. Scale bar is 50 μm.
(A) Expression of *rx3* in the Y472 wild type brain.
(B) Expression of *rx3* in the Y472 mutant brain.
(C) Expression of *rx3* in the L115 wild type brain.
(D) Expression of *rx3* in the L115 mutant brain.

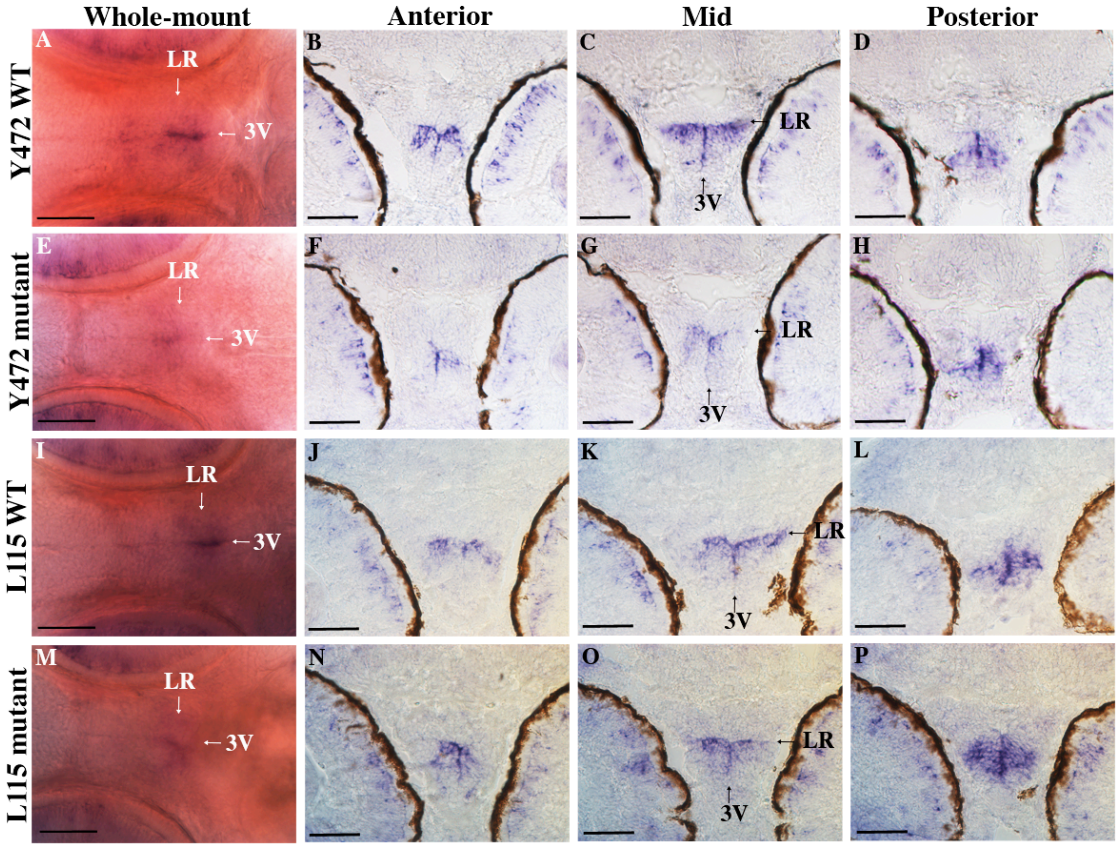

Figure 6.3. Expression of *rx3* in the 52 hpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *rx3* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle. N=6 per group, 2 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *rx3* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *rx3* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *rx3* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *rx3* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

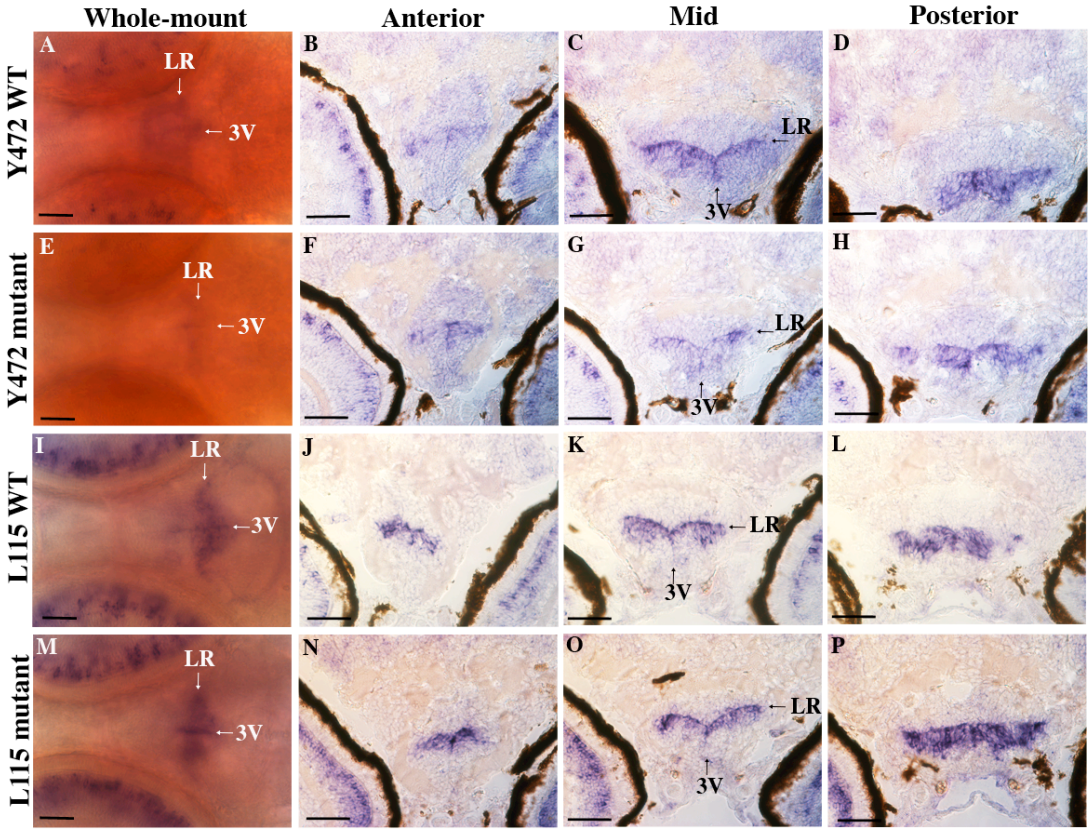

Figure 6.4. Expression of *rx3* in the 3 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *rx3* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle. N=6 per group, 2 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *rx3* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *rx3* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *rx3* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *rx3* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

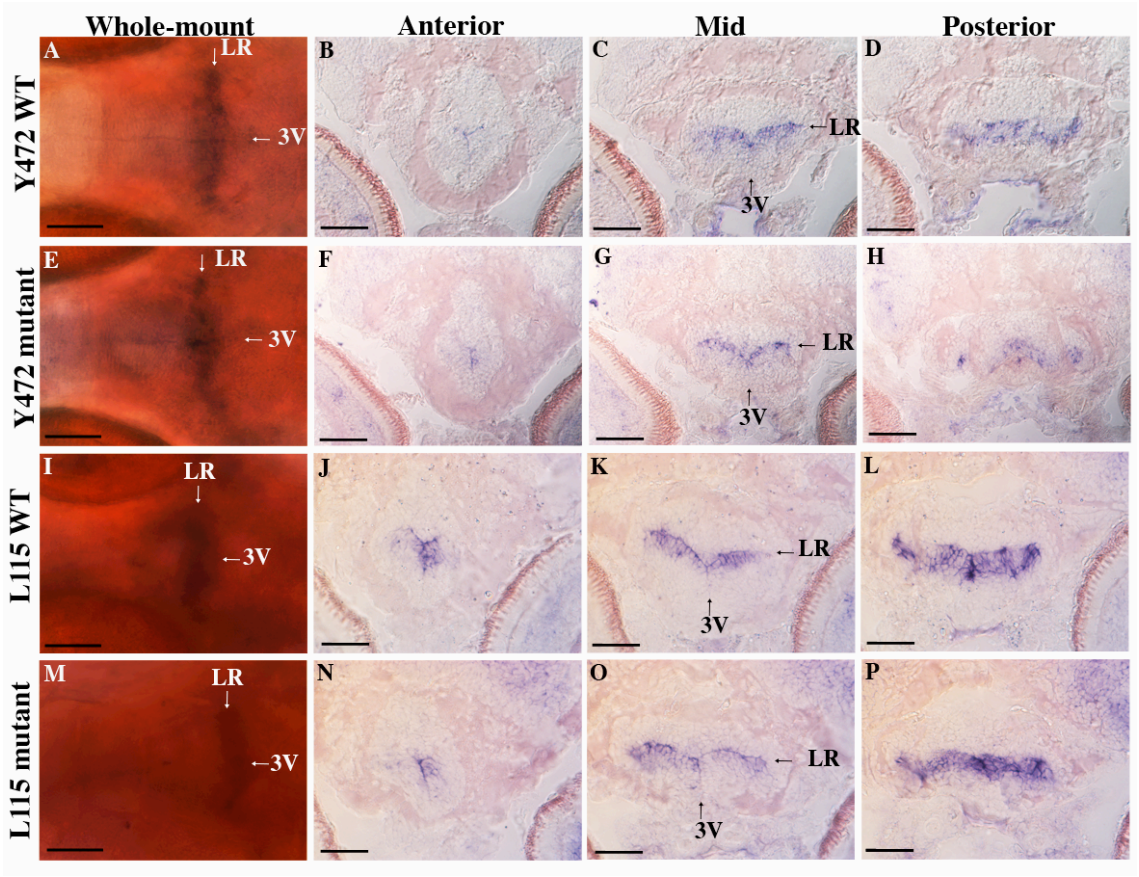

Figure 6.5. Expression of *rx3* in the 4 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *rx3* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle. N=6 per group, 1 technical replicate. Scale bar is 50 µm.

(A-D) Expression of *rx3* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *rx3* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *rx3* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *rx3* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

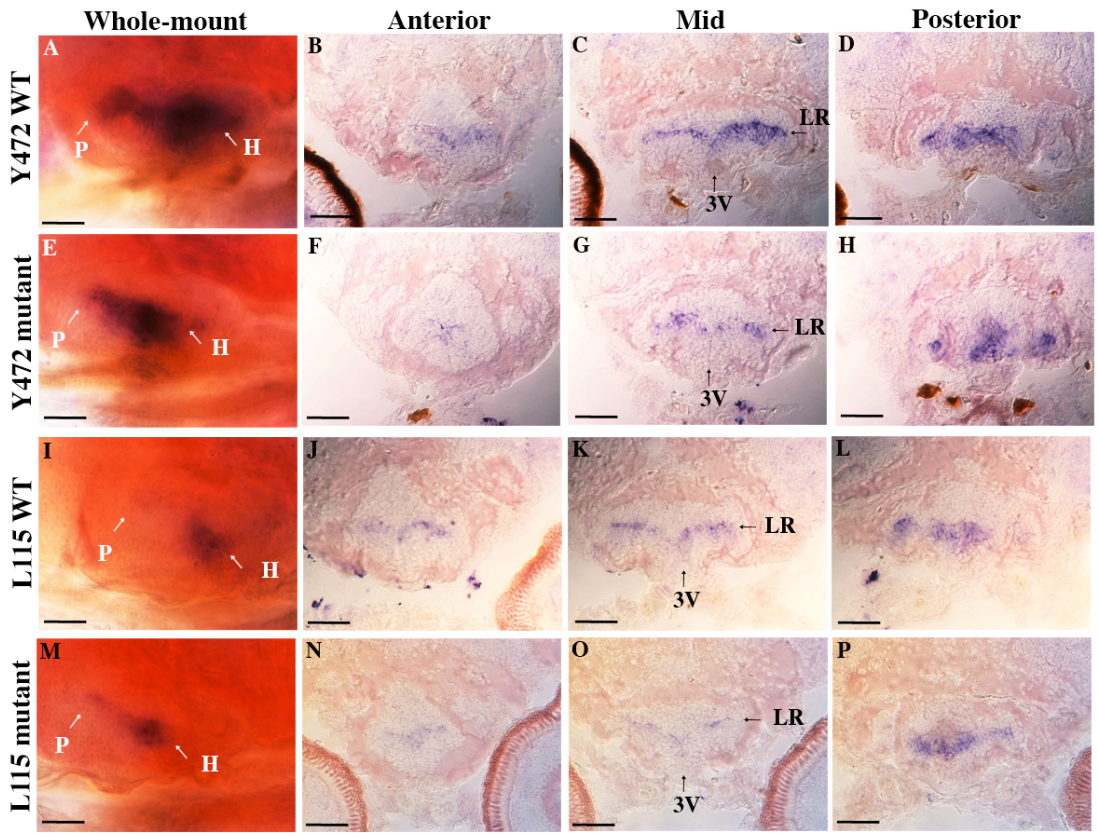

Figure 6.6. Expression of *rx3* in the 5 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (lateral view, anterior left) of *rx3* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle; P, pre-optic region; H, hypothalamus. N=6 per group, 1 technical replicate. Scale bar is 50 µm.

(A-D) Expression of *rx3* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *rx3* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *rx3* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *rx3* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

Expression of *rx3* in the L115 mutant hypothalamus appeared identical to that of wild types in earlier (24 hpf – 3 dpf) stages (Figure 6.2 – 6.4), but appeared to show a subtle reduction in the mid hypothalamus of 4 and 5 dpf larva (Figure 6.5, 6.6). Expression of *rx3* in the retina appeared unaffected by the *disc1* mutation, except in the Y472 mutant larvae at 52 hpf and 3 dpf, in which a reduction was observed (Figure 6.3 C, G; 6.4 A, E).

6.2.3 Expression of *sf1* in *disc1* larvae

At 24 hpf expression of *sf1* was observed in the anterior ventral diencephalon and the interrenal tissue (Figure 6.7 and data not shown). There was no apparent difference in expression of *sf1* between wild types and mutants at 24 hpf (Figure 6.7). Between 52 hpf and 5 dpf expression can be seen in a region described as the the ventral-medial nucleus (VMN) of the hypothalamus [42] (Figure 6.8-6.11) and in the interrenal gland (data not shown). At each of these stages there was a marked increase in expression of *sf1* in the hypothalamus of both Y472 and L115 mutants, in comparison to wild types (Figure 6.8-6.11). The area of *sf1* expression appeared larger in the mutant hypothalamus and the signal appeared stronger, particularly in the mid hypothalamus (Figure 6.8-6.11). No difference in expression of *sf1* was observed between the wild type and mutant interrenal gland at any stage (data not shown).

6.2.5 Expression of *sf1* in the *disc1* L115 adult brain

In the adult zebrafish brain, expression of *sf1* was detected in cells adjacent to the 3rd ventricle in the anterior and mid hypothalamus (Figure 6.12). Comparison with previous expression analyses in the literature suggests that these cells form part of the periventricular hypothalamus [47, 113, 114]. Preliminary analysis of expression in *disc1* L115 adults indicated that whilst expression of *sf1* was fairly consistent between wild type individuals, expression in the homozygous mutant appeared reduced in some individuals and increased in others, in comparison to the wild types (Figure 6.12).

