Published in final edited form as:

Rev Neurosci. 2011 ; 22(1): 49–62. doi:10.1515/RNS.2011.007.

Stressing Zebrafish for Behavioral Genetics

Karl J. Clark*, Nicole J. Boczek, and Stephen C. Ekker

Mayo Clinic, Department of Biochemistry and Molecular Biology, Rochester, MN 55901

Synopsis

The stress response is a normal reaction to a real or perceived threat. However, stress response systems that are overwhelmed or out of balance can increase both the incidence and severity of diseases including addiction and mood and anxiety disorders. Using an animal model with both genetic diversity and large family size can help discover the specific genetic and environmental contributions to these behavioral diseases. The stress response has been studied extensively in teleosts because of their importance in food production. The zebrafish (*Danio rerio*) is a major model organism with a strong record for use in developmental biology, genetic screening, and genomic studies. More recently, the stress response of larval and adult zebrafish has been documented. High-throughput automated tracking systems make possible behavioral readouts of the stress response in zebrafish. This non-invasive measure of the stress response can be combined with mutagenesis methods to dissect the genes involved in complex stress response behaviors in vertebrates. Understanding the genetic and epigenetic basis for the stress response in vertebrates will help to develop advanced screening and therapies for stress-aggravated diseases like addiction and mood and anxiety disorders.

Introduction

The stress response is an adaptive reaction to a real or perceived threat, or stressor. The role of the response is to maintain or restore normal balance or homeostasis. When homeostasis is threatened by a stressor, a diverse suite of neuronal, endocrine, and autonomic response mechanisms can be utilized to re-establish homeostasis. This process of changing to stay the same has been referred to as allostasis [130]. A normal stress response is required for healthy living. However, intense acute, repetitive, or chronic stress may overwhelm the ability to regain homeostasis leading to overloaded or imbalanced stress pathways. The biological cost or burden of responding to stressors, allostatic load [90], can occur over time and leads to an increase in disease incidence and severity- including but not limited to cardiovascular disease [58], immunosuppression [38], weight-gain and metabolic disease [101], memory loss and dementia [48, 144], aging [45, 117], addiction [87, 145] [22, 124], and mood and anxiety disorders [69] [47].

The clinical significance of stress-aggravated disorders is extremely high. In particular, the stress-induced changes to brain function that contribute to addiction and mood disorders impact some of the major causes of death and disability in the world. Smoking and alcohol use are the single and third largest preventable causes of death in the United States, respectively [93]. About one in every four people suffers from a diagnosable anxiety, mood, impulse, or substance abuse disorders each year [79]. The worldwide disease burden of neuropsychiatric disorders nearly matches that of cardiovascular disease and cancer combined [146]. Specifically, major depressive disorder is projected to be the leading cause of disability worldwide within the next twenty years [146]. Understanding the central role of

*Corresponding Author: Karl J. Clark, Dept of Biochemistry and Molecular Biology, Guggenheim 13, Mayo Clinic, 221 4th Ave. SW, Rochester, MN 55902, clark.karl@mayo.edu, 507-538-5311.

stress in the onset and perpetuation of disease will help alleviate the prevalence and severity of addictive and mood disorders in the future.

Genetic variations within the human population along with life experiences make people more or less susceptible to the debilitating effects of stress. The portion of phenotypic variance (e.g. anxiety, mood, or addiction disorders) that is attributable to genetic variation within the population is often referred to as heritability. On the contrary, the remainder of the phenotypic variance is attributable to the environmental variation of the population. The heritability of both major depressive disorder (MDD) and anxiety disorders is predicted to be a minimum of 30–40% [65, 131]. However, those values increase to ranges of 50–80% in longitudinal studies that represent reliable diagnosis of disease [49, 76]. Therefore, disease onset is dependent on both heritable (genetic) and environmental (epigenetic) factors.

Many of the genetic effects, especially those associated with stress response and coping mechanisms, may be shared across neuropsychological disorders. Swedish National Twin Samples show that the heritable or genetic predisposition for MDD and generalized anxiety disorder (GAD) are indistinguishable in females and about 74% shared in males [74]. The shared genetic predisposition (74% in males and 100% in females) of MDD and GAD indicates that the genetic basis of these two diseases is highly shared and the onset of one or the other is dependent primarily on environmental cues. Indeed, the two diseases are often somewhat difficult to distinguish in some patients [143]. Addiction shares comorbidity with psychiatric disorders like MDD and GAD, often being diagnosed in the same patient [59, 72, 123]. The genetic predisposition to addiction within the population, is similar to MDD and GAD, with heritability scores of ~72% and 50–60% for nicotine and alcohol dependence, respectively [75, 77, 78]. The genetic influence and comorbidity of addiction with mood and anxiety disorders suggests some shared causes- either genetic, environmental, or a combination of the two.

Understanding the role of the stress response in the onset and progression of addiction and mood disorders will permit advanced screening for indicators of disease potential and the development of new therapies. Indeed, developing genetic or even epigenetic tests is a goal of individualized medicine wherein each person may seek care and therapeutic intervention tailored to their own genetic background. The gene and environment interactions of the stress response and its role in disease progression are dependent on heritable genetic factors, life priming events (epigenetics), and later life events that trigger disease onset [37]. Life-priming events occur during critical time periods that are especially plastic and responsive to environmental cues to set future response thresholds; in mammalian models this includes gestational, early-childhood, and adolescent time periods [88, 89].

The biological tissues and pathways that contribute to a successful stress response are diverse and numerous. Unbiased forward genetic screens that make no assumption about what pathways are involved in the process of stress response make it possible to discover the involvement of genes in both suspected and unknown or unsuspected pathways. These systems can study the genetic or epigenetic contribution to multigenic disease. Quantitative genetic approaches in animal models with moderate genetic diversity are ideal tools to uncover small contributions of individual genes to overall disease progression [81, 85]. Therefore, using a cost-effective genetic model organism to study the stress response will allow substantial insight into the proper functioning of stress response systems and potential avenues of dysregulation.

Stress axis dysregulation impacts the balance of core components of the hypothalamic-pituitary- adrenal (HPA) axis. Corticotropin releasing factor (CRF) is secreted by the hypothalamus following perception of a stressor. CRF activates the pituitary to release

adrenocorticotrophic hormone (ACTH) and ACTH travels through the circulation to the adrenal glands to stimulate the release of the glucocorticoid-cortisol. In addition to direct mutagenesis of these core HPA gene products, gene dosages and an individual's complement of genetic modifiers can together shift the delicate balance of the stress response and related hormones in the body to create genetic predispositions to stress-related diseases. Two documented examples of genetic influence on stress and disease are the serotonin transporter gene (5HTT) and the corticotropin releasing factor receptor 1 gene (CRHR1). Both genes have alleles that affect gene dosage, or the amount of protein produced, but not protein function. The resulting changes in protein concentration of these genes influence the likelihood of an early life stressor inducing depression in adulthood [28] or stressor induced alcohol relapse in a rat model [64], respectively. Behavioral disorders like addiction, major depressive disorder, and general anxiety disorder, are complex multi-system diseases that require whole animal models to be effectively assessed [113, 127].

The teleosts equivalent of the HPA, the hypothalamic-pituitary-interrenal (HPI) axis, and the stress response have been studied in fish for over 20 years with a frequent goal to improve aquaculture conditions and avoid production losses in these important food commodities [13, 73, 118]. The primary organs of the HPA axis - the hypothalamus, pituitary, and adrenal cortex - serve similar functions and structure in fish with the exception that the adrenal gland is instead made up of interrenal cells (adrenal cortex) that intermingle with chromaffin cells (adrenal medulla). These cells are loosely associated with the posterior cardinal vein and the anterior portion of the kidney, referred to as the head kidney [53]. Beyond anatomy, higher-order behaviors including dominant and submissive behavior [32, 50, 83, 106, 126], aggression [67, 96], displaced aggression [97], and alternate coping styles [98, 104, 106, 116] are clearly evident in numerous fish species. The core genes involved in the stress response are conserved within teleosts [3, 5]. However, like their mammalian counterparts, most of these "large" fish species have little or no genetic tractability for mutagenesis studies to identify genes involved in the perception, propagation, proper attenuation, or epigenetic modifications of the stress response.

The zebrafish (*Danio rerio*) is a major model organism with a rich assembly of methods and tools to study the developmental, physiological, and genetic basis of the stress response. The high fecundity and genetic diversity of the zebrafish provides a platform to discover gene mutations that quantitatively affect the stress response in larval and adult fish. Longitudinal studies in zebrafish will help to elicit the mechanisms by which early life experiences impact adult phenotypes. In the past four years, considerable efforts have been made to make initial descriptions of the stress response in the zebrafish. Here we will review these advances as well as current methods of genetic screening in zebrafish that will promote our understanding of the subtle interplay between genes and environment focusing on stress, the neuroendocrine system, and behavior.

Measuring the Stress Response

Stressors

There is a rich history of studying stressors and the stress response in teleosts (for review, [13, 50, 73, 84, 118]). Physical stressors- including handling, capture, net chasing, and other physical disturbances- are among the most common acute stressors in the laboratory. In addition, fish crowding and confinement are partially physical stressors that can be effective as both acute and chronic stressors. Social stressors include dominant and submissive interactions among fish. Many fish are highly social in nature and interact closely with shoals of conspecifics and heterospecifics, fish of the same species or differing species, respectively. Crowding and isolation impact these social interactions making them both social stressors in addition to physical stressors. Environmental stressors include water

quality measures like dissolved oxygen, nitrogen cycle, hardness, pH, temperature, and salinity as well as heavy metals and xenobiotics like insecticides, herbicides, and pharmaceuticals. In addition, infection and pathogens can act as a stressor, yet chronic stress can lead to a reduction in immunity and an increase in infection rates [39].

Zebrafish are native to the floodplains of India, where they are subject to rapid changes in water movement and qualities during the monsoon season when the majority of reproduction occurs [44, 63, 129]. The ability of larvae and fish to adjust to these environmental variables is due in part to endocrine responses to these stressors. Several stressors have been directly tied to an increase in cortisol production (see Table 1). Alsop and Vijayan were able to effectively elicit a larval stress response by swirling embryos for 30 seconds [4], a mild physical stress that represents temporary loss of control of the resting embryo after traffic through the waterway or changes in water currents. Alderman and Bernier used a hyperosmotic shock to stimulate cortisol production in larval zebrafish [1], the ability to adjust to various salinity conditions is important to zebrafish which although a freshwater species, can acclimate to brackish water. In adult zebrafish, a cortisol response from fish handling, tank transfer, or water and shoal disturbance has been demonstrated [105, 112]. Overcrowding [111] and turbulence from heavy aeration of a bubbler [137] effectively elicit a stress response in adult zebrafish. Predators and alarm pheromone cause a strong behavioral response in many fish species including zebrafish. Barcellos et al. measured the cortisol response in the presence of a predator and with visualization of the predator [10]. This is consistent with the significant behavioral work using predators and alarm pheromone in zebrafish (for review, [56]). Measuring cortisol production in response to a novel tank has demonstrated that zebrafish are more susceptible to this novelty stressor when going through drug withdrawal [24] and antidepressants/anxiolytics like fluoxetine can reduce cortisol production and alter the behavior indicating less anxiety to the novel tank [43]. Environmental toxicology studies in zebrafish are rapidly increasing, as small laboratory friendly fish are viewed as good models of both acute and latent toxicology [27, 66]. As demonstrated by Craig et al., toxicants like waterborne copper induce strong cortisol production [33]. Thus, stress responses in zebrafish can be reproducibly triggered by a number of stressors in the laboratory under controlled conditions.

Physiological Measures of the Stress Response

Two of the major physiological changes that occur during a stress response are the activation of the sympathetic nervous system resulting in release of epinephrine and activation of the HPA axis releasing cortisol from the adrenal glands. The release of catecholamines (primarily epinephrine) from chromaffin cells (adrenal medulla) is a rapid response occurring within seconds of the perceived stress. In contrast, the signaling cascade that releases corticotropin releasing factor (CRF) from the hypothalamus to release adrenocorticotropin hormone (ACTH) from the pituitary into the bloodstream to activate the interrenal cells (adrenal cortex) to release cortisol is a slower process generally occurring within minutes rather than seconds. The timing of these two responses enables one to rapidly collect plasma or tissue without handling stress associated with the collection process interfering with cortisol measurements, but direct measurement of epinephrine requires the use of cannula. When catecholamine release has been studied in teleosts (for review, [102]), changes in catecholamine are not generally observed following light or moderate stressors. Cortisol release however is generally responsive to a wide range of stressors [141]. Other metabolites, like glucose are sometimes used as an indirect measurement of the activity of epinephrine and cortisol on the system. For example, Cofiel and Mattioli have used glucose as a measure of confinement stress in adult zebrafish [31].

Cortisol measurements in mammals can be obtained from saliva, plasma, or urine by radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), or gas chromatography-

mass spectrophotometry [68]. In larger teleosts, blood can be collected for measuring cortisol in the plasma. Unfortunately, the amount of plasma available in developing fish and smaller fish species like the zebrafish precludes routine detection of cortisol by these standard methods. Consequently, an alternative method to measure cortisol has been implemented for these small fish or larvae. For example, the whole-body cortisol assay [36] extracts cortisol from all of the fish tissue. Measurements of cortisol in both adult [10, 24, 33, 43, 105, 111, 112, 137] and larval zebrafish [1, 4] have used whole-body cortisol measurements (See Table 1). One potential drawback to using whole-body cortisol or even total cortisol measurements in plasma is that this approach does not account for the bioavailability of the cortisol that is measured. In mammals, the vast majority of circulating cortisol is bound by proteins, with about 6–13% of the circulating cortisol freely available for signaling [54]. Although not well studied in teleosts, the percentage of cortisol that is bioavailable under non-stressed conditions ranges from 10–45%, and that percentage increases significantly following an acute stressor [11, 25, 46, 70]. The amount of bound cortisol in teleosts is significantly lower than in mammals. This discrepancy may result from the lack of a true ortholog to the primary protein that binds cortisol in mammals, corticosteroid-binding protein or transcortin, in teleosts fishes. [11, 25, 46, 70] Therefore, measuring total plasma cortisol or whole-body cortisol is an effective tool. Although these measurements may underestimate the cortisol response to stress because the assays are unable to distinguish changes in the bioavailability of cortisol, the impact is far less significant in teleosts fishes relative to mammals. Free cortisol and other steroids travel across the gills to and from the water [119]. This permits non-invasive, multiple measurements of cortisol release over time. For smaller fish this allows non-lethal measurements of free cortisol. This technique has not yet been applied to the zebrafish, but it has been successfully implemented on a similarly sized fish, the three-spined stickleback [121]. Unfortunately due to the much smaller levels of cortisol in larval fish, measuring free cortisol release into the water from individual larvae or even groups of larvae may not be possible.