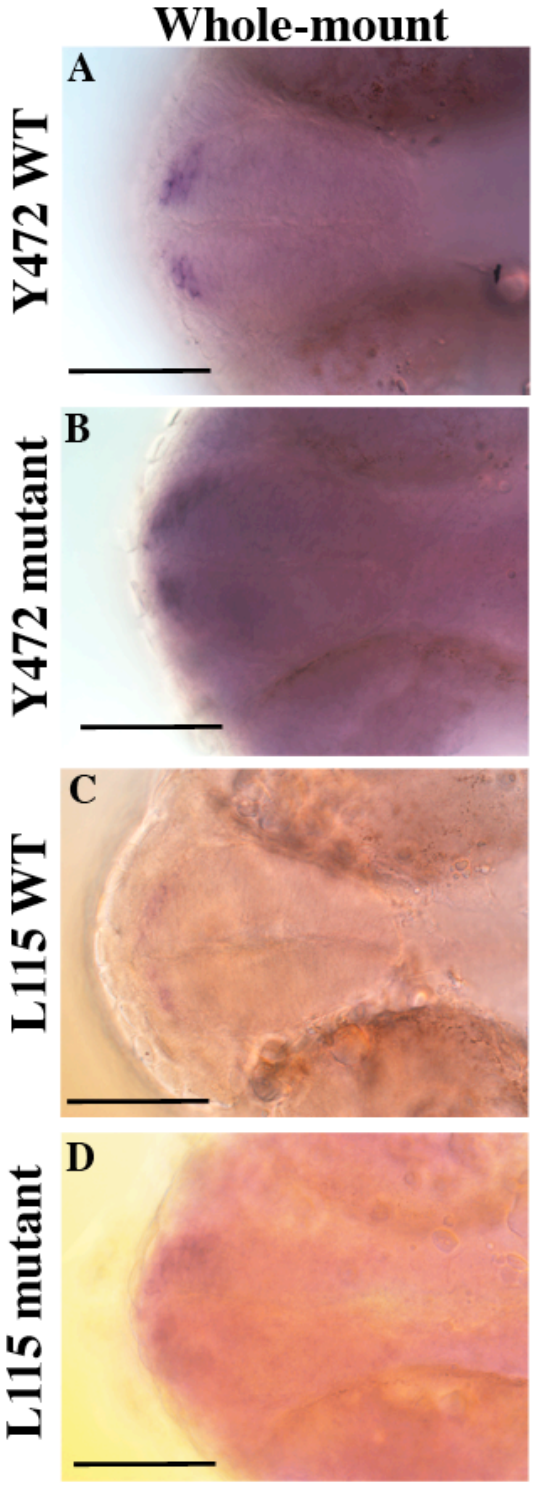

Figure 6.7. Expression of *sf1* in the 24 hpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larvae. N= 6 per group, 1 technical replicate. Scale bar is 50 µm.
(A) Expression of *sf1* in the Y472 wild type brain.
(B) Expression of *sf1* in the Y472 mutant brain.
(C) Expression of *sf1* in the L115 wild type brain.
(D) Expression of *sf1* in the L115 mutant brain.

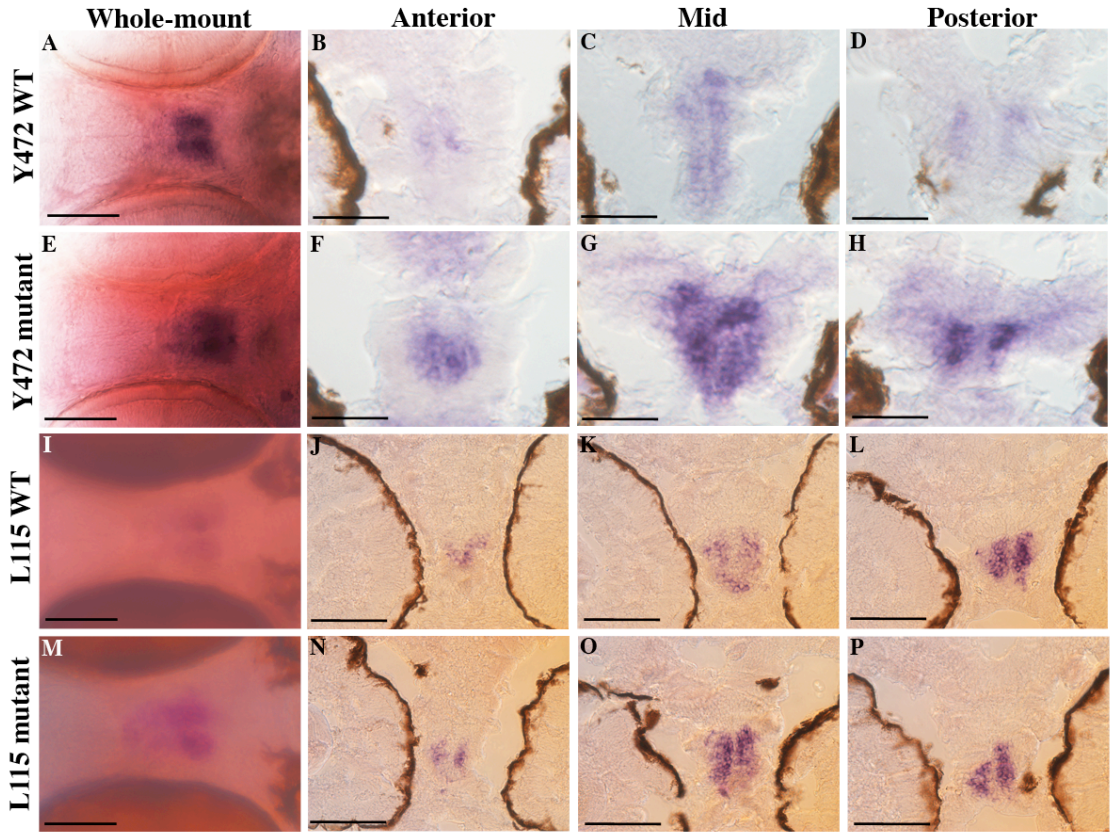

Figure 6.8. Expression of *sf1* in the 52 hpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle. N= 6 per group, 2 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *sf1* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *sf1* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *sf1* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *sf1* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

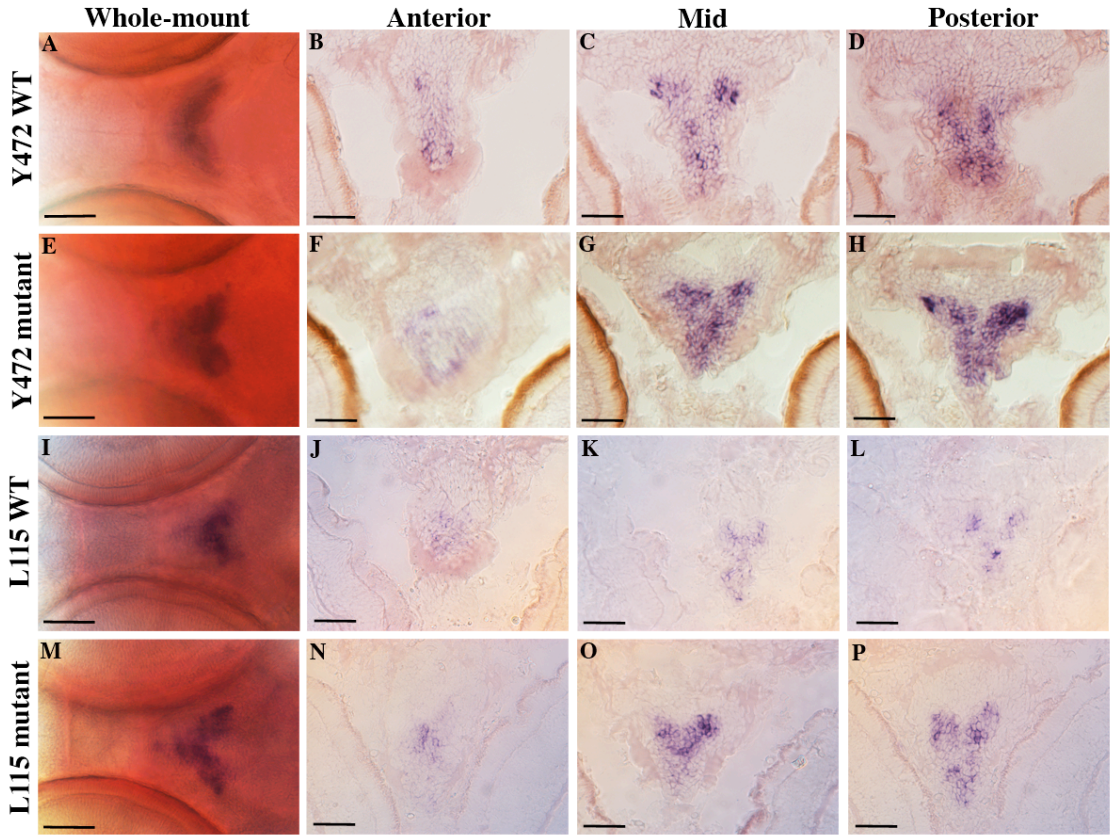

Figure 6.9. Expression of *sf1* in the 3 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 2 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *sf1* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *sf1* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *sf1* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *sf1* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

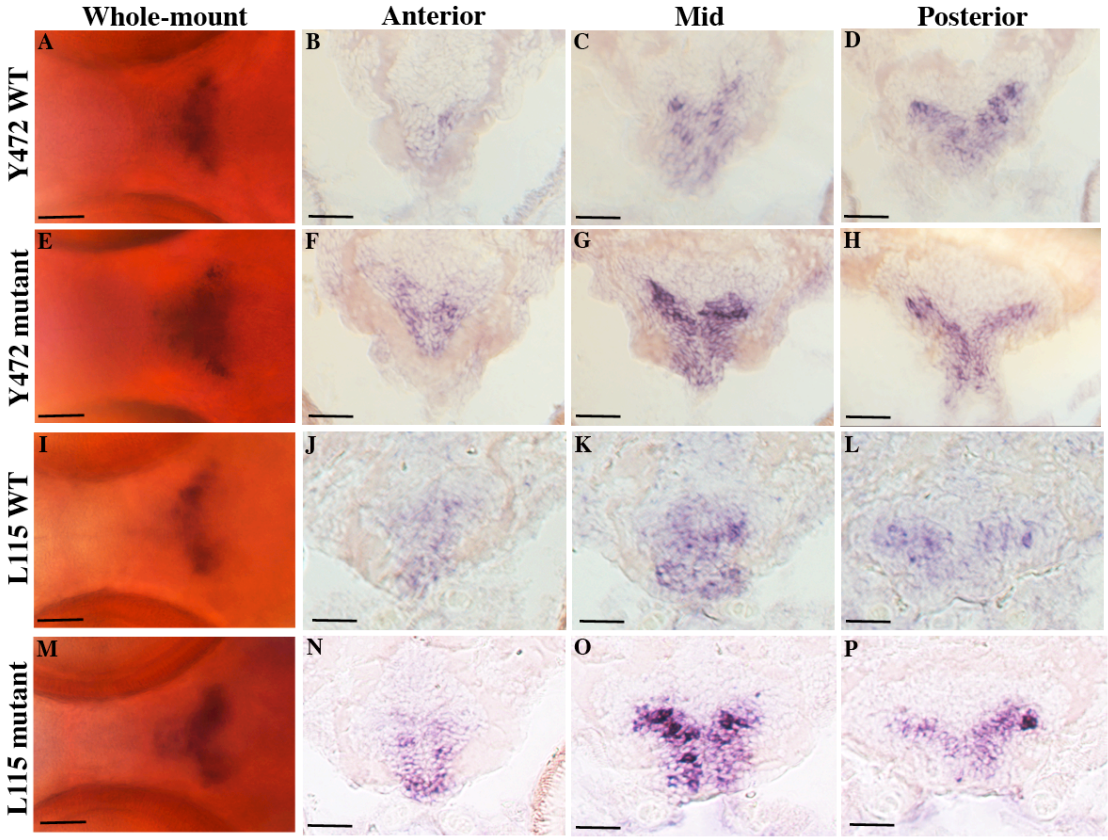

Figure 6.10. Expression of *sf1* in the 4 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 3 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *sf1* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *sf1* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *sf1* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *sf1* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

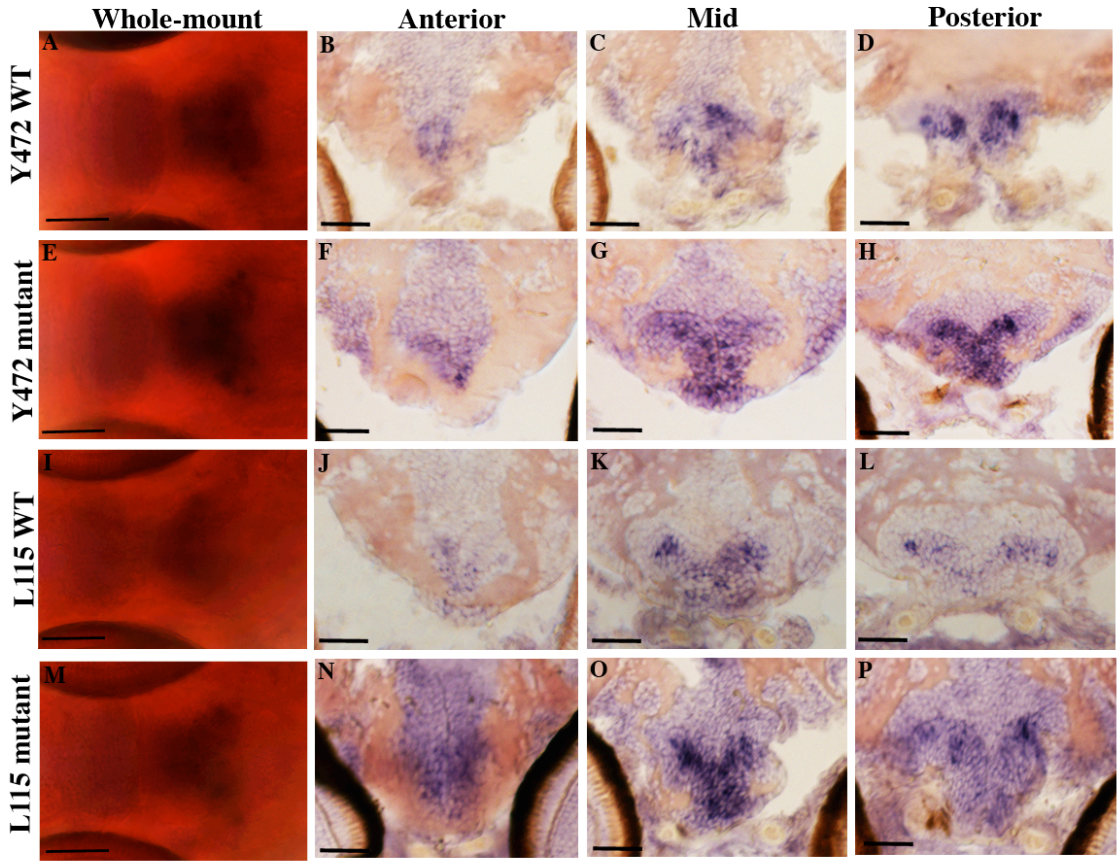

Figure 6.11. Expression of *sf1* in the 5 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *sf1* in the zebrafish brain and sections through the anterior, mid and posterior hypothalamus. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 3 technical replicates. Scale bar is 50 µm.

(A-D) Expression of *sf1* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus.

(E-H) Expression of *sf1* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus.

(I-L) Expression of *sf1* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus.

(M-P) Expression of *sf1* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus.

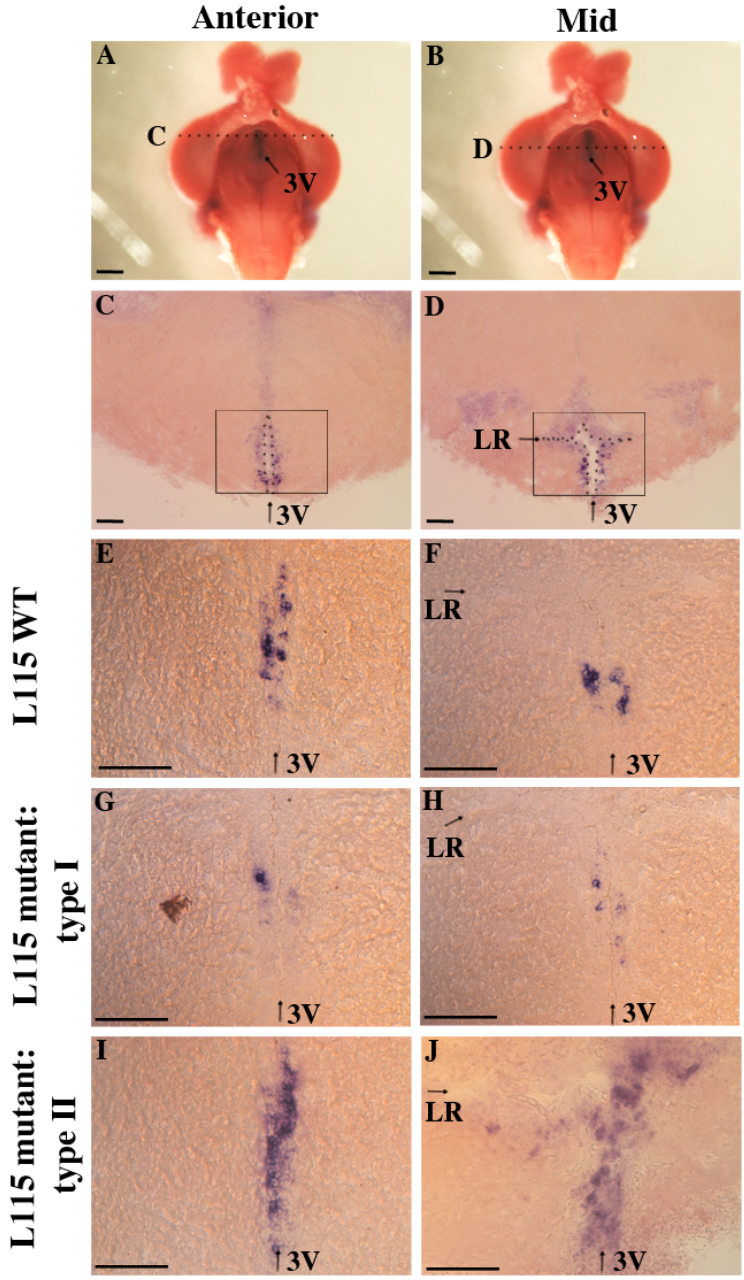

Figure 6.12. Expression of *sf1* in the adult *disc1* L115 zebrafish hypothalamus. WT, wild type sibling; mutant, homozygous mutant; 3V, 3rd ventricle; LR, lateral recess. N= 2 per group, 2 technical replicates.

(A-B) Whole-mount *in situ* hybridization of *sf1*. Dotted line indicates plane of sectioning for anterior and mid hypothalamic transverse sections. Scale bar is 1000 µm.

(C-D) Transverse sections through the zebrafish brain, showing expression of *sf1*, at levels indicated in A-B. Dotted line indicates outline of the 3rd ventricle, boxed region indicates region depicted in E-J. Scale bar is 100 µm.

(E-F) Expression of *sf1* in the anterior and mid hypothalamus in the L115 wild type zebrafish. Scale bar is 100 µm.

(G-H) Expression of *sf1* in the anterior and mid hypothalamus in L115 mutants showing reduced expression relative to wild type siblings.

(I-J) (G-H) Expression of *sf1* in the anterior and mid hypothalamus in L115 mutants showing increased expression relative to wild type siblings. Scale bar is 100 µm.

6.2.6 Stress-induced changes in *sf1* expression

In 5 dpf L115 larval brains, expression of *sf1* in the hypothalamus appeared stronger in mutants than in wild types (Figure 6.13-6.14), as previously observed. Acute exposure to alarm pheromone (protocol 2)(Figure 6.13) or sodium chloride (Figure 6.14) did not have any clear effect on the expression of *sf1* in the hypothalamus in either wild type or mutant larvae.

6.2.3 Expression of *crf* in *disc1* larvae

In 52 hpf and 3 dpf larvae, expression of *crf* was observed in the preoptic region and dorsal-lateral tuberal (mid) hypothalamus and at 3 dpf, in additional cells in the ventral mid hypothalamus (Figure 6.15). At 52 hpf, expression of *crf* was increased in Y472 mutants in comparison to wild type controls, but this increase was not significant to either the pre-optic region or the mid hypothalamus (Figure 6.16, Table 6.1). At 3 dpf, there were again more *crf* positive cells in the Y472 mutant, when compared to wild type controls, and this difference was detected predominantly in the mid hypothalamus (Figure 6.16, Table 6.1).

6.2.7 Expression of *pomc*

Between 52 hpf and 5 dpf, expression of *pomc* was observed in the arcuate nucleus of the hypothalamus and in the pituitary gland (Figure 6.17-6.24). Some reduction in *pomc* expression was noted, but in contrast to the result with *sf1*, where the *disc1* mutation appeared fully-penetrant, the effect of *disc1* mutation on *pomc* expression was variable. In the 52 hpf and 3 dpf and 4 dpf larvae, no difference in *pomc* cell count was observed in the Y472 and L115 larvae (Figure 6.17-6.22, Tables 6.2-6.4). Similarly, the length of the hypothalamus (defined in Materials and Methods) was not altered in comparison to that of the wild type, and consequently, there was no change in *pomc* cell density in the Y472 and L115 larvae, compared to wild types (Figures 6.17-6.22, Tables 6.2-6.4). No difference in expression of *pomc* in the L115 larvae was observed at 5 dpf (Figure 6.23 & 6.24; Table 6.5), however, in the 5 dpf Y472 larvae, there were significantly fewer *pomc* positive cells in the mutant brain (Figure 6.23 & 6.24, Table 6.5). No significant reduction in region size or cell density was observed in the Y472 mutant hypothalamus at 5 dpf (Figure 6.24, Table 6.5).

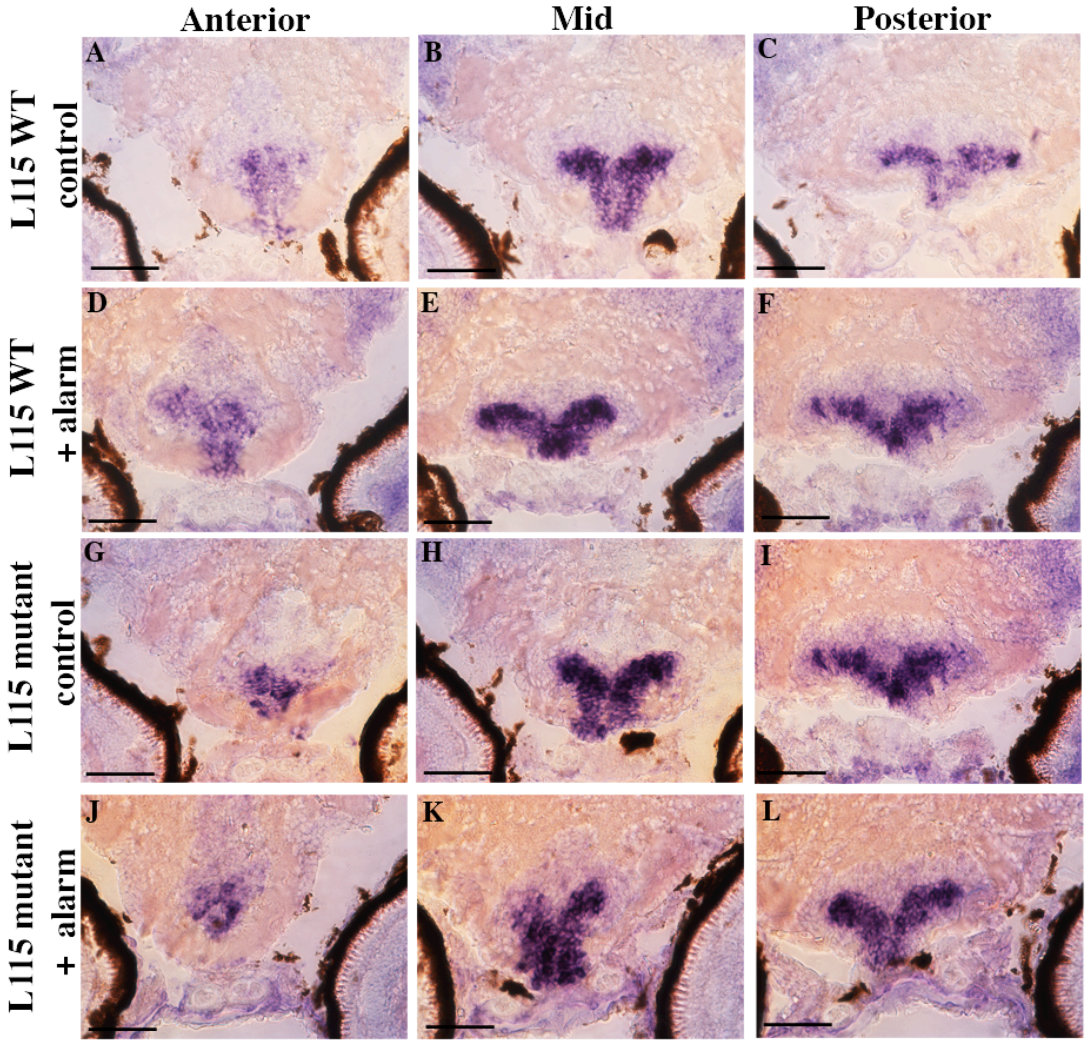

Figure 6.13. Expression of *sf1* in the 5 dpf *disc1* L115 zebrafish brain after acute alarm pheromone exposure (protocol 2). Sections through the anterior, mid and posterior hypothalamus after whole-mount *in situ* hybridization of *sf1*. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 1 technical replicate. Scale bar is 50 µm.
(A-C) Expression of *sf1* in the L115 wild type brain in sections through the hypothalamus, when fish were exposed to a control solution.
(D-F) Expression of *sf1* in the L115 wild type brain in sections through the hypothalamus, when fish were exposed to alarm pheromone.
(G-I) Expression of *sf1* in the L115 mutant brain in sections through the hypothalamus, when fish were exposed to a control solution.
(J-L) Expression of *sf1* in the L115 mutant brain in sections through the hypothalamus, when fish were exposed to alarm pheromone.

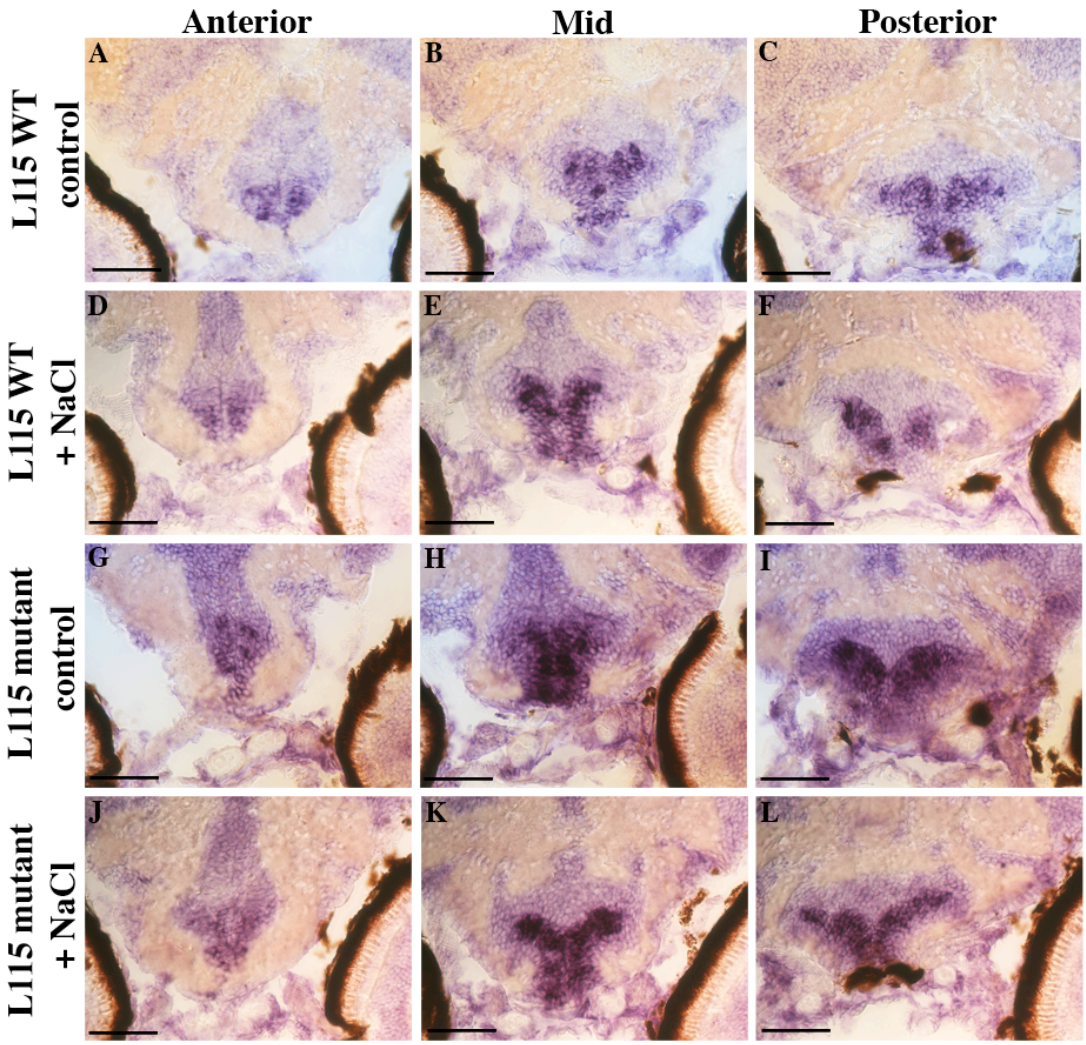

Figure 6.14. Expression of *sf1* in the 5 dpf *disc1* L115 zebrafish brain after acute sodium chloride exposure. Sections through the anterior, mid and posterior hypothalamus after whole-mount *in situ* hybridization of *sf1*. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 1 technical replicate. Scale bar is 50 µm.

(A-C) Expression of *sf1* in the L115 wild type brain in sections through the hypothalamus, when fish were exposed to a control solution.

(D-F) Expression of *sf1* in the L115 wild type brain in sections through the hypothalamus, when fish were exposed to sodium chloride.

(G-I) Expression of *sf1* in the L115 mutant brain in sections through the hypothalamus, when fish were exposed to a control solution.

(J-L) Expression of *sf1* in the L115 mutant brain in sections through the hypothalamus, when fish were exposed to sodium chloride.