Behavioral Measure of Stress

Altered behavior is another outcome or measure of stress. This can be as simple a change in the place preference of the fish, locomotion style and/or speed, or social interactions. The effects of stress can alter cognition and learning. The specific behavioral responses of larval and adult zebrafish are covered in more detail below.

Other Measures of the Stress Response

In addition to physiological and behavioral measures of the stress response, gene signals or transgenic zebrafish that will produce a reporter signal following exposure to a stressor can be used. Kishi et al. showed that senescence-associated β -galactosidase (SA- β -gal), a marker commonly associated with aging, increases during oxidative stress in zebrafish [80]. Using this marker and lacZ staining, they identified 11 recessive mutants from a library of 306 retroviral insertional mutants [8] that had elevated levels of SA- β -gal. Kishi et al. went on to identify an additional eight mutant lines using a Chemically Assisted Screening in Heterozygotes (CASH) method. Briefly, N-ethyl-N-nitrosourea (ENU) mutagenized males were outcrossed, and the resultant heterozygous larvae were treated with a low level of a reactive oxygen chemical, tert-butyl hydroperoxide (BHP), to induce oxidative stress. Screening 150 mutagenized F1 families lead to the identification of eight candidate genetic modifiers of the oxidative stress pathway.

Increased expression of heat shock proteins during cellular stress responses occur in response to some stressors in fish, in particular for many environmental or toxicological stressors [73]. The hsp70 promoter sequence has been cloned from zebrafish and used to create numerous transgenic reporter lines including green fluorescent protein (GFP) [62].

Waterborne cadmium exposure in Rainbow Trout results in accumulation of cadmium in olfactory tissue and a loss of normal response to alarm pheromone in juveniles [120]. Blechinger et al. exposed hsp70-eGFP transgenic zebrafish larvae to Cadmium for 3 hours and saw specific activation of eGFP in the olfactory sensory neurons and neuromasts [17]. Like the rainbow trout data, this early exposure resulted in a loss of the alarm pheromone response in juvenile zebrafish. Cadmium is capable of eliciting an acute cortisol response in tilapia within 5 hours of exposure [149]. However, the activation of the hsp70 promoter within specific cells of the zebrafish larvae demonstrated a localized cellular response to the cadmium and not a systemic response to cortisol. This exemplifies both the usefulness and limitations of using heat shock promoters as a marker for cellular stress.

Alternatively, transgenic lines of zebrafish could be developed that produce a reporter like green fluorescent protein during the stress response. The glucocorticoid receptor is a member of the nuclear hormone receptors that function as a transcriptional regulator after binding of their ligand. The upstream activating sequences of several glucocorticoid responsive genes have led to the identification of a consensus glucocorticoid response element [92, 94]. Alternatively, transcriptome analysis following a stressor is likely to identify specific transcriptional targets of the stress response [6]. Either a generic promoter composed of glucocorticoid response elements or the promoter regions from specific genes identified in transcriptome analysis can be used to drive a reporter gene in a transgenic fish model. Another related approach is the development of glucocorticoid receptor sensors that are similar to nuclear hormone trap vectors established by Tiefenbach et al. [135]. All of these approaches could lead to spatial and temporal visualization of the genomic activities of cortisol or stress response signaling in larval fish.

The Ontogeny of the Stress Response in Zebrafish

The development and onset, or ontogeny, of the stress response in zebrafish [1, 4] reflects those in other fish species [12, 71, 133]. In particular, oocytes and newly fertilized eggs contain some cortisol that was deposited by the mother. The levels of cortisol then gradually fall until just before hatching when *de novo* synthesis of cortisol begins. Around hatching or just after, the young larvae are able to respond to a stressor with increased production of cortisol. Using whole-body cortisol measurements in zebrafish, two groups were able to detect clear production of cortisol at 4 days post-fertilization following either a physical or hyperosmotic stressor [1, 4]. This relatively early onset of the stress response indicates that high-throughput genetic or drug screens could be used to identify altered stress response phenotypes in larval or juvenile zebrafish. Beyond the onset of HPI axis function in developing zebrafish, little is known about the long-term effects of acute or chronic stress during its development. These “early life experiences” generally help to “set” the HPA axis, but traumatic events or chronic stress can displace or alter this “set” point and lead to long-term changes in brain function, behavior, and the increase the risk of systemic or neural disorders [For review [89]]. Pioneering work using the rainbow trout has shown that early life stressors or exposure to cortisol even before the development of the central nervous system and HPI axis can alter the stress response in juvenile trout [9]. This suggests that the mechanisms observed in mammals are conserved in developing teleosts and could be identified using genetic and/or drug screening in zebrafish.

Behavioral Screening for Stress Response Phenotypes

Behavioral Measures of Adult Fish

The behavioral repertoire currently identified in adult zebrafish is far richer than what is currently known about larval behaviors. This is due in part to the three dimensional and richer movement patterns as well as fully developed social behaviors. Behavior in a novel

tank to examine anxiety or boldness is the most direct measure of stress in adult zebrafish to date (Table 2). Spending more time in the bottom of the tank, erratic movements or jumping, as well as freezing or creeping are all increased with increased anxiety or fear-like exposure to a predator or alarm pheromone [17, 43, 128]. In other work, exposure to a predator increases cortisol production [10], and use of fluoxetine reduces anxiety or increases “boldness” while decreasing cortisol levels [43]. This assay therefore allows measurement of baseline anxiety to the novel tank itself with the ability to increase or decrease the intensity of the stressor by addition of alarm pheromone or fluoxetine, respectively. This assay should be able to identify genetic modifiers of the stress response by comparing mutant carriers to non-carrier siblings that have been raised in similar or the same environment. Using a novel object is similar in function, but has been used less frequently with adult zebrafish [147, 148].