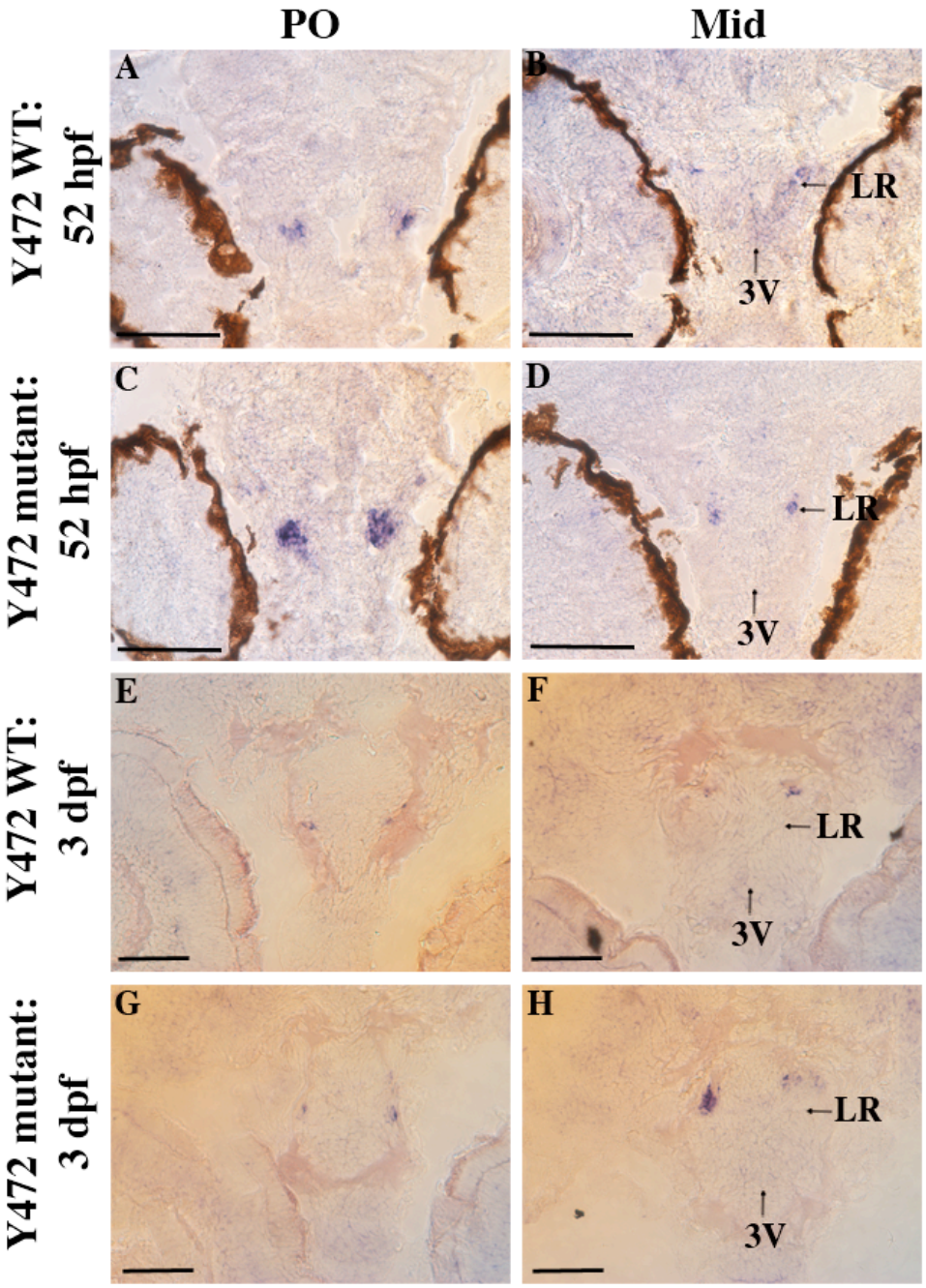

Figure 6.15. Expression of *crf* in the *disc1* Y472 larval zebrafish brain. Transverse sections through the pre-optic region and hypothalamus after whole-mount *in situ* hybridization of *crf.* Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva; LR, lateral recess; 3V, 3rd ventricle. N= 5 per 52 hpf group, 1 technical replicate; 5 per 3 dpf group, 2 technical replicates. See Figure 6.16 and Table 6.1 for cell counts. Scale bar is 50 μm.

(A-B) Expression of *crf* in the pre-optic region and mid hypothalamus of 52 hpf Y472 wild type zebrafish.

(C-D) Expression of *crf* in the pre-optic region and mid hypothalamus of 52 hpf Y472 mutant zebrafish.

(E-F) Expression of *crf* in the pre-optic region and mid hypothalamus of 3 dpf Y472 wild type zebrafish.

(G-H) Expression of *crf* in the pre-optic region and mid hypothalamus of 3 dpf Y472 mutant zebrafish.

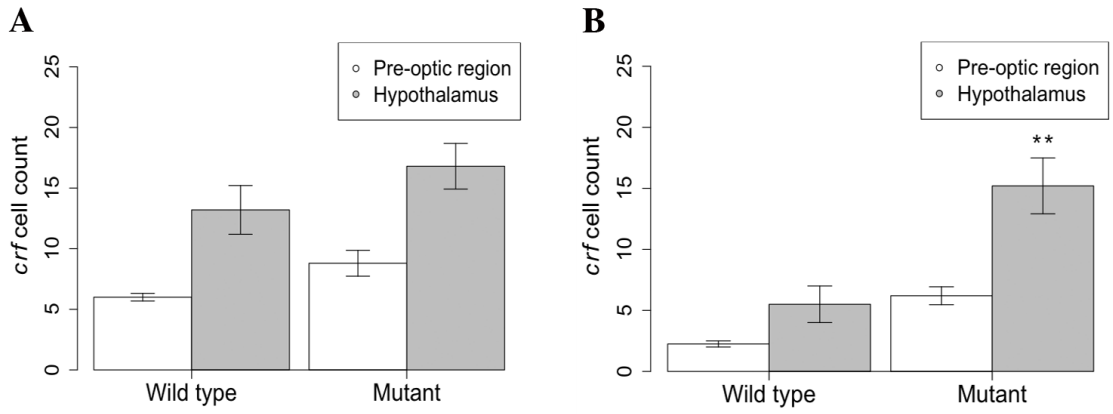

Figure 6.16. Counts of *crf* positive cells in the *disc1* Y472 larval hypothalamus. Points and bars represent mean ± standard error. Wild type, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. ** indicates statistically significant difference from wild type hypothalamus at $p<0.01$. N= 5 per 52 hpf group, 1 technical replicate; 5 per 3 dpf group, 2 technical replicates. See Table 6.1 for statistics.
(A) Mean count of *crf* positive cells in 52 hpf Y472 wild type mutant larval pre-optic region and hypothalamus.
(B) Mean count of *crf* positive cells in 3 dpf Y472 wild type mutant larval pre-optic region and hypothalamus.

Table 6.1. Statistical analysis of *crf* cell counts in *disc1* Y472 larval hypothalamus. The results of one-way ANOVA tests and Tukey's Multiple Comparison test are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae; hyp, hypothalamus; PO, pre-optic region. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 5 per 52 hpf group, 1 technical replicate; 5 per 3 dpf group, 2 technical replicates. See Figures 6.15 and 6.16.

Stage	Factor	F value	d.f	p value	
52 hpf	Genotype	4.64	1, 16	0.047	*
	Region	26.20	1, 16	0.0001	***
	Genotype: region	0.07	1, 16	0.791	
3 dpf	Genotype	20.22	1, 14	0.0005	***
	Region	18.25	1, 14	0.0008	***
	Genotype: region	3.59	1, 14	0.079	

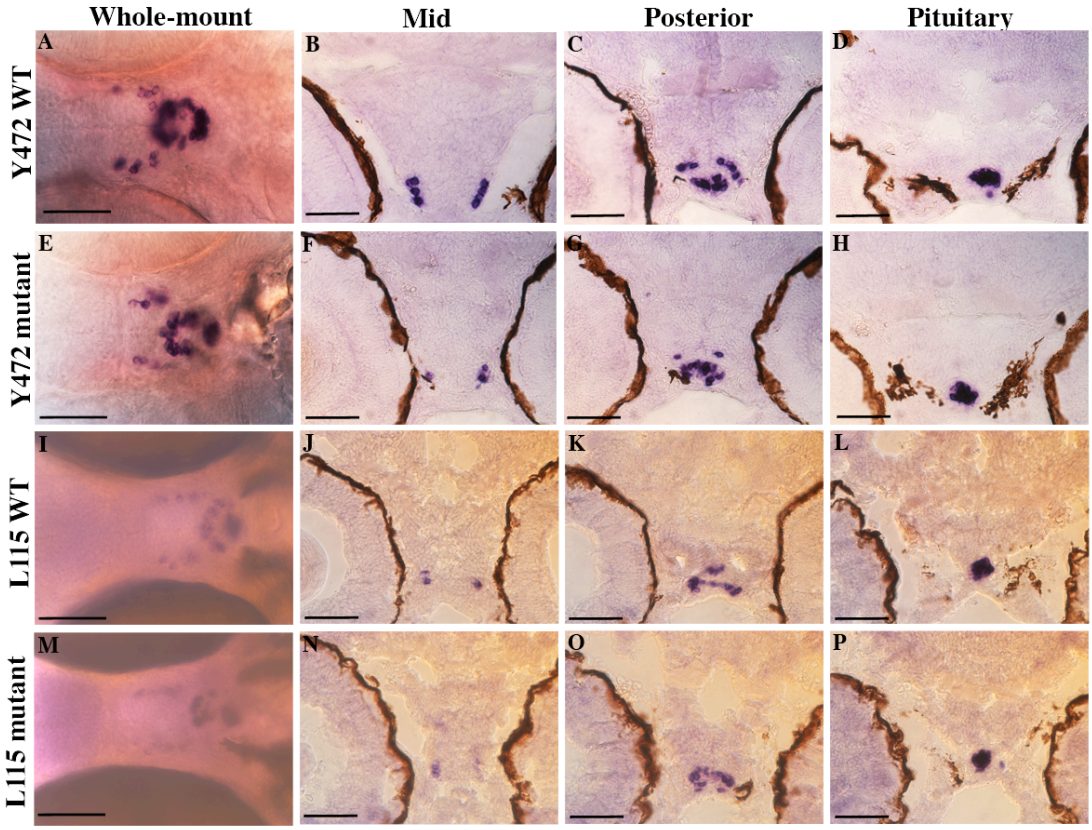

Figure 6.17. Expression of *pomc* in the 52 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *pomc* in the zebrafish brain and sections through the mid and posterior hypothalamus and the pituitary gland. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per Y472 group, 3 technical replicates; 3 per L115 group, 1 technical replicate. See Figure 6.18 and Table 6.2 for cell counts. Scale bar is 50 µm.

(A-D) Expression of *pomc* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(E-H) Expression of *pomc* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

(I-L) Expression of *pomc* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(M-P) Expression of *pomc* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

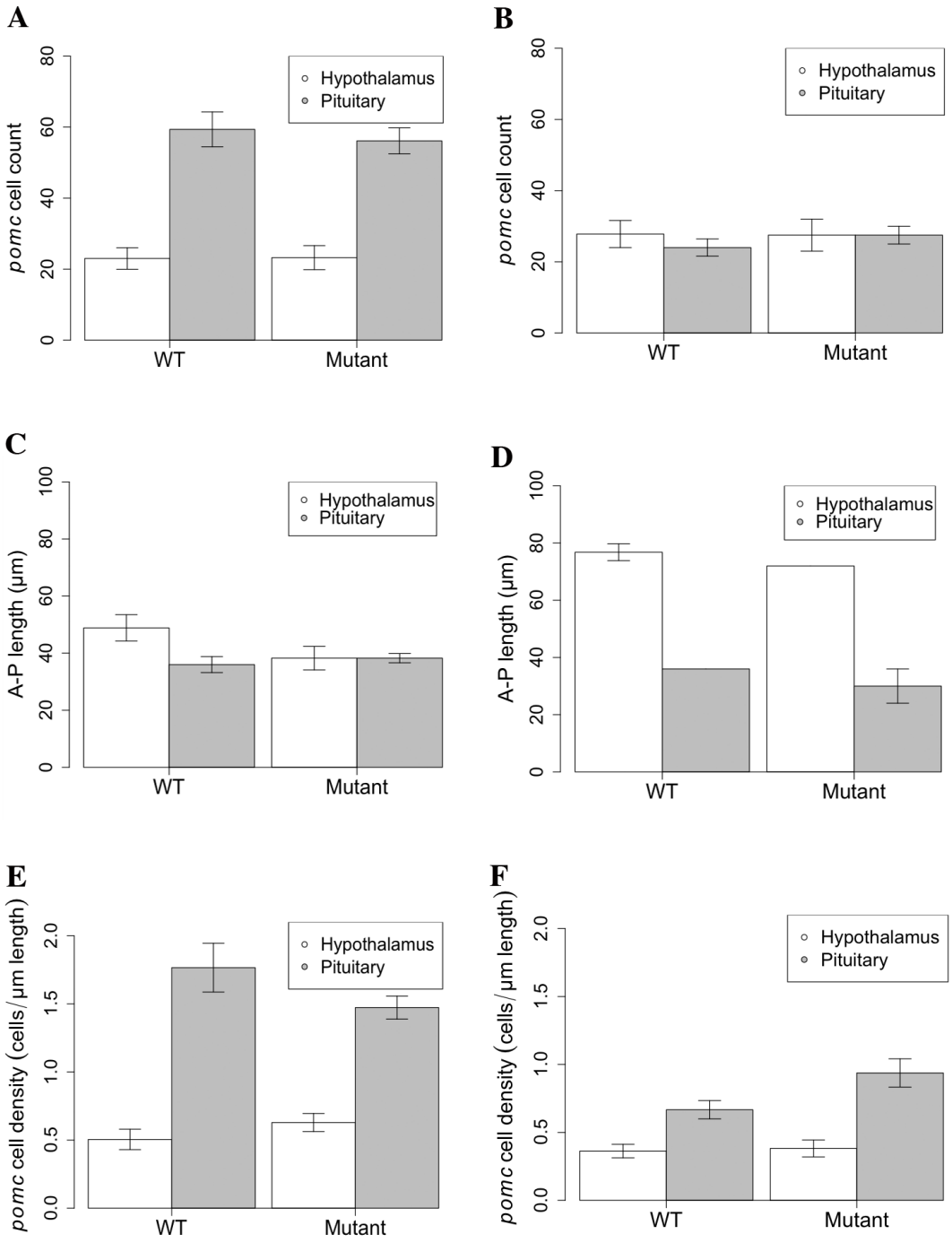

Figure 6.18. Counts of *pomc* positive cells in *disc1* 52 hpf hypothalamus and pituitary. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. N= 6 per Y472 group, 3 technical replicates; 3 per L115 group, 1 technical replicate. See Table 6.2 for statistics.

(A-B) Mean count of *pomc* positive cells in Y472 (A) and L115 (B) wild type mutant larval hypothalamus and pituitary.

(C-D) Mean anterior-posterior length of Y472 (C) and L115 (D) wild type mutant larval hypothalamus and pituitary.

(E-F) Mean density of *pomc* positive cells in Y472 (E) and L115 (F) wild type and mutant larval hypothalamus and pituitary.

Table 6.2. **Statistical analysis of *pomc* cell counts in *disc1* L115 and Y472 52 hpf larval hypothalamus and pituitary.** The results of one-way ANOVA tests and Tukey's Multiple Comparison test are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae; hyp, hypothalamus; pit, pituitary gland; A-P length, anterior-posterior length of region. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 6 per Y472 group, 3 technical replicates; 3 per L115 group, 1 technical replicate. See Figure 6.18 for plots.

	Parameter	Factor	F value	d.f	p value	
L115	**Cell count**	Genotype	0.16	1, 10	0.698	
		Region	0.56	1, 10	0.47	
		Genotype: region	0.22	1, 10	0.646	
	A-P length	Genotype	3.40	1, 10	0.095	
		Region	242.02	1, 10	<0.0001	***
		Genotype: region	0.04	1, 10	0.842	
	Cell density	Genotype	3.53	1, 10	0.089	
		Region	28.99	1, 10	0.0003	***
		Genotype: region	2.65	1, 10	0.134	
Y472	**Cell count**	Genotype	0.15	1, 56	0.69	
		Region	82.89	1, 56	<0.0001	***
		Genotype: region	0.21	1, 56	0.65	
	A-P length	Genotype	1.45	1, 56	0.233	
		Region	3.01	1, 56	0.088	
		Genotype: region	3.44	1, 56	0.069	
	Cell density	Genotype	0.62	1, 56	0.435	
		Region	93.87	1, 56	<0.0001	***
		Genotype: region	3.77	1, 56	0.057	

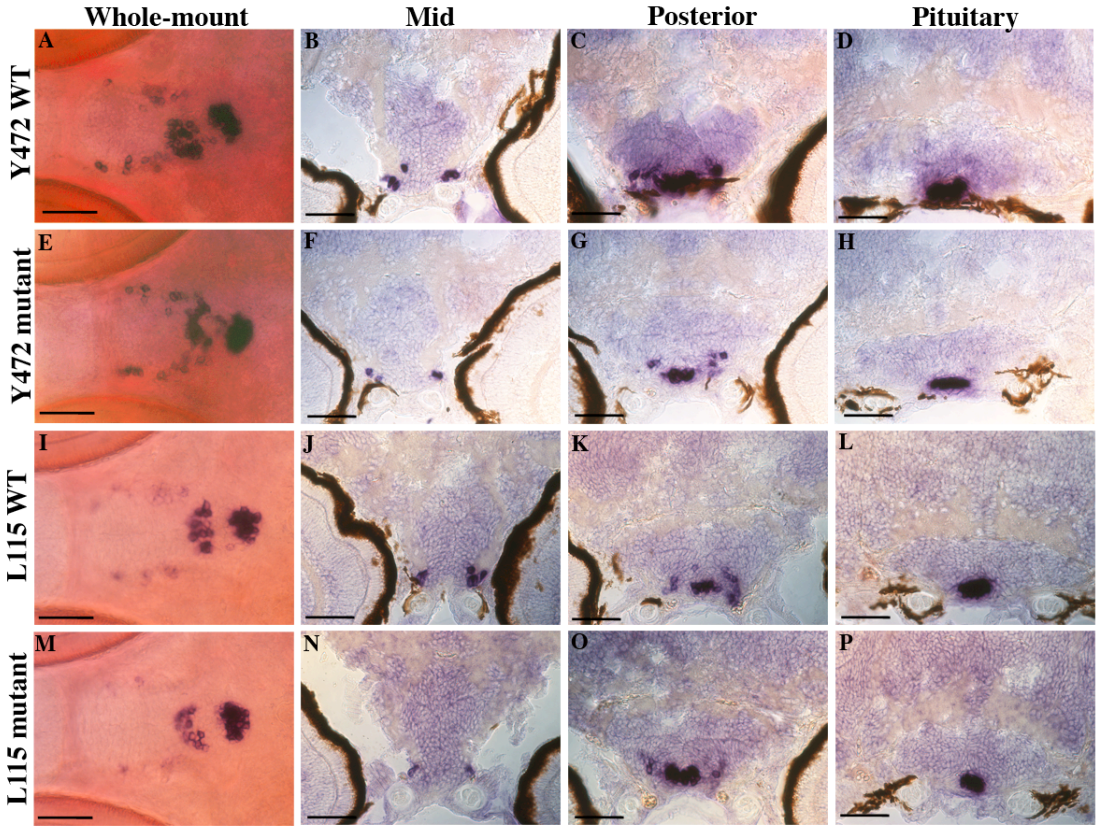

Figure 6.19. Expression of *pomc* in the 3 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *pomc* in the zebrafish brain and sections through the mid and posterior hypothalamus and the pituitary gland. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 2 technical replicates. See Figure 6.19 and Table 6.3 for cell counts. Scale bar is 50 µm.

(A-D) Expression of *pomc* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(E-H) Expression of *pomc* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

(I-L) Expression of *pomc* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(M-P) Expression of *pomc* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

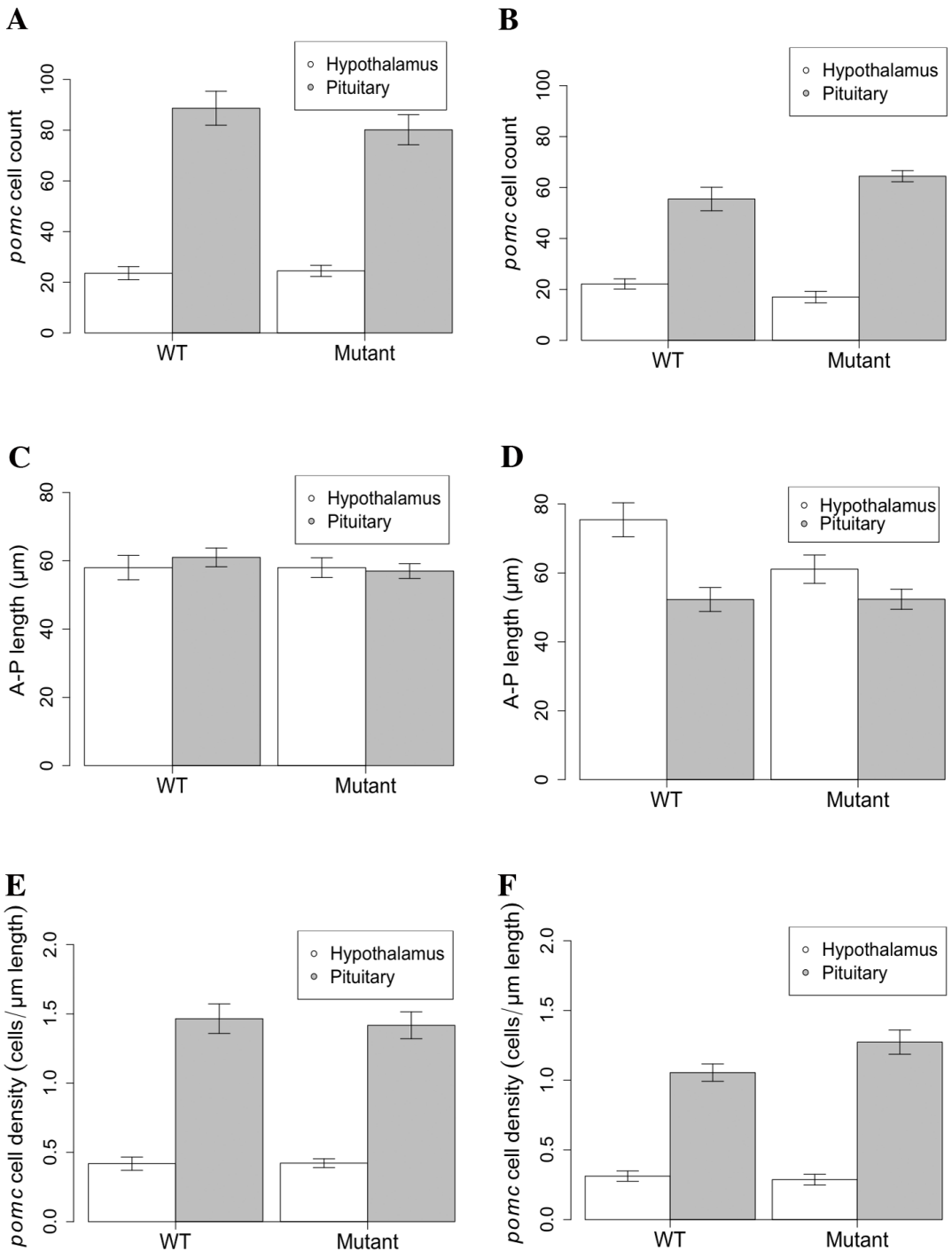

Figure 6.20. Counts of *pomc* positive cells in *disc1* 3 dpf hypothalamus and pituitary. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. N= 6 per group, 2 technical replicates. See Table 6.3 for statistics.

(A-B) Mean count of *pomc* positive cells in Y472 (A) and L115 (B) wild type mutant larval hypothalamus and pituitary.

(C-D) Mean anterior-posterior length of Y472 (C) and L115 (D) wild type mutant larval hypothalamus and pituitary.

(E-F) Mean density of *pomc* positive cells in Y472 (E) and L115 (F) wild type and mutant larval hypothalamus and pituitary.

Table 6.3. **Statistical analysis of *pomc* cell counts in *disc1* L115 3 dpf larval hypothalamus and pituitary.** The results of one-way ANOVA tests and Tukey's Multiple Comparison test are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae; hyp, hypothalamus; pit, pituitary gland; A-P length, anterior-posterior length of region. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 6 per group, 2 technical replicates. See Figure 6.20 for plots.

	Parameter	Factor	F/t value	d.f	p value	
L115	Cell count	Genotype	0.36	1, 46	0.550	
		Region	158.54	1, 46	<0.0001	***
		Genotype: region	4.96	1, 46	0.031	*
		WT hyp: mut hyp	-	-	0.661	
		WT pit: mut pit	-	-	0.203	
	A-P length	Genotype	3.05	1, 46	0.087	
		Region	17.20	1, 46	0.0001	***
		Genotype: region	3.12	1, 46	0.084	
	Cell density	Genotype	2.72	1, 46	0.106	
		Region	213.81	1, 46	<0.0001	***
		Genotype: region	4.36	1, 46	0.042	*
		WT hyp: mut hyp	-	-	0.989	
		WT pit: mut pit	-	-	0.053	
Y472	Cell count	Genotype	0.62	1, 44	0.433	
		Region	158.95	1, 44	<0.0001	***
		Genotype: region	0.97	1, 44	0.331	
	A-P length	Genotype	0.48	1, 44	0.492	
		Region	0.12	1, 44	0.730	
		Genotype: region	0.48	1, 44	0.492	
	Cell density	Genotype	0.08	1, 44	0.780	
		Region	173.10	1, 44	<0.0001	***
		Genotype: region	0.11	1, 44	0.742	

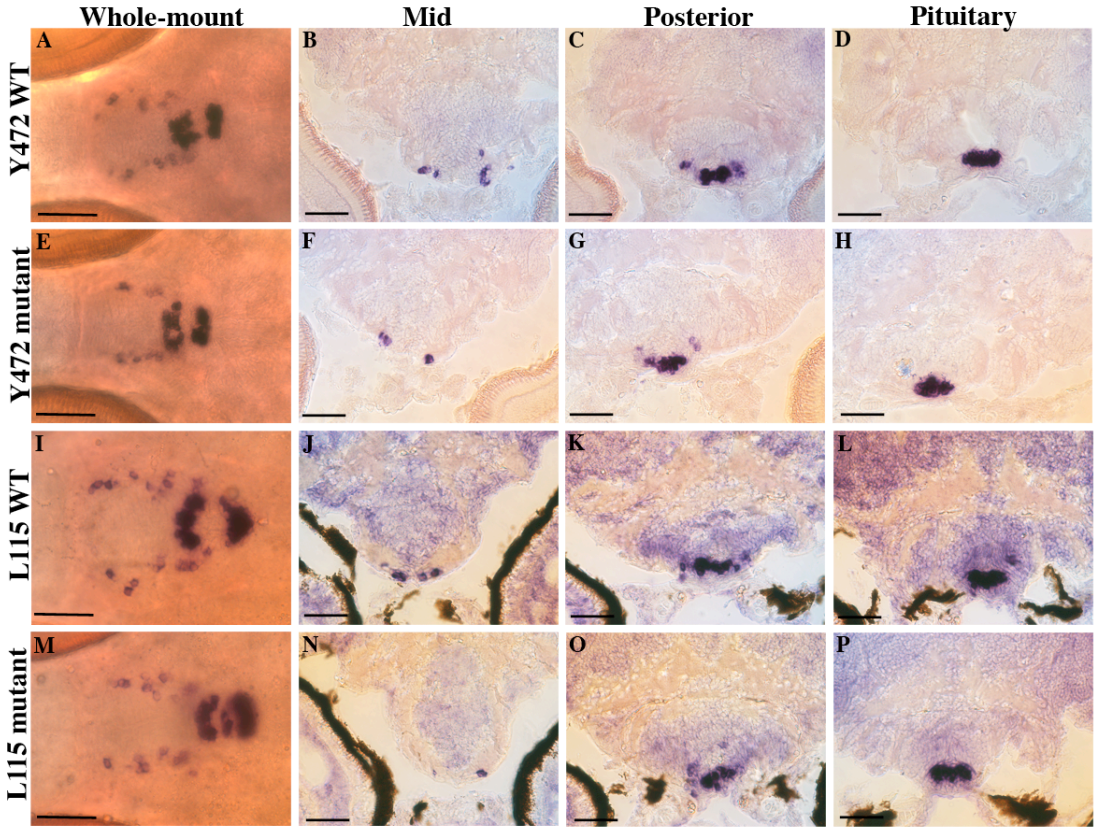

Figure 6.21. Expression of *pomc* in the 4 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *pomc* in the zebrafish brain and sections through the mid and posterior hypothalamus and the pituitary gland. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 2 technical replicates. See Figure 6.22 and Table 6.4 for cell counts. Scale bar is 50 µm.

(A-D) Expression of *pomc* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(E-H) Expression of *pomc* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

(I-L) Expression of *pomc* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(M-P) Expression of *pomc* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

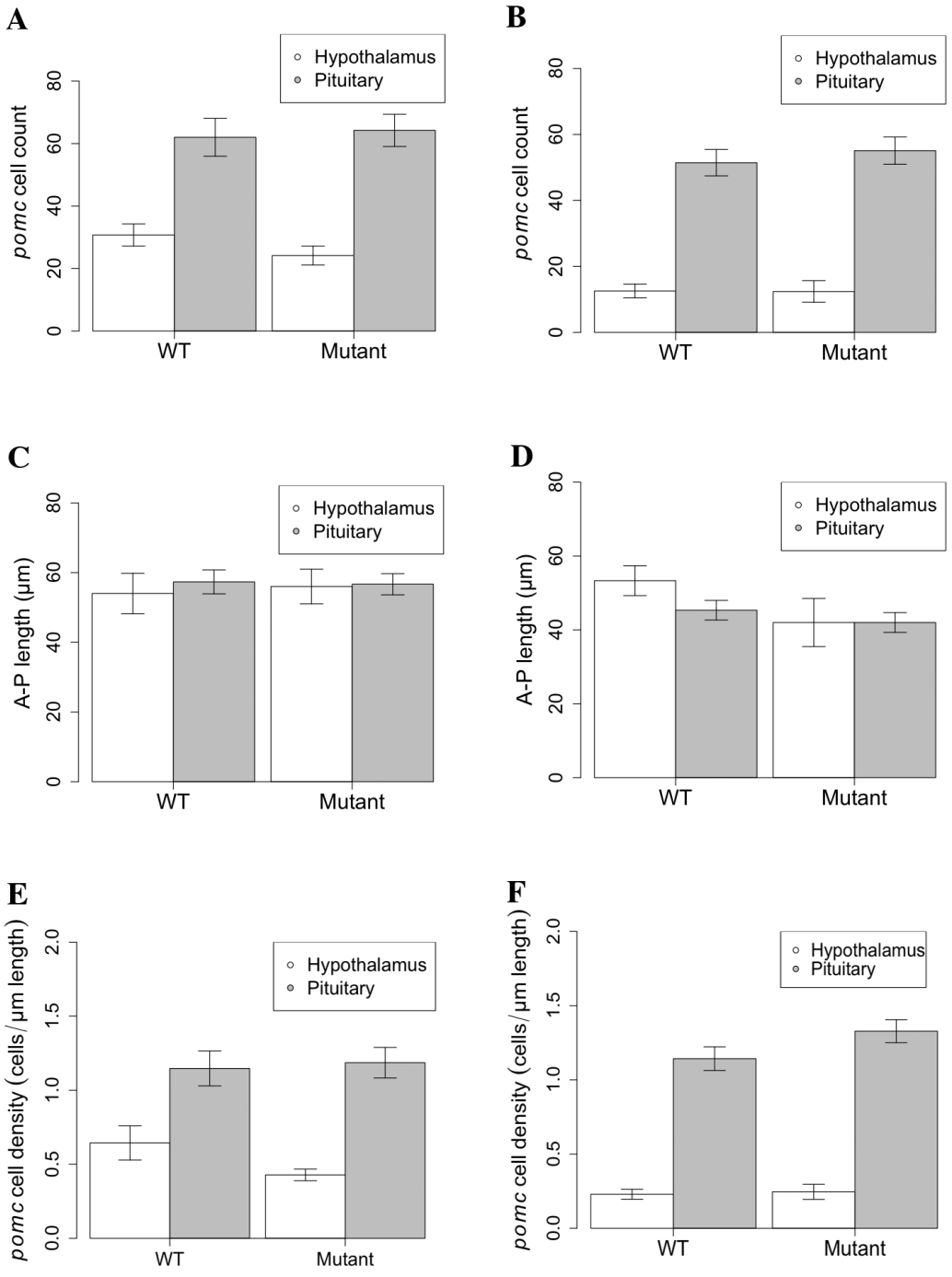

Figure 6.22. Counts of *pomc* positive cells in *disc1* 4 dpf hypothalamus and pituitary. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. N= 6 per Y472 group, 2 technical replicates; 6 per L115 group, 2 technical replicate. See Table 6.4 for statistics.

(A-B) Mean count of *pomc* positive cells in Y472 (A) and L115 (B) wild type mutant larval hypothalamus and pituitary.

(C-D) Mean anterior-posterior length of Y472 (C) and L115 (D) wild type mutant larval hypothalamus and pituitary.

(E-F) Mean density of *pomc* positive cells in Y472 (E) and L115 (F) wild type and mutant larval hypothalamus and pituitary.