Larval Stress Response Assays

When available, larval assays are both cost- and space-saving for genetic screening, as a whole generation of adult fish need not be raised. Therefore, a number of specific behavioral paradigms have been developed for larval zebrafish that rely on changes in movement. Many take advantage of specific reflex responses of the developing embryo that occur before the full onset of the stress response; other assays are based on startle responses that occur within seconds and can only be captured using high-speed video capture (For review see, [23, 26, 95]). These assays are robust, but they may not be ideal for capturing behavioral responses to stress. More recently, extended activity monitoring of larval zebrafish, generally in 96-well microtiter plates has become a reality, as automated methods of larval detection and motion analysis are developed using readily available software [34] or commercially available behavior monitoring software systems. At least two products are active in the zebrafish larval market- ZebraLab software and a zebraBox imaging system (ViewPoint Life Sciences, Montreal, Canada) [109, 110, 114] and EthoVision software combined with an imaging tower (Noldus Information Technology, Wageningen, Netherlands) [107]. Current behavioral monitoring of larval zebrafish work requires a single embryo per well or dish to facilitate tracking movement. However, new methods of detection and tracking in *Drosophila* [18, 35] may lead to similar developments for zebrafish larvae.

Table 3 summarizes published behavioral assays of zebrafish larvae that could be adapted to primary stress response assays. Key aspects for assay design to monitor the larval stress response at 4 days post-fertilization and beyond include capturing the full course of an acute stress response while delivering reproducible results in an inexpensive yet high-throughput manner. The primary aspect of larval monitoring is measuring movement. Some initial studies examine place preference in larval zebrafish [20, 21]. This is a first step towards both conditioned place preference, conditioned place aversion, and could identify visual/spatial cues that are discernable to larval zebrafish. Cognition and learning are affected by stress, and these assays could be a secondary or indirect measure of the stress response in larval fish. To date, behavioral assays to measure the stress response in any larval stage fish have not been reported. However, cortisol assays in other species as well as the zebrafish indicate that they respond to stressors at this time [1, 4, 12, 71, 133]. We hypothesized that the larval movement would be altered during a stress response. To test this, we recorded the movements of larval zebrafish at four days post-fertilization both before and after hyperosmotic shock. As shown in Figure 1, the activity rate of water (control) addition to the well with each embryo did not significantly alter the movement of the larval zebrafish relative to pretest levels. However, addition of salt had a dose-dependent effect on the movement of the larval fish. In this test, we saw activity peaking between 3 and 10 minutes following the hyperosmotic shock, suggesting that much of the observed behavioral changes

was due to direct brain signaling in response to stress [136] or non-genomic actions of cortisol are driving this early behavior [61]. The increased locomotion had not completely extinguished in the first fifteen minutes following application of the stressor, suggesting that longer recording times could be desirable as some mutants may effect the timing of stress response extinction. In addition to altering the activity rate of larval fish, stress may alter the movement distance, turning frequency, or speed bursts. Incorporating other measures of larval movement into these assays will only help to clarify the response to stressors. Robust behavioral assays of larval fish could be used to detect changes in the perception, propagation, and proper attenuation of the stress response, including direct brain signaling and both non-genomic and genomic signaling of corticosteroids.

Mutagenesis and Screening Considerations

Mutagenesis Strategies

Mutagenesis techniques in zebrafish permit multiple avenues for both forward and reverse genetic screens. The traditional method of ENU chemical mutagenesis is a powerful and efficient way to produce single nucleotide changes and very small deletions throughout the genome [42, 60]. Unfortunately, even with clear Mendelian phenotypes, cloning the genes that are mutated is a slow and challenging process [55]. An alternative approach to traditional ENU mutagenesis, TILLING (Targeting Induced Local Lesions in Genomes), pairs the random mutagenesis of ENU with high throughput screening methods to identify mutants in the coding regions of selected “target” genes [41, 142]. Another approach for targeted gene mutation uses zinc finger nucleases in zebrafish [40, 91] and provides a direct method for reverse genetic testing of genes of interest. Alternatively, insertional mutagenesis using retroviral vectors has proved to be an efficient technique to mutagenize zebrafish [2, 7, 51, 52]. Moreover, Tol2 transposons also efficiently integrate into the genome, and mutagenic gene-breaking transposons have produced mutant lines [30, 103, 125]. When used properly, morpholino oligonucleotides effectively knockdown gene expression to reveal gene function during embryonic and early larval development of zebrafish (for review, [16]). However, non-specific toxicity attributed to activation of the p53 pathway commonly leads to neural defects in morpholino embryos [115]. The non-specific toxicity can be overcome by co-injection of a p53 morpholino [115]. Unfortunately, the onset of the stress response at 4 DPF is at or beyond the effective window of activity of most morpholinos [16].

Considerations for Polygenic Traits

In searching for genes that play a role in the stress response and/or the adaptation to stressors, many if not most will be represented by quantitative trait loci (QTLs). QTLs are genetic loci that contribute to polygenic phenotypes in a cooperative manner, like human skin color. A single QTL is only a contributor to a polygenic phenotype, and disruption or loss of a QTL is only apparent when quantifying the phenotype across a population. This means that one will not be able to merely rely on simple Mendelian numbers and qualitative segregation in recessive screens. Dominant heterozygous screens may be as informative as recessive screens as alterations in the dosing of single genes can lead to behavior changes [28, 64, 103]. In complex polygenic systems like the stress response, alterations in one gene can directly or indirectly alter other components of the system. Reducing a gene in one genetic context may have little consequence on its own, but in another context the gene reduction can directly affect the phenotype. Unlike many other model organisms, zebrafish strains are generally maintained as outcrossed stocks with a high degree of genetic diversity. This genetic variability is essential for efficiently uncovering QTLs that are found within a subset of the population. In these kinds of analyses, mutant carriers need to be directly compared to non-carrier siblings to collect the most information from behavioral

mutagenesis screens in zebrafish. This is one reason gene-break transposons serve as an excellent mutagen for such behavioral mutagenesis screens [103].

Tracking Mutant Carriers in Behavioral Assays

There are multiple methods to identify mutant carriers, including sequencing, PCR, and visualization. Arguably, the easiest and most cost-effective is the direct observation of a dominant reporter (e.g. a fluorescent protein). Of the available mutagens in zebrafish, gene-breaking transposons uniquely permit visual sorting of carriers versus non-carriers (fluorescent larvae versus non-fluorescent larvae) in a heterozygous screen (Fig. 2). In addition, gene-break transposons are highly mutagenic, allow rapid cloning of the mutagenized gene, and new editions of this system permit rapid confirmation of the phenotype by reversion of the allele via Cre recombinase [30, 103]. This approach was recently used by Petzold et al. to identify two genes involved in the nicotine response [103].