Table 6.4. Statistical analysis of *pomc* cell counts in *disc1* L115 and Y472 4 dpf larval hypothalamus and pituitary. The results of one-way ANOVA tests and Tukey's Multiple Comparison test are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae; hyp, hypothalamus; pit, pituitary gland; A-P length, anterior-posterior length of region. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 6 per Y472 group, 2 technical replicates; 6 per L115 group, 2 technical replicate. See Figure 6.22 for plots.

	Parameter	Factor	F value	d.f	p value	
L115	Cell count	Genotype	0.25	1, 34	0.662	
		Region	135.73	1, 34	<0.0001	***
		Genotype: region	0.29	1, 34	0.591	
	A-P length	Genotype	2.81	1, 34	0.103	
		Region	0.75	1, 34	0.392	
		Genotype: region	0.84	1, 34	0.367	
	Cell density	Genotype	2.49	1, 56	0.124	
		Region	247.36	1, 56	<0.0001	***
		Genotype: region	1.74	1, 56	0.196	
Y472	Cell count	Genotype	0.06	1, 56	0.802	
		Region	45.97	1, 56	<0.0001	***
		Genotype: region	0.70	1, 56	0.406	
	A-P length	Genotype	0.01	1, 56	0.923	
		Region	0.23	1, 56	0.635	
		Genotype: region	0.10	1, 56	0.752	
	Cell density	Genotype	0.36	1, 56	0.554	
		Region	33.93	1, 56	<0.0001	***
		Genotype: region	1.38	1, 56	0.245	

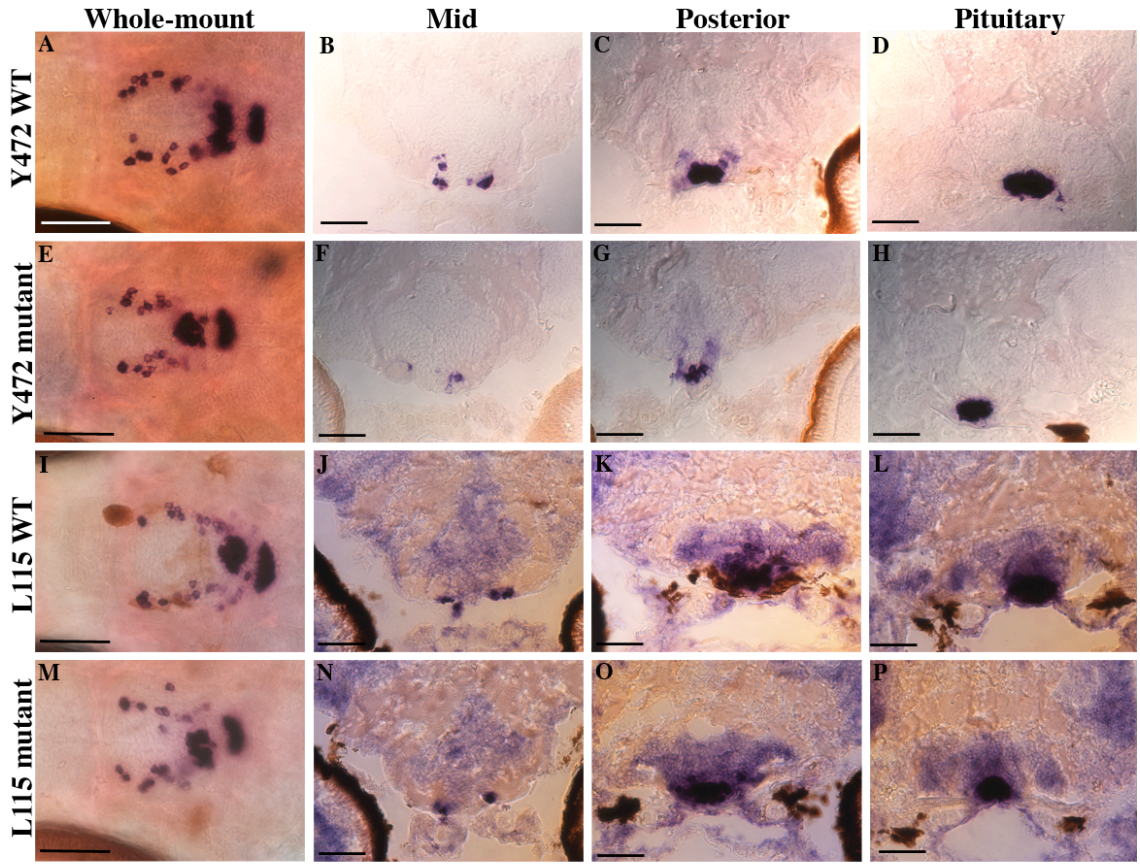

Figure 6.23. Expression of *pomc* in the 5 dpf *disc1* zebrafish brain. Whole-mount *in situ* hybridization (ventral view, anterior left) of *pomc* in the zebrafish brain and sections through the mid and posterior hypothalamus and the pituitary gland. Wild type, wild type in-cross larva; mutant, homozygous mutant in-cross larva. N= 6 per group, 2 technical replicates. See Figure 6.24 and Table 6.5 for cell counts. Scale bar is 50 µm.

(A-D) Expression of *pomc* in the Y472 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(E-H) Expression of *pomc* in the Y472 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

(I-L) Expression of *pomc* in the L115 wild type brain in a whole-mount view and sections through the hypothalamus and pituitary.

(M-P) Expression of *pomc* in the L115 mutant brain in a whole-mount view and sections through the hypothalamus and pituitary.

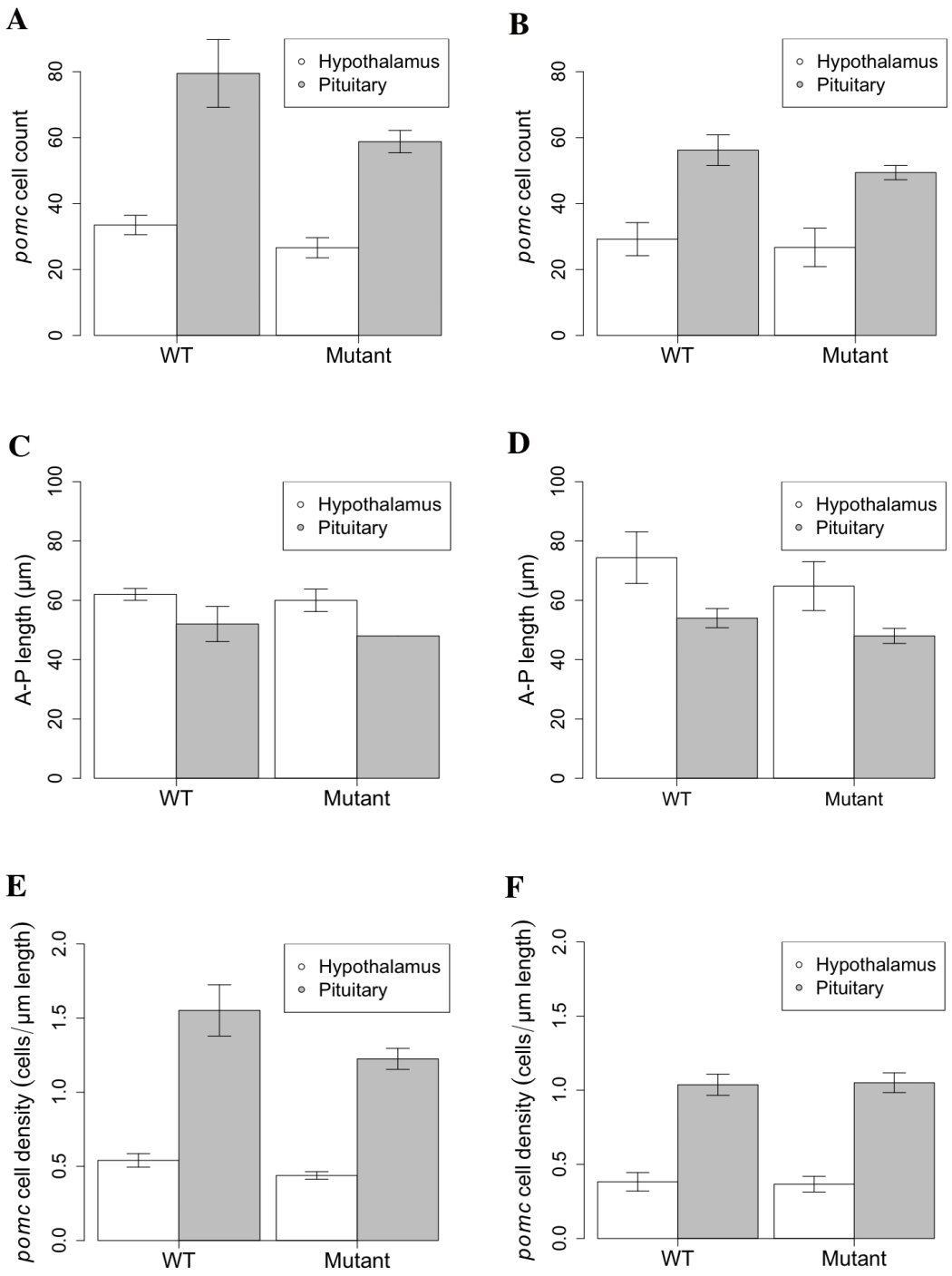

Figure 6.24. Counts of *pomc* positive cells in *disc1* 5 dpf hypothalamus and pituitary. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. N= 6 per group, 2 technical replicates. See Table 6.5 for statistics.

(A-B) Mean count of *pomc* positive cells in Y472 (A) and L115 (B) wild type mutant larval hypothalamus and pituitary.

(C-D) Mean anterior-posterior length of Y472 (C) and L115 (D) wild type mutant larval hypothalamus and pituitary.

(E-F) Mean density of *pomc* positive cells in Y472 (E) and L115 (F) wild type and mutant larval hypothalamus and pituitary.

Table 6.5. Statistical analysis of *pomc* cell counts in *disc1* L115 and Y472 5 dpf larval hypothalamus and pituitary. The results of one-way ANOVA tests and Tukey's Multiple Comparison test are shown. d.f, degrees of freedom; WT, wild type in-cross larvae; mut, homozygous mutant in-cross larvae; hyp, hypothalamus; pit, pituitary gland; A-P length, anterior-posterior length of region. * indicates a statistically significant difference at $p < 0.05$, ** $p<0.01$, *** $p<0.001$. N= 6 per group, 2 technical replicates. See Figure 6.24 for plots.

	Parameter	Factor	F value	d.f	p value	
L115	**Cell count**	Genotype	1.01	1, 36	0.321	
		Region	28.93	1, 36	<0.0001	***
		Genotype: region	0.23	1, 36	0.645	
	A-P length	Genotype	1.51	1, 36	0.227	
		Region	8.61	1, 36	0.006	**
		Genotype: region	0.08	1, 36	0.778	
	Cell density	Genotype	0.0	1, 36	0.982	
		Region	109.89	1, 36	<0.0001	***
		Genotype: region	0.05	1, 36	0.818	
Y472	**Cell count**	Genotype	4.84	1, 18	0.041	*
		Region	40.47	1, 18	<0.0001	***
		Genotype: region	1.21	1, 18	0.286	
	A-P length	Genotype	0.60	1, 18	0.447	
		Region	8.05	1, 18	0.011	*
		Genotype: region	0.07	1, 18	0.799	
	Cell density	Genotype	4.21	1, 18	0.055	
		Region	76.53	1, 18	<0.0001	***
		Genotype: region	1.16	1, 18	0.295	

6.2.8 Stress induced changes in *pomc* expression

In 5 dpf L115 larval brains, there were significantly fewer *pomc* positive cells in the mutant, compared to that of wild types (Figure 6.25 A, Table 6.6) and the length of the mutant hypothalamus was also significantly reduced (Figure 6.25 B, Table 6.6) and no significant reduction in density of *pomc* cells within the mutant hypothalamus was seen (Figure 6.25 C, Table 6.6). There was no significant difference in the number of *pomc* cells in the wild type and mutant pituitary or size of the pituitary (Figure 6.25 A-B, Table 6.6); however, the density of *pomc* cells in the mutant was significantly lower than that of wild types (Figure 6.25 C, Table 6.6). There was a significant main effect of the alarm treatment (protocol 2) on *pomc* cell count, however treatment did not have a significant effect on the number of *pomc* cells in wild type or mutant brains in either region (Figure 6.25 A, Table 6.6). Fish treated with alarm pheromone had a significantly shorter hypothalamus but this did not correspond with a difference in count or density of *pomc* cells (Figure 6.25 A-C, Table 6.6).

6.3 Discussion

6.3.1 Expression of *disc1* in wild type larvae

In line with other animal studies, *disc1* mRNA appeared to be expressed in the early zebrafish hypothalamus. This data raises the possibility that *disc1* plays a functional role in the hypothalamus, potentially in the modulation of the HPI axis. Intriguingly, *disc1* appeared to be expressed in a similar fashion to *rx3* in the hypothalamus. Previous studies have shown that both *disc1* and *rx3* are regulated by Shh [43, 115] prompting me to analyse *rx3* in wild type and *disc1* larvae. This might indicate an interaction between *disc1* and *rx3* in the hypothalamus.

6.3.2 Expression of *rx3* in *disc1* larvae

Expression of *rx3* appeared reduced in both the Y472 mutant, and more subtly in the L115 mutant, particularly in the mid hypothalamus. The reduction was most apparent in the 52 hpf Y472 larva, in which expression was absent from the lateral recess and ventral most cells lining the 3rd ventricle of the mid hypothalamus.

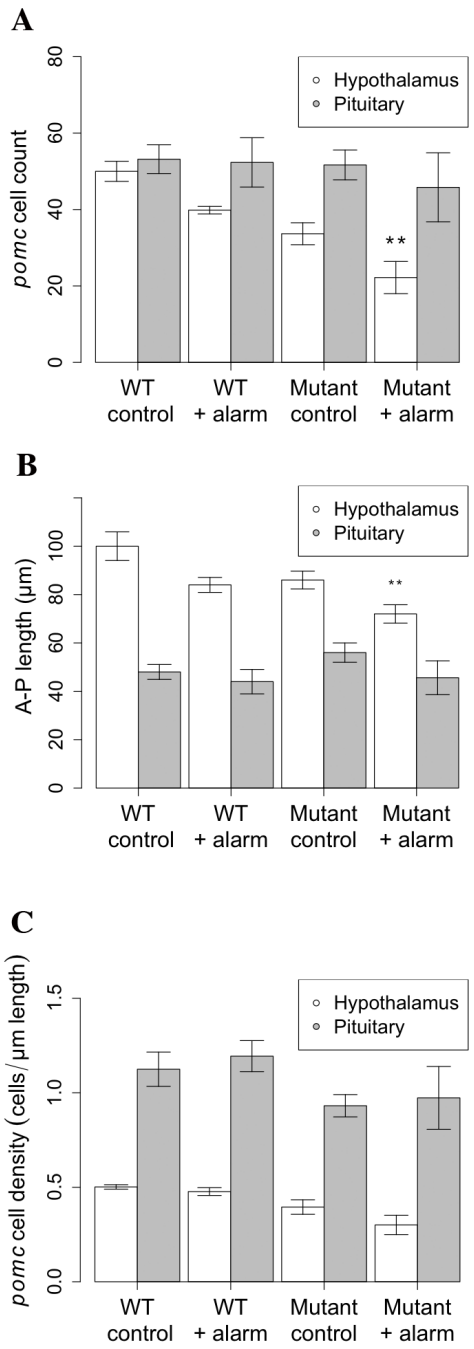

Figure 6.25. The effect of alarm pheromone (protocol 2) exposure on counts of *pomc* positive cells in *disc1* L115 5 dpf hypothalamus and pituitary. Points and bars represent mean ± standard error. WT, wild type in-cross larvae; mutant, homozygous mutant in-cross larvae. ** indicates significant difference from WT control hypothalamus at $p<0.01$. N = 6 per group, 1 technical replicate. See Table 6.6 for statistics.

(E) Mean count of *pomc* positive cells in L115 wild type mutant larval hypothalamus and pituitary, when exposed to alarm pheromone or control solution.

(F) Mean anterior-posterior length of L115 wild type mutant larval hypothalamus and pituitary, when exposed to alarm pheromone or control solution.

(G) Mean density of *pomc* positive cells in L115 wild type and mutant larval hypothalamus and pituitary, when exposed to alarm pheromone or control solution.

Table 6.6. Statistical analysis of *pomc* cell counts in *disc1* L115 5 dpf larval hypothalamus and pituitary after exposure to alarm pheromone (protocol 2). The results of two-way ANOVA tests and Tukey's Multiple Comparison test are shown. WT, wild type in-cross; mut, homozygous mutant in-cross; hyp, hypothalamus; pit, pituitary gland; A-P length, anterior-posterior length of region. * indicates a statistically significant difference at $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N= 6 per group, 1 technical replicate. See Figure 6.25 for plots.

Parameter	Factor	F value	d.f	p value	
Cell count	Genotype	9.67	1, 38	0.004	**
	Treatment	4.64	1, 38	0.038	*
	Region	18.37	1, 38	0.0001	***
	Genotype: treatment	0.24	1, 38	0.630	
	Genotype: region	3.83	1, 38	0.058	
	Treatment: region	1.35	1, 38	0.253	
A-P length	Genotype	1.20	1, 38	0.280	
	Treatment	11.66	1, 38	0.002	**
	Region	135.38	1, 38	<0.0001	***
	Genotype: treatment	0.12	1, 38	0.736	
	Genotype: region	7.45	1, 38	0.009	**
	WT hyp: mut hyp	-	-	0.048	*
	WT pit: mut pit	-	-	0.659	
	Treatment: region	1.53	1, 38	0.224	
Cell density	Genotype	10.70	1, 38	0.002	**
	Treatment	0.0	1, 38	0.959	
	Region	144.45	1, 38	<0.0001	***
	Genotype: treatment	0.21	1, 38	0.648	
	Genotype: region	0.46	1, 38	0.502	
	Treatment: region	1.16	1, 38	0.289	

In the mouse, *Rax* positive cells are thought to be progenitor cells, which are essential for normal development of the *Pomc* and *Sf-1* positive cell populations that usually occupy the arcuate and VMN respectively [51]. A reduction in expression of *rx3* in the *disc1* mutant might therefore suggest a reduction in the progenitor cell population, which would have consequent effects for development of the hypothalamus and establishment of the *sf1*- and *pomc*- defined VMN and arcuate nuclei. There was also a subtle reduction in expression of *rx3* in the retina of the Y472 mutant at 52 hpf and 3 dpf. Expression of *rx3* is essential for development of the eyes in zebrafish [50]. Although superficially the eye appears morphologically normal in mutant larvae, this data suggests that *disc1* could also play a role in eye development. Given the reduction of *rx3* expression in the *disc1* mutant larvae, I therefore asked whether *sf1* (a marker of the VMN), *crf* (a marker of the DMN) or *pomc* (a marker of the arcuate nucleus) are altered in *disc1* mutant larvae compared to wild types.

6.3.4 Expression of *sf1* in *disc1* larvae

The expression of *sf1* was increased in *disc1* mutant zebrafish between 52 hpf and 5 dpf, in terms of the size of the expression area and the signal intensity, particularly in the mid hypothalamus. No difference in expression was seen in the interrenal gland, which is inline with the normal baseline cortisol levels observed in the *disc1* mutant larvae. Evidence has suggested that *Sf-1* has a role in neuronal migration and is essential for normal development of the VMN [42], however, it is not clear what effect an upregulation of *sf1* might have. It is not clear whether the VMN itself is expanded or whether there are additional *sf1* positive cells outside of this region, because the boundaries of the hypothalamic nuclei are not well established at this early developmental stage. Analysis of multiple markers might help to uncover this.

6.3.5 Expression of *sf1* in the *disc1* adult brain

Expression of *sf1* in the adult zebrafish was detected in a small number of cells adjacent to the 3rd ventricle of the anterior hypothalamus. The pattern of expression differs from that seen in the mouse brain, in which *Sf1* is expressed more laterally in the well defined ventral-medial nucleus [42]. This nucleus has not

been described in the adult zebrafish brain and expression of *sf1* appears to be restricted more medially, to a region that has been described as the periventricular nucleus [47, 113, 114]. This region is reported to express *crf* and *crf* binding protein, where they play a role in hypophysial secretion [47]. Altered gene expression in this region suggests that *disc1* may play a role in the modulation of the HPI axis. The large amount of variation detected in the expression of *sf1* in the mutant hypothalamus suggests either an inability to regulate *sf1* or an abnormal development of this region. This could be tested in part by performing a late knock-out of *disc1* and subsequently analysing expression of *sf1*. As previously described, knock-out of *Sf1* in the adult mouse hypothalamus has detrimental consequences for the organisation of the VMN and consequences for energy homeostasis [56]. Altered expression of *sf1* in the *disc1* adult hypothalamus might suggest disorganisation of the hypothalamus, as hypothesised in the *disc1* larva, and could be responsible for the observed difference in adult body size, given the role of *sf1* in energy homeostasis.

6.3.6 Stress-induced changes in *sf1* expression

Acute exposure to a chemical stressor had no effect on the expression of *sf1* in the hypothalamus, as determined by *in situ* hybridisation. There was no previous evidence to suggest that *sf1* might be activated by a stressor, but I hypothesised that if *sf1* were upregulated by stress in the wild type larva, then increased expression observed in the mutant might suggest increased baseline stress levels. qPCR would have been a useful technique to determine if any more subtle changes in mRNA expression occur.

6.3.3 Expression of *crf* in *disc1* larvae

Expression of *crf* was detected in the *disc1* Y472 larval preoptic region and in the hypothalamus, as previously described [46]. Expression of *crf* appeared increased in the early *disc1* mutant hypothalamus in particular. When the HPI axis is activated by a stressor, *crf* mRNA is transiently upregulated [96, 105], presumably as it signals to the pituitary to release ACTH. If *crf* was permanently upregulated in the *disc1* mutant zebrafish, this might suggest increased activity of the HPI axis, however, an increase in expression of *pomc* and baseline cortisol levels was not

observed. This relationship could suggest an impairment in the regulation of a HPI circuit, in which *crf* fails to activate downstream HPI targets in the mutant zebrafish, and therefore a positive feedback loop signals for its increased expression. This hypothesis would require a more thorough investigation.

6.3.7 Expression of *pomc*

There were fewer *pomc* positive cells in the Y472 mutant brain at 5 dpf and in one analysis of 5 dpf L115 larvae, there were fewer *pomc* cells in the hypothalamus specifically, and this coincided with a smaller mutant hypothalamus. In this analysis, there was also a reduction in *pomc* cell density in the L115 mutant pituitary. These data suggest that the pituitary, as well as hypothalamus might be affect by the *disc1* mutation, which is unsurprising given their link. Increased *pomc* expression can be triggered by stress [96] and so a reduction in *pomc* expression might suggest a disregulation of the HPI axis. On the other hand, reduced *pomc* expression in the mutant hypothalamus was concurrent with a smaller hypothalamus, suggesting that growth and cell migration is affected.

6.3.8 Stress induced changes in *pomc* expression

Acute alarm pheromone exposure had no effect on expression of *pomc* in 5 dpf larvae. Previous studies have demonstrated that expression of *pomc* was upregulated after adult zebrafish were exposed to a vortex stressor after 60 minutes [96]; it is possible that the 10 minute exposure in my experiment was not sufficient to trigger a transcriptional response.

6.4 Concluding remarks

Work in mice has shown that a loss of *Rax* results in a loss of *Sf-1* and *Pomc* positive cells in the hypothalamus. In the *disc1* mutant zebrafish, *rx3* and *pomc* expression appeared reduced in some cases, yet expression of *sf1* was increased. An increase in *crf* and *pomc* expression is seen in stressed fish, concurrent with an activation of the HPI axis [96, 105]. Whilst an increased number of *crf* positive cells was observed, there were fewer *pomc* positive cells in the *disc1* mutant hypothalamus, which is not indicative of increased HPI activity. Taken together, these data suggest an imbalance of cell types in the developing hypothalamus. A

deregulation of cell populations in the early hypothalamus could have powerful effects on the development of this region and wide-ranging functional consequences, given the key role of the hypothalamus in regulating homeostasis.

7 General discussion

7.1 Summary of results

Given the evidence from mouse models that *DISC1* modulates the stress response [7], its expression in the hypothalamus [40, 41, 110] and its documented roles in neuronal development [5], I hypothesised that mutation in *disc1* would cause abnormal behavioural and endocrine responses to stress, mediated at least in part by altered hypothalamic development. I have carried out an initial investigation of this hypothesis using the two lines of *disc1* mutant zebrafish, L115 and Y472, at both larval and adult stages. My data show that *disc1* mutants exhibit hypomotility, reduced endocrine and behavioural responsiveness to acute stress and altered expression of key developmental and HPI-related genes in the hypothalamus. These findings suggest that *disc1* mutation modulates the HPI axis in zebrafish, at least in part via altered development of the hypothalamus.

7.2 Use of the zebrafish model

The zebrafish offers ease of genetic manipulation, a variety of quantifiable behaviours and a relatively simple system for neurodevelopmental studies. This makes the zebrafish an attractive system, in which behavioural phenotypes can be correlated with molecular and cellular changes caused by genetic variation. In contrast to laboratory mouse lines, which are strongly inbred, and therefore exhibit more limited phenotypic variability, laboratory zebrafish are typically outbred. This expansion of the gene pool means that zebrafish exhibit large amounts of phenotypic variation, both between strains and within strains. The diverse genetic background means that siblings carrying the same mutant genotype can exhibit large individual differences. Arguably, this makes them a good model system for human phenotypes and complex trait analyses.

This phenotypic variation is apparent in my analyses in which variation, particularly in behaviour, was observed in fish of the same *disc1* genotype, between offspring cohorts that were produced from the same parent group, between offspring cohorts produced from different parent groups (e.g. different generations) and also within individual cohorts. The first scenario is relevant to all

analyses that utilised larval zebrafish, in which each technical replicate represents a different 'batch' of offspring, which were produced from the same parent group but on a separate occasion. These offspring were obtained via the marbling technique, and so although the parent pool was identical for each batch, the contribution of individual parent fish to the offspring batch will likely vary. This genetic variation is likely responsible for variation observed in some measures, such as the number of *pomc* positive cells at 5 dpf, and suggests that the interaction of *disc1* with other background genes, or with small environmental differences, modulates phenotypes. In addition, each offspring batch was raised and analysed at a separate time point, and so although rearing conditions were controlled, it is possible that some environmental differences also occurred, and contributed to phenotypic differences between batches. The environmental parameters that could have inferred small changes between experiments were temperature, humidity, swimming medium, frequency of disturbance.

The second scenario, variation in offspring from different parent groups, was detected in behavioural assays utilising different generations of adult zebrafish. Each generation was produced by parents that were outcrossed to different wild type groups, meaning that the genetic background of each cohort was different and relatively diverse. An example of the contribution of background genetics to phenotypic differences can be seen in analyses of different zebrafish strains, which exhibit significant differences in behaviour [81, 116, 117]. A different example of this type of variation in my data set was in the comparison of wild type and homozygous mutant larvae that were obtained via an in-cross of wild type or homozygous mutant fish respectively, versus sibling wild type and homozygous mutant larvae that were obtained via an in-cross of heterozygotes. Mutants from each cross exhibited hypomotility in the basic locomotion assay, but far fewer animals from the heterozygous in-cross were required to detect a statistically significant difference between genotypes in this parameter, relative to the other cross. This increase in strength of the mutant phenotype in the sibling comparison is likely due to them being relatively more outbred. Outbreeding is used to 'purify' desired genotypes induced by ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis, as in the *disc1* fish, by removing additional unwanted mutations.

Variation between individuals of the same cohort was also apparent, particularly in behavioural assays, which are notorious for high levels of variation [118]. Inter-individual differences in zebrafish activity have been previously reported [116, 119]. One study reported that females demonstrate a higher level of consistency than males and that some inter-individual differences in behaviour (distance swam and bottom dwell) were stable over time and in different testing environments, suggestive of so-called personality traits [16]. These data suggest that the reported individual differences in behaviour were caused by genetic variation, rather than environmental change, in this case a different testing tank or prior exposure. In contrast, some behaviours are known to be context-dependent [1, 79]. The interaction of the *DISC1* mutation with the environment means that environmental control is critical for experiments, but controlling the environmental context of fish prior to behavioural analysis can be difficult.

7.3 Linking behaviour with molecular changes

One of the difficulties in behavioural science is linking behavioural phenotypes with molecular and cellular changes. It has been postulated that hundreds or perhaps thousands of genes might interact in order to generate a given behaviour [119], although it has also been demonstrated that individual genes can provide a large contribution to specific behaviours, for example the fibroblast growth factor receptor 1A (fgfr1a) in aggression [120].

In my data, an impaired cortisol response to stress correlates with an impaired behavioural response in the *disc1* mutant. Although evidence shows that stress exerts significant effects on behaviour, these effects are likely mediated through the action of numerous hormones. Until recently the direct effect of cortisol on behavior has been difficult to test. The recent development of optogenetic tools to manipulate neuronal activity in living animals [106] represents an exciting method via which the neural circuits underpinning zebrafish behaviour can be investigated. Recently, optogenetically enhanced cortisol levels were shown to increase locomotion in zebrafish [106]. This data is supported by the evidence that cortisol mobilises energy [15], which will allow for the increased demands of

kinetic energy that can be associated with stress. In my data set, wild type larvae exposed to the alarm pheromone and NaCl stressors exhibited an increase in cortisol levels and a concurrent reduction in activity, as determined by swimming speed. This result suggests that the action of hormones other than cortisol, or other neurological changes are responsible for the observed reduction in activity in response to stress.

Both a reduction in swimming activity [22, 105, 121] and an increase in swimming speed have been previously reported in response to stress [80], and this likely depends on the stressor. The behavioural stress response has not been widely studied in groups of individuals and thus the effect on polarization has not been previously reported. Zebrafish group swimming behaviour can be categorized as shoaling or schooling, and fish will move from one to the other depending on context [122]. Shoaling can be defined merely as aggregation of individuals, whilst schooling can be defined by higher polarization, higher swimming speed and reduced density, and is thought to infer anti-predatory advantages which may carry metabolic and attentional costs [122]. The functional significance of the change to a less dense, less polarized and slower group by 5 dpf wild type larvae upon exposure to alarm pheromone in my experiments is unclear, but suggests a clear developmental difference in anti-predatory behaviour.

7.4 Zebrafish *disc1* mutation

A key problem with the use of the here-described zebrafish mutants is the undetermined functional effect of the mutations. The L115 and Y472 point mutations both code for stop codons in the *N*-terminal domain of the polypeptide, a region which is not well conserved. A lack of zebrafish-specific *disc1* antibodies has meant that determining whether or not the mutants are null alleles is difficult, although preliminary Western blots suggest that some protein is synthesized. Utilization of the CRISPR/Cas9 system to delete the conserved arginine-rich motif (ARM) of the *N*-terminal domain, the conserved coiled-coil domain or even the entire gene would allow for validation of the described effects of *disc1* mutation on behaviour, endocrine function and hypothalamic development.

7.5 DISC1 alters hypothalamic development and functions in cell fate decisions

Previous DISC1 GXE models have revealed impaired behavioural and endocrine stress responses in DISC1 mice [38, 123], and demonstrated a mechanism in which elevated stress-induced corticosterone levels in the GXE mouse are responsible for alterations in epigenetic control of dopaminergic neurons and behavioural abnormalities [123]. However, these studies do not directly address how DISC1 interacts with the HPA axis to cause the impaired endocrine stress response in GXE mice. Given the previously described wide-ranging roles for DISC1 in neuronal development [5, 32], its effects on the HPI axis could be equally wide-ranging. Based on the initial data discussed in this thesis, I can only speculate as to the mechanism(s) via which *disc1* might interact with the HPI axis to modulate the stress response in zebrafish.