However, as the number of cloned zebrafish mutants produced by ENU mutagenesis and TILLING efforts [140, 150], zinc-finger nucleases, or retroviral insertions [139] grows, a modified “shelf” or “freezer” screen could be used to screen for modifiers of the stress response. The convenience of fluorescent sorting could be applied to these mutagenized fish lines if dominant fluorescent reporters are used in the production of carrier fish from cryopreserved sperm. For example, a large number of enhancer trap lines are available that are mapped to each of the 25 linkage groups of the zebrafish [29, 82, 99]. Mapped mutants from TILLING, zinc-finger nuclease, or non-fluorescent retroviral insertions can be matched with a fluorescently tagged homologous chromosome (Fig. 3). Carriers or recessive larvae can be sorted by segregation away from the fluorescently-tagged chromosome. A small percentage of the larvae may undergo crossing over at homologous chromosomes and be miscategorized. However when restricted to analytical genetic test crosses, these rare events in a population-based screen are likely to be insignificant. As an alternative to pairing a carrier to a dominant fluorescent marker, PCR (retroviral insertions) or sequencing (ENU or zinc-finger nuclease) can be used to distinguish carriers. Another consideration when using ENU mutagenized fish is the thousands of nucleotide changes in the genome of ENU mutagenized fish. Many of these are not null or lethal per se; however, they could contribute in small but quantifiable ways in the larvae’s response to stress. The impact of these bystander mutations can be limited by using alleles that have been outcrossed and selected for multiple generations. However, there may still be some bystander mutations located on the same linkage group and near the targeted gene that impact the assay. Unlike gene-break transposon mutations, mutations from ENU, zinc-finger nuclease, or current retroviral insertion strategies are not revertible. However, having multiple alleles for each gene provides a check and balance to insure the measured phenotype was associated with the target gene. This greatly reduces the throughput, and, unfortunately, many mutated genes do not have multiple alleles identified yet.

Longitudinal Studies in Zebrafish

Precise transitions from larval to adult behaviors have yet to be determined, but the onset of some of the more common behaviors has been examined. In particular, alarm pheromone elicits the specific escape responses characterized in adult fish starting at about 50 days post-fertilization in juveniles [138]. To begin to understand the mechanisms and genes involved in the epigenetics of the stress response, development of longitudinal studies will be required as part of the full complement of zebrafish genetic screening. As shown in Figure 4, it would be possible to raise the screened larval fish with the “stressed” larvae representing fish with an adverse life history. Larval stressors- type, intensity, and timing- that effectively modify the stress response of adult zebrafish to act as a model of adverse life history need to be identified prior to initiation of this work. However, as Auperin and Geslin have shown,

exogenous cortisol is sufficient for long-term modification of the stress response in rainbow trout [9]. This screening scenario could identify genes involved in the stress response of larval fish and adult fish, but this approach also uniquely identifies genes responsible for any epigenetic programming in response to the initial stressor.

Conclusion

The gene and environment interactions of the stress response and their roles in disease onset and progression are largely unknown. Behavioral disorders including addiction and mood and anxiety disorders are susceptible to both genetic and environmental influences. Unbiased forward genetic screens make it possible to discover the genetic or epigenetic contribution to polygenic disease. Rapid identification of mutant carriers versus non-carriers in both larval and adult zebrafish promotes phenotype analysis. Gene-break transposon mutagenesis strategies or selectively breeding cloned mutants to fluorescently marked linkage groups makes visual sorting possible. This critical step allows subtraction of the contribution of a diverse genome on complex behavioral phenotypes and permits focusing on the contribution of a single gene. Combining mutagenesis with robust, reliable behavioral assays in both the larval and adult zebrafish will permit genome-wide investigations into the genes that modify the stress response, including perception, propagation, and attenuation of the stress response. To discover genetic systems that are critical for sensing environmental cues and epigenetic programming of the stress response, longitudinal screens can be used that examine the stress response as fish develop from larvae to adults. The zebrafish model system has both genetic diversity and large familial populations, two key elements to successfully study complex polygenic behaviors. Stress contributes to the onset and severity of a number of diseases. Among these are addiction and mood and anxiety disorders-the leading causes of preventable death and disability in the world, respectively. Understanding the genetic backgrounds that either increase or decrease the susceptibility to stress and disease is a critical component of the development of future screening and health care. The zebrafish is poised to combat the dangers of stress above and beyond its star role in the hobby aquarium.

References

1. Alderman SL, Bernier NJ. Ontogeny of the corticotropin-releasing factor system in zebrafish. *General and Comparative Endocrinology*. 2009; 164:61–69. [PubMed: 19366623]
2. Allende ML, Amsterdam A, Becker T, Kawakami K, Gaiano N, Hopkins N. Insertional mutagenesis in zebrafish identifies two novel genes, *pescadillo* and *dead eye*, essential for embryonic development. *Genes Dev*. 1996; 10:3141–3155. [PubMed: 8985183]
3. Alsop D, Vijayan M. The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. *General and Comparative Endocrinology*. 2009; 161:62–66. [PubMed: 18930731]
4. Alsop D, Vijayan MM. Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Regul Integr Comp Physiol*. 2008; 294:R711–719. [PubMed: 18077507]
5. Alsop D, Vijayan MM. Molecular programming of the corticosteroid stress axis during zebrafish development. *Comp Biochem Physiol, Part A Mol Integr Physiol*. 2009; 153:49–54.
6. Aluru N, Vijayan MM. Stress transcriptomics in fish: a role for genomic Cortisol signaling. *Gen Comp Endocrinol*. 2009; 164:142–150. [PubMed: 19341738]
7. Amsterdam A, Burgess S, Golling G, Chen W, Sun Z, Townsend K, Farrington S, Haldi M, Hopkins N. A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev*. 1999; 13:2713–2724. [PubMed: 10541557]
8. Amsterdam A, Nissen RM, Sun Z, Swindell EC, Farrington S, Hopkins N. Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci USA*. 2004; 101:12792–12797. [PubMed: 15256591]

9. Auperin B, Geslin M. Plasma Cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg Cortisol content. *General and Comparative Endocrinology*. 2008; 158:234–239. [PubMed: 18671977]
10. Barcellos L, Ritter F, Kreutz L, Quevedo R, da Silva L, Bedin A, Finco J, Cericato L. Whole- body Cortisol increases after direct and visual contact with a predator in zebrafish, *Danio rerio*. *Aquaculture*. 2007; 272:774–778.
11. Barry T, Unwin M, Malison J, Quinn T. Free and total Cortisol levels in semelparous and iteroparous chinook salmon. *Journal of Fish Biology*. 2001; 59:1673–1676.
12. Barry TP, Malison JA, Held JA, Parrish JJ. Ontogeny of the Cortisol stress response in larval rainbow trout. *General and Comparative Endocrinology*. 1995; 97:57–65. [PubMed: 7713384]
13. Barton BA. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*. 2002; 42:517. [PubMed: 21708747]
14. Bencan Z, Levin ED. The role of alpha7 and alpha4beta2 nicotinic receptors in the nicotine-induced anxiolytic effect in zebrafish. *Physiol Behav*. 2008; 95:408–412. [PubMed: 18671990]
15. Bencan Z, Sledge D, Levin ED. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacol Biochem Behav*. 2009; 94:75–80. [PubMed: 19643124]
16. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. *Zebrafish*. 2009; 6:69–77. [PubMed: 19374550]
17. Blechinger SR, Kusch RC, Haugo K, Matz C, Chivers DP, Krone PH. Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. *Toxicol Appl Pharmacol*. 2007; 224:72–80. [PubMed: 17706735]
18. Branson K, Robie AA, Bender J, Perona P, Dickinson MH. High-throughput ethomics in large groups of *Drosophila*. *Nat Methods*. 2009; 6:451–457. [PubMed: 19412169]
19. Breteau S, Lee S, Guo S. Sensitivity of zebrafish to environmental toxins implicated in Parkinson's disease. *Neurotoxicology and teratology*. 2004; 26:857–864. [PubMed: 15451049]
20. Breteau S, Li Q, Lockwood BL, Kobayashi K, Lin E, Guo S. A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. *Neuroscience*. 2007; 146:1109–1116. [PubMed: 17428610]
21. Brouwerhoff SE, Hurley JB, Janssen-Bienhold U, Neuhauss SC, Driever W, Dowling JE. A behavioral screen for isolating zebrafish mutants with visual system defects. *Proc Natl Acad Sci USA*. 1995; 92:10545–10549. [PubMed: 7479837]
22. Bruijnzeel AW, Repetto M, Gold MS. Neurobiological mechanisms in addictive and psychiatric disorders. *Psychiatr Clin North Am*. 2004; 27:661–674. [PubMed: 15550286]
23. Brustein E, Saint-Amant L, Buss RR, Chong M, McDearmid JR, Drapeau P. Steps during the development of the zebrafish locomotor network. *J Physiol Paris*. 2003; 97:77–86. [PubMed: 14706693]
24. Cachat J, Canavello P, Elegante M, Bartels B, Hart P, Bergner C, Egan R, Duncan A, Tien D, Chung A, Wong K, Goodspeed J, Tan J, Grimes C, Elkhayat S, Suci C, Rosenberg M, Chung KM, Kadri F, Roy S, Gaikwad S, Stewart A, Zapolsky I, Gilder T, Mohnot S, Beeson E, Amri H, Zukowska Z, Soignier RD, Kalueff AV. Modeling withdrawal syndrome in zebrafish. *Behav Brain Res*. 2010; 208:371–376. [PubMed: 20006651]
25. Caldwell CA, Kattesh HG, Strange RJ. Distribution of Cortisol among its free and protein- bound fractions in rainbow trout (*Oncorhynchus mykiss*): evidence of control by sexual maturation. *Comp Biochem Physiol A Comp Physiol*. 1991; 99:593–595. [PubMed: 1679695]
26. Carmean V, Ribera A. Genetic Analysis of the Touch Response in Zebrafish (*Danio rerio*). *International Journal of Comparative Psychology*. 2010; 23:91–102.
27. Carvan MJ, Dalton TP, Stuart GW, Nebert DW. Transgenic zebrafish as sentinels for aquatic pollution. *Ann N Y Acad Sci*. 2000; 919:133–147. [PubMed: 11083105]
28. Caspi A, Sugden K, Moffitt TE, Taylor A, Craig IW, Harrington H, McClay J, Mill J, Martin J, Braithwaite A, Poulton R. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. *Science*. 2003; 301:386–389. [PubMed: 12869766]

29. Choo BGH, Kondrichin I, Parinov S, Emelyanov A, Go W, Toh W-c, Korzh V. Zebrafish transgenic Enhancer TRAP line database (ZETRAP). *BMC Dev Biol.* 2006; 6:5. [PubMed: 16478534]
30. Clark KJ, Balciunas D, Pogoda H-M, Ding Y, Westcot SE, Bedell V, Greenwood TM, Urban MD, Skuster KJ, Petzold AM, Ni J, Nielson AL, Patowary A, Scaria V, Sivasubbu S, Xu X, Hammerschmidt M, Ekker SC. Protein trapping produces a functional expression codex of the vertebrate proteome in preparation. *Nature Methods.* 2011; 8:506–512. [PubMed: 21552255]
31. Cofiel LPV, Mattioli R. L-histidine enhances learning in stressed zebrafish. *Braz J Med Biol Res.* 2009; 42:128–134. [PubMed: 19219306]
32. Corrêa SA, Fernandes MO, Iseki KK, Negrão JA. Effect of the establishment of dominance relationships on Cortisol and other metabolic parameters in Nile tilapia (*Oreochromis niloticus*). *Braz J Med Biol Res.* 2003; 36:1725–1731. [PubMed: 14666258]
33. Craig PM, Hogstrand C, Wood CM, McClelland GB. Gene expression endpoints following chronic waterborne copper exposure in a genomic model organism, the zebrafish, *Danio rerio*. *Physiol Genomics.* 2009; 40:23–33. [PubMed: 19789285]
34. Creton R. Automated analysis of behavior in zebrafish larvae. *Behavioural Brain Research.* 2009; 203:127–136. [PubMed: 19409932]
35. Dankert H, Wang L, Hoopfer ED, Anderson DJ, Perona P. Automated monitoring and analysis of social behavior in *Drosophila*. *Nat Methods.* 2009; 6:297–303. [PubMed: 19270697]
36. de Jesus EG, Hirano T. Changes in whole body concentrations of cortisol, thyroid hormones, and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. *General and Comparative Endocrinology.* 1992; 85:55–61. [PubMed: 1563618]
37. de Kloet ER, Derijk RH, Meijer OC. Therapy Insight: is there an imbalanced response of mineralocorticoid and glucocorticoid receptors in depression? *Nature clinical practice Endocrinology & metabolism.* 2007; 3:168–179.
38. Dhabhar FS. Acute stress enhances while chronic stress suppresses skin immunity. The role of stress hormones and leukocyte trafficking. *Ann N Y Acad Sci.* 2000; 917:876–893. [PubMed: 11268419]
39. Dhabhar FS. Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. *Neuroimmunomodulation.* 2009; 16:300–317. [PubMed: 19571591]
40. Doyon Y, McCallum JM, Miller JC, Faraji F, Ngo C, Katibah GE, Amora R, Hocking TD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Amacher SL. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat Biotechnol.* 2008; 26:702–708. [PubMed: 18500334]
41. Draper BW, McCallum CM, Stout JL, Slade AJ, Moens CB. A high-throughput method for identifying N-ethyl-N-nitrosourea (ENU)-induced point mutations in zebrafish. *Methods Cell Biol.* 2004; 77:91–112. [PubMed: 15602907]
42. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development.* 1996; 123:37–46. [PubMed: 9007227]
43. Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavella PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav Brain Res.* 2009; 205:38–44. [PubMed: 19540270]
44. Engeszer RE, Patterson LB, Rao AA, Parichy DM. Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish.* 2007; 4:21–40. [PubMed: 18041940]
45. Epel ES. Psychological and metabolic stress: a recipe for accelerated cellular aging? *Hormones (Athens).* 2009; 8:7–22. [PubMed: 19269917]
46. Fast MD, Hosoya S, Johnson SC, Afonso LOB. Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress. *Fish Shellfish Immunol.* 2008; 24:194–204. [PubMed: 18065240]
47. Feder A, Nestler EJ, Charney DS. Psychobiology and molecular genetics of resilience. *Nat RevNeurosci.* 2009; 10:446–457.