A key question is whether the gene expression changes detected in the *disc1* mutant hypothalamus constitute developmental changes rather than reflecting dynamic changes in gene expression, for instance as a result of abnormal responses. There are three mechanisms that could be responsible for the observed alteration in the expression of *sf1,* for example, in the *disc1* mutant hypothalamus. One is that the mutated disc1 protein (truncated or absent) signals for additional *sf1* positive cells to be specified, thereby expanding this region of the hypothalamus. The second is that the mutated protein signals to additional cells to start expressing *sf1*. The third is that mutated disc1 protein signals to *sf1* positive cells to upregulate *sf1* expression. Although my studies do not directly distinguish between these, a number of lines of evidence favour the idea that the *disc1* mutation results in aberrant hypothalamic development. First, recent work in zebrafish has shown that the morphogen sonic hedgehog (*shh*), which is required for hypothalamic development [124, 125], is upstream of both *disc1* [115], and the transcription factor *rx3* [21]. Recent studies show that *rx3* interacts both positively and negatively with shh to govern hypothalamic progenitor cells and their differentiation [21]. Together with my observations that *disc1* and *rx3* co-localise in the hypothalamus and that *rx3* is reduced in the *disc1* mutant, this suggests that *disc1* is functioning downstream of *shh* but upstream of *rx3* and leads me to

hypothesise that *disc1* modulates the HPI axis via a direct or indirect interaction with *rx3* (Figure 7.1).

In the mouse, the *rx3* homologue, *Rax,* is implicated in the differentiation of tuberal progenitor cells into three separate mature neuronal populations including *sf1* neurons in the VMN, *pomc* neurons in the Arc and *otp (orthopedia)*/*dlx* positive neurons in the DMN (Figure 1.4) [14]. The gene regulatory networks that govern the differentiation of *Rax* progenitors appear to be conserved in the zebrafish [14] and studies show that, as in mouse, *rx3* is required for the differentiation of neurons in the tuberal hypothalamus, including *sf1* and *pomc* neurons [43, 126]. Together, these studies suggest that dysregulation of *rx3* could have wide-ranging effects on hypothalamic development and hence function. In addition to *sf1* and *pomc*, my studies reveal that the numbers of *crf* positive cells are increased in the *disc1* mutant hypothalamus. Studies in other vertebrates suggest that the *crf* neurons of the paraventricular nucleus are specified from a separate pool of progenitor cells in the anterior hypothalamus [14]. *Rax* has not been previously implicated in this pathway, suggesting that *disc1* is involved in the differentiation of separate progenitor cell populations, or suggesting a novel role for *Rax* in anterior hypothalamic progenitor cell differentiation.

In mice and zebrafish a loss of *Rax*/*rx3* progenitor cells results in downregulation of both *sf1* and *pomc* [43, 51, 126]. My results suggest that a reduction in *rx3* leads to an increase in *sf1* expression, suggesting a previously unrecognised aspect of its control. More experiments are needed to uncover the mechanism behind this apparent paradox but one possibility is suggested by the finding that *rx3* downregulation is needed for the further differentiation of *rx3* progenitors [43] (Figure 7.2). Potentially, then, the low levels of *rx3* in the *disc1* mutant fish may lead to two separate effects: (a) an increased differentiation of *rx3* positive progenitors into *sf1* positive cells, and then (b) a reduction in numbers of progenitors, and hence a reduction in *pomc* positive cells. This mechanism might explain both the upregulation of *sf1*, and concurrent downregulation of *pomc* in the *disc1* mutant.

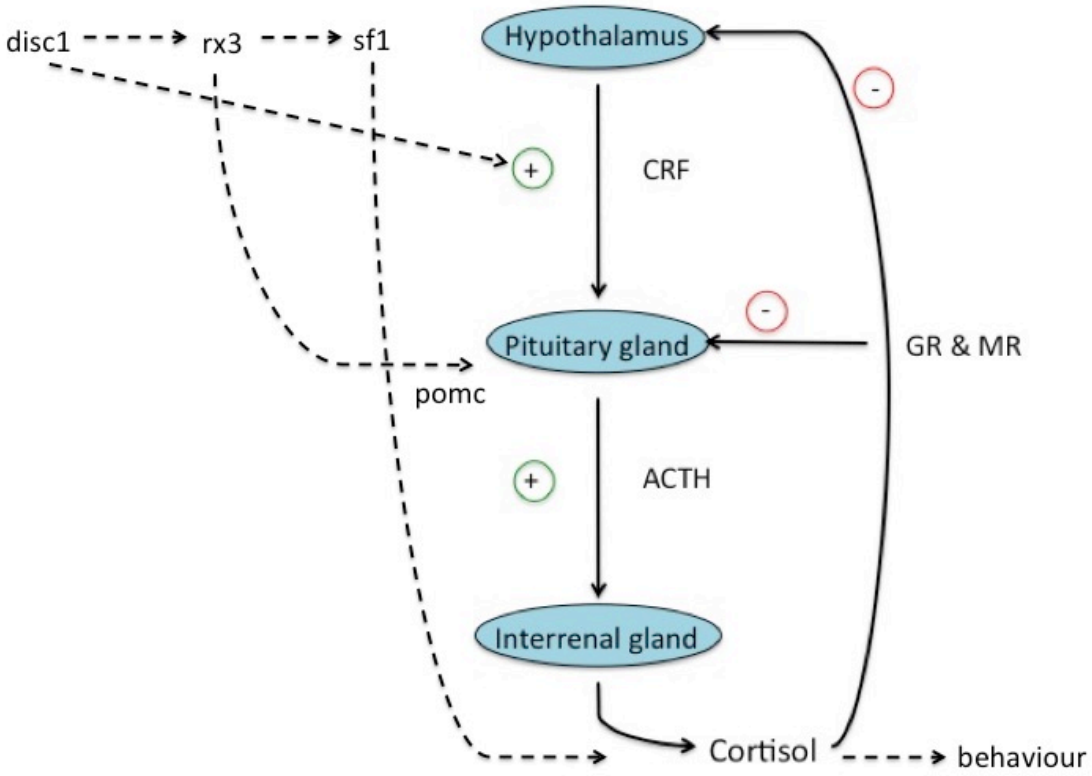

Figure 7.1. Schematic of possible pathways via which *disc1* might modulate the HPI axis. Figure adapted from [1]. *disc1* likely modulates expression of *sf1* and *pomc* via *rx3*. Whilst the role of *pomc* in the HPI axis is known, a potential role for *sf1* has not been identified. *disc1* also appears to modulate *crf*, either directly or indirectly, which is a key component of the HPI axis. Solid lines indicate well-established interactions of the HPI axis. Dashed lines indicate speculative and/or uncharacterised interactions. CRF, corticotropin releasing factor; ACTH, adenocorticotropic hormone; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; +, positive signals; -, negative feedback.

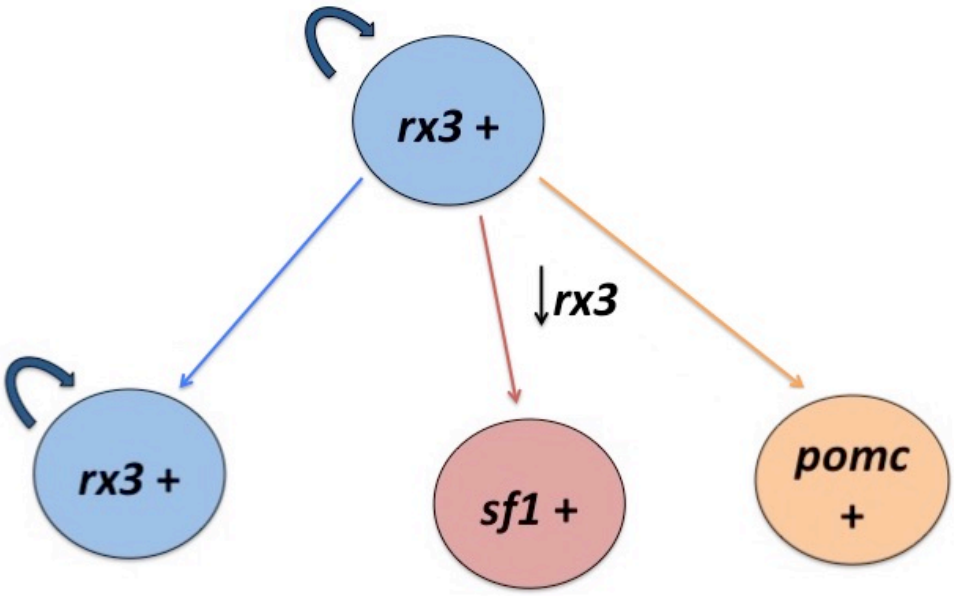

Figure 7.2. Schematic of possible mechanism via which *rx3* might specify *sf1* and *pomc* cells in the hypothalamus. *rx3* positive progenitor cells may divide and give rise to more *rx3* positive progenitor cells, or, via a downregulation of *rx3*, differentiate into *sf1* positive and *pomc* positive neurons of the ventral-medial nucleus and arcuate nucleus respectively. *sf1* positive neurons are likely specified earlier than *pomc* positive neurons.

In support of a role for *disc1* in cell fate decisions, a recent study implicated DISC1 in the transport of specific mRNAs in dendrites [127]. A short (15 amino acid) arginine-rich motif in the *N*-terminal region of DISC1 was shown to be essential for mRNA binding [127]. This region is the only part of the *N*-terminal domain of the protein that is conserved, thereby suggesting that it is critical for normal DISC1 function. The transport of mRNA has been shown to be important in neural cell fate decisions [128, 129], suggesting that DISC1 might effect cell fate decisions via altered transport of mRNA granules. Based on these data, I hypothesise that *disc1* effects development of the hypothalamus via an alteration in cell fate decisions.

7.6 Increased hypothalamic expression of *sf1* does not appear to directly correlate with altered stress

There is abundant evidence demonstrating the importance of SF1 in development of the adrenal and interrenal glands [55, 58, 61]. Interestingly, work in *Sf1* heterozygous mice, which have small adrenal glands, has shown that the role *Sf1* in adrenal development is independent of the HPA axis, and that the reduced expression in the adrenal gland is solely responsible for the impaired stress response in these mice [130]. Despite reduced expression of *Sf1* in the heterozygous mouse, the hypothalamic-pituitary response to stress is normal, however, the architecture of the VMN was not analysed. These data suggest that altered expression of *sf1* in the hypothalamus is not directly responsible for the impaired stress response in the *disc1* mutant zebrafish, but may be an artifact of an upstream mechanism which causes more widespread alterations to hypothalamic development. The posterior hypothalamus is known in mouse and fish to harbor progenitor and stem-like cells [131]. The observed altered expression of *sf1* in this region may therefore suggest more general changes in neurogenesis and development of the hypothalamus, with consequences for wider disruption of the HPI axis in the *disc1* mutant. Impaired development of the hypothalamus and HPI axis may explain why *disc1* mutant larvae appear desensitized to stress, in terms of their endocrine response.

7.7 DISC1 as a GXE model

My findings suggest that *disc1* modulates the stress response in zebrafish, supporting previous studies in mice that propose that *DISC1* is a good GXE model. In one study, mice expressing mutant human *DISC1* were hyper-responsive to a stressor and this was mediated by epigenetic control of gene expression [7]. Whilst in another study, mutant *DISC1* mice exposed pre-natally to poly I:C infection showed a reduced endocrine response to the stressor, suggesting possible genetic control of sensitivity to the environment [38]. These studies, along with my data, demonstrate genetic moderation of individual susceptibility to the effect of the environment, albeit by seemingly varied pathways.

Evidence for the involvement of epigenetic mechanisms in stress-induced changes in gene expression, endocrine function and behaviour is well established in rodent models [132] and in human studies [133], but has not yet been investigated in the zebrafish. *DISC1* has been implicated in the control of *TH* gene expression via DNA methylation in GXE mice [7] and it has been previously demonstrated in mice that a vast array of different genes are epigenetically altered by stress [132]. It would therefore be relevant to investigate a possible epigenetic control of gene expression by *disc1* in the zebrafish.

A potential weakness in the mouse GXE field is the use of behavioural tests and stressors that are not particularly species relevant [3]. When testing models for a disease such as schizophrenia, examination of behaviours such as PPI, which are disease-relevant [36], are obviously meaningful, whilst evaluation of the natural murine behavioural repertoire will also be beneficial. Similarly the analysis of stressors linked to schizophrenia such are infection is clearly valuable, but the combination of such clinically-relevant stimuli alongside species-specific stressors, as the alarm pheromone is to the fish, might provide more accurate and sensitive analyses of GXE models [3]. Exposure to a live predator or predator cues in zebrafish has benefits over other common lab techniques to stimulate stress as it has etiological relevance.

Sex differences appear to be less well recognized in the mouse field and indeed only one of the *DISC1* GXE models analysed both sexes [134], but did not mention any difference. Mental illnesses such as schizophrenia and depression are reported to have gender-biases [35]. My analyses revealed a sex difference in the behavioural response of wild type fish to a stressor, although no differential effect on mutants was observed. This suggests that the concerned pathways might be modulated by sex hormones. Sex-dependent interactions should be considered in further analyses.

The zebrafish *disc1* model strongly complements the GXE *DISC1* mouse work. The mutant fish do not show any strong phenotypes under baseline conditions, therefore avoiding any ceiling effect, allowing for an accurate analysis of an environmental interaction. The availability of the two different mutant lines and exposure to multiple different stressors allows for a comprehensive analysis of GXE interactions. A reporting of negative results will be of vital importance in this field, in which it will be important to know under which circumstances no adverse phenotypes emerge.

The *disc1* mutant zebrafish demonstrated a 'muted' response to stress, in that whole body cortisol levels were barely elevated and the behavioural response was also reduced in comparison to wild types. Muted responses to stress are also reported in mental illness [2], and although excess cortisol is a risk factor in humans for the development of many diseases, both cardio-metabolic and psychiatric, the endocrine response of human psychiatric patients to stress has not been studied in detail. The implications of an inability to elevate cortisol levels in the human are not entirely clear, although the muted behavioural response to the alarm substance stressor in zebrafish may have fitness consequences.

7.8 Implications

Stress related conditions place a massive burden on individuals, society and the National Health Service. Understanding the mechanisms via which responses to stress are modulated by genetic factors will be important for the design of therapeutics and preventative measures.

Thus far, GXE studies have focused on the analysis of genetic risk factors of human diseases. But the study of protective genetic factors and environmental resilience will also be useful in identifying novel molecular targets and new therapeutic interventions [3]. Additionally there is a growing appreciation that the emergence of many psychiatric illnesses might be due to the combined action of many genes of small effect, suggesting that the investigation of multiple factors in the same model will also be useful. A key question is how the *DISC1* mutation leads to the manifestation of different psychiatric disorders. This can be investigated by comparing phenotypes after exposing the different *DISC1* animal models to the same environmental factor, and conversely exposing each *DISC1* model to various different environmental factors.

Inducible genotypes will also be useful in testing whether phenotypes are reversible. One *DISC1* GXE study demonstrated that turning off expression later in development led to elimination of the adverse behavioural phenotypes [38], encouraging the idea that phenotypes, that are neurodevelopmental in origin, might be removable via therapeutic intervention. It is not possible to replicate the defining features of a disease like schizophrenia, such as hallucinations and delusions, in animal models, but modelling brain circuitry, cellular and molecular phenotypes associated with disease can provide mechanistic insight [3].

7.9 Concluding remarks

The work presented in this thesis describes a zebrafish gene-environment interaction model, which operates via a novel mechanism. Mutant *disc1* zebrafish have impaired behavioural and endocrine responses to stress, which are likely mediated by abnormal hypothalamic development. This novel zebrafish model will complement traditional mammalian models and has potential to mediate a much deeper understanding of the molecular mechanisms via which psychiatric disease manifest themselves through the interplay between genes and environment. These studies provide insight into a previously undescribed role for *disc1* in hypothalamic development and the resultant behavioural and endocrine abnormalities. The zebrafish research field is developing rapidly, and the

combined force of emerging and future innovations in genetic and molecular techniques with advances in behavioural methodology and technology will play a role in the understanding, treatment and prevention of psychiatric illness.

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