48. Ferrari E, Magri F. Role of neuroendocrine pathways in cognitive decline during aging. *Ageing Res Rev.* 2008; 7:225–233. [PubMed: 18672097]
49. Foley DL, Neale MC, Kendler KS. Reliability of a lifetime history of major depression: implications for heritability and co-morbidity. *Psychological Medicine.* 1998; 28:857–870. [PubMed: 9723141]
50. Fox HE, White SA, Kao MH, Fernald RD. Stress and dominance in a social fish. *J Neurosci.* 1997; 17:6463–6469. [PubMed: 9236253]
51. Gaiano N, Allende M, Amsterdam A, Kawakami K, Hopkins N. Highly efficient germ-line transmission of proviral insertions in zebrafish. *Proc Natl Acad Sci USA.* 1996; 93:7777–7782. [PubMed: 8755552]
52. Gaiano N, Amsterdam A, Kawakami K, Allende M, Becker T, Hopkins N. Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature.* 1996; 383:829–832. [PubMed: 8893009]
53. Gallo VP, Civinini A. Survey of the adrenal homolog in teleosts. *Int Rev Cytol.* 2003; 230:89–187. [PubMed: 14692682]
54. Gayrard V, Alvinerie M, Toutain PL. Interspecies variations of corticosteroid-binding globulin parameters. *Domest Anim Endocrinol.* 1996; 13:35–45. [PubMed: 8625614]
55. Geisler R, Rauch G-J, Geiger-Rudolph S, Albrecht A, van Bebber F, Berger A, Busch-Nentwich E, Dahm R, Dekens MPS, Dooley C, Elli AF, Gehring I, Geiger H, Geisler M, Glaser S, Holley S, Huber M, Kerr A, Kirn A, Knirsch M, Konantz M, Kuchler AM, Maderspacher F, Neuhauss SC, Nicolson T, Ober EA, Praeg E, Ray R, Rentzsch B, Rick JM, Rief E, Schauerte HE, Schepp CP, Schönberger U, Schonthaler HB, Seller C, Sidi S, Söllner C, Wehner A, Weiler C, Nüsslein-Volhard C. Large-scale mapping of mutations affecting zebrafish development. *BMC genomics.* 2007; 8:11. [PubMed: 17212827]
56. Gerlai R. Zebrafish antipredatory responses: a future for translational research? *Behav Brain Res.* 2010; 207:223–231. [PubMed: 19836422]
57. Gerlai R, Lahav M, Guo S, Rosenthal A. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. *Pharmacol Biochem Behav.* 2000; 67:773–782. [PubMed: 11166068]
58. Girod JP, Brotman DJ. Does altered glucocorticoid homeostasis increase cardiovascular risk? *Cardiovasc Res.* 2004; 64:217–226. [PubMed: 15485680]
59. Graham NA, Frost-Pineda K, Gold MS. Tobacco and psychiatric dual disorders. *J Addict Dis.* 2007; 26 (Suppl 1):5–12. [PubMed: 19283970]
60. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nüsslein-Volhard C. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development.* 1996; 123:1–36. [PubMed: 9007226]
61. Haller J, Mikics E, Makara GB. The effects of non-genomic glucocorticoid mechanisms on bodily functions and the central neural system. A critical evaluation of findings. *Front Neuroendocrinol.* 2008; 29:273–291. [PubMed: 18054070]
62. Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, Shoji W. Laser-induced gene expression in specific cells of transgenic zebrafish. *Development.* 2000; 127:1953–1960. [PubMed: 10751183]
63. Hamilton (formerly Buchanan.) F. An account of the fishes found in the River Ganges and its branches. Edinburgh: Archibald Constable and Company; 1822. p. 323–324.
64. Hansson AC, Cippitelli A, Sommer WH, Fedeli A, Björk K, Soverchia L, Terasmaa A, Massi M, Heilig M, Cicciocioppo R. Variation at the rat *Crhr1* locus and sensitivity to relapse into alcohol seeking induced by environmental stress. *Proc Natl Acad Sci USA.* 2006; 103:15236–15241. [PubMed: 17015825]
65. Hettema JM, Neale MC, Kendler KS. A review and meta-analysis of the genetic epidemiology of anxiety disorders. *The American Journal of Psychiatry.* 2001; 158:1568–1578. [PubMed: 11578982]

66. Hinton DE, Kullman SW, Hardman RC, Volz DC, Chen P-J, Carney M, Bencic DC. Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? *Mar Pollut Bull.* 2005; 51:635–648. [PubMed: 16154600]
67. Höglund E, Kolm N, Winberg S. Stress-induced changes in brain serotonergic activity, plasma cortisol and aggressive behavior in Arctic charr (*Salvelinus alpinus*) is counteracted by L-DOPA. *Physiol Behav.* 2001; 74:381–389. [PubMed: 11714503]
68. Holder G. Measurement of glucocorticoids in biological fluids. *Methods Mol Biol.* 2006; 324:141–157. [PubMed: 16761376]
69. Holsboer F, Ising M. Central CRH system in depression and anxiety--evidence from clinical studies with CRH1 receptor antagonists. *European Journal of Pharmacology.* 2008; 583:350–357. [PubMed: 18272149]
70. Hosoya S, Johnson SC, Iwama GK, Gamperl AK, Afonso LOB. Changes in free and total plasma cortisol levels in juvenile haddock (*Melanogrammus aeglefinus*) exposed to long-term handling stress. *Comp Biochem Physiol, Part A Mol Integr Physiol.* 2007; 146:78–86.
71. Hwang PP, Wu SM, Lin JH, Wu LS. Cortisol content of eggs and larvae of teleosts. *General and Comparative Endocrinology.* 1992; 86:189–196. [PubMed: 1601267]
72. Iñiguez SD, Warren BL, Parise EM, Alcantara LF, Schuh B, Maffeo ML, Manojlovic Z, Bolaños-Guzmán CA. Nicotine exposure during adolescence induces a depression-like state in adulthood. *Neuropsychopharmacology.* 2009; 34:1609–1624. [PubMed: 19092782]
73. Iwama GK, Afonso LOB, Todgham A, Ackerman P, Nakano K. Are hsp90s suitable for indicating stressed states in fish? *J Exp Biol.* 2004; 207:15–19. [PubMed: 14638828]
74. Kendler KS, Gardner CO, Gatz M, Pedersen NL. The sources of co-morbidity between major depression and generalized anxiety disorder in a Swedish national twin sample. *Psychological medicine.* 2007; 37:453–462. [PubMed: 17121688]
75. Kendler KS, Heath AC, Neale MC, Kessler RC, Eaves LJ. A population-based twin study of alcoholism in women. *JAMA.* 1992; 268:1877–1882. [PubMed: 1404711]
76. Kendler KS, Karkowski LM, Prescott CA. Fears and phobias: reliability and heritability. *Psychological Medicine.* 1999; 29:539–553. [PubMed: 10405076]
77. Kendler KS, Neale MC, Heath AC, Kessler RC, Eaves LJ. A twin-family study of alcoholism in women. *Am J Psychiatry.* 1994; 151:707–715. [PubMed: 8166312]
78. Kendler KS, Neale MC, Sullivan P, Corey LA, Gardner CO, Prescott CA. A population-based twin study in women of smoking initiation and nicotine dependence. *Psychol Med.* 1999; 29:299–308. [PubMed: 10218922]
79. Kessler RC, Chiu WT, Dernier O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry.* 2005; 62:617–627. [PubMed: 15939839]
80. Kishi S, Bayliss PE, Uchiyama J, Koshimizu E, Qi J, Nanjappa P, Imamura S, Islam A, Neuberger D, Amsterdam A, Roberts TM. The identification of zebrafish mutants showing alterations in senescence-associated biomarkers. *PLoS Genet.* 2008; 4:e1000152.
81. Koolhaas JM, de Boer SF, Coppens CM, Buwalda B. Neuroendocrinology of coping styles: towards understanding the biology of individual variation. *Frontiers in Neuroendocrinology.* 2010; 31:307–321. [PubMed: 20382177]
82. Kotani T, Nagayoshi S, Urasaki A, Kawakami K. Transposon-mediated gene trapping in zebrafish. *Methods.* 2006; 39:199–206. [PubMed: 16814563]
83. Larson ET, O'Malley DM, Melloni RH. Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish. *Behavioural Brain Research.* 2006; 167:94–102. [PubMed: 16213035]
84. Leatherland J, Li M, Barkataki S. Stressors, glucocorticoids and ovarian function in teleosts. *Journal of Fish Biology.* 2010; 76:86–111. [PubMed: 20738701]
85. Mackay TFC, Stone EA, Ayroles JF. The genetics of quantitative traits: challenges and prospects. *Nat Rev Genet.* 2009; 10:565–577. [PubMed: 19584810]
86. MacPhail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S. Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. *Neurotoxicology.* 2009; 30:52–58. [PubMed: 18952124]

87. McCormick CM. An animal model of social instability stress in adolescence and risk for drugs of abuse. *Physiology & behavior*. 2010; 99:194–203. [PubMed: 19419678]
88. McCormick CM, Mathews IZ, Thomas C, Waters P. Investigations of HPA function and the enduring consequences of stressors in adolescence in animal models. *Brain Cogn*. 2010; 72:73–85. [PubMed: 19616355]
89. McEwen BS. Understanding the potency of stressful early life experiences on brain and body function. *Metab Clin Exp*. 2008; 57 (Suppl 2):S11–15. [PubMed: 18803958]
90. McEwen BS, Stellar E. Stress and the individual. Mechanisms leading to disease. *Arch Intern Med*. 1993; 153:2093–2101. [PubMed: 8379800]
91. Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol*. 2008; 26:695–701. [PubMed: 18500337]
92. Merkulov VM, Merkulova TI. Structural variants of glucocorticoid receptor binding sites and different versions of positive glucocorticoid responsive elements: Analysis of GR-TRRD database. *J Steroid Biochem Mol Biol*. 2009; 115:1–8. [PubMed: 19429454]
93. Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. *JAMA*. 2004; 291:1238–1245. [PubMed: 15010446]
94. Montoliu L, Blendy JA, Cole TJ, Schütz G. Analysis of perinatal gene expression: hormone response elements mediate activation of a lacZ reporter gene in liver of transgenic mice. *Proc Natl Acad Sci USA*. 1995; 92:4244–4248. [PubMed: 7753790]
95. Neuhauss SCF. Behavioral genetic approaches to visual system development and function in zebrafish. *J Neurobiol*. 2003; 54:148–160. [PubMed: 12486702]
96. Øverli Ø, Korzan WJ, Höglund E, Winberg S, Bollig H, Watt M, Forster GL, Barton BA, ØVerli E, Renner KJ, Summers CH. Stress coping style predicts aggression and social dominance in rainbow trout. *Horm Behav*. 2004; 45:235–241. [PubMed: 15053939]
97. Øverli Ø, Korzan WJ, Larson ET, Winberg S, Lepage O, Pottinger TG, Renner KJ, Summers CH. Behavioral and neuroendocrine correlates of displaced aggression in trout. *Horm Behav*. 2004; 45:324–329. [PubMed: 15109906]
98. Øverli Ø, Pottinger TG, Carrick TR, Øverli E, Winberg S. Brain monoaminergic activity in rainbow trout selected for high and low stress responsiveness. *Brain Behav Evol*. 2001; 57:214–224. [PubMed: 11641559]
99. Parinov S, Kondrichin I, Korzh V, Emelyanov A. *Tol2* transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes *in vivo*. *Dev Dyn*. 2004; 231:449–459. [PubMed: 15366023]
100. Parra KV, Adrian JC, Gerlai R. The synthetic substance hypoxanthine 3-N-oxide elicits alarm reactions in zebrafish (*Danio rerio*). *Behavioural Brain Research*. 2009; 205:336–341. [PubMed: 19583985]
101. Pasquali R, Vicennati V, Cacciari M, Pagotto U. The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. *Ann N Y Acad Sci*. 2006; 1083:111–128. [PubMed: 17148736]
102. Perry S, Bernier N. The acute humoral adrenergic stress response in fish: facts and fiction. *Aquaculture*. 1999; 177:285–295.
103. Petzold AM, Balciunas D, Sivasubbu S, Clark KJ, Bedell V, Westcot S, Myers S, Moulder G, Thomas M, Ekker S. Nicotine response genetics in the zebrafish. *Proc Natl Acad Sci USA*. 2009
104. Pottinger TG, Balm PH, Pickering AD. Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *General and Comparative Endocrinology*. 1995; 98:311–320. [PubMed: 7628690]
105. Pottinger TG, Calder GM. Physiological stress in fish during toxicological procedures - a potentially confounding factor. *Environ Toxic Water*. 1995; 10:135–146.
106. Pottinger TG, Carrick TR. Stress responsiveness affects dominant-subordinate relationships in rainbow trout. *Horm Behav*. 2001; 40:419–427. [PubMed: 11673915]
107. Powers CM, Yen J, Linney EA, Seidler FJ, Slotkin TA. Silver exposure in developing zebrafish (*Danio rerio*): persistent effects on larval behavior and survival. *Neurotoxicology and Teratology*. 2010; 32:391–397. [PubMed: 20116428]

108. Pradel G, Schmidt R, Schachner M. Involvement of L1.1 in memory consolidation after active avoidance conditioning in zebrafish. *J Neurobiol.* 2000; 43:389–403. [PubMed: 10861564]
109. Prober DA, Rihel J, Onah AA, Sung R-J, Schier AF. Hypocretin/orexin overexpression induces an insomnia-like phenotype in zebrafish. *J Neurosci.* 2006; 26:13400–13410. [PubMed: 17182791]
110. Prober DA, Zimmerman S, Myers BR, McDermott BM, Kim S-H, Caron S, Rihel J, Solnica-Krezel L, Julius D, Hudspeth AJ, Schier AF. Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. *J Neurosci.* 2008; 28:10102–10110. [PubMed: 18829968]
111. Ramsay J, Feist G, Varga Z, Westerfield M, Kent M, Schreck C. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. *Aquaculture.* 2006; 258:565–574.
112. Ramsay JM, Watral V, Schreck CB, Kent ML. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. *Dis Aquat Org.* 2009; 88:69–84. [PubMed: 20183967]
113. Redei EE, Ahmadiyeh N, Baum AE, Sasso DA, Slone JL, Solberg LC, Will CC, Volenec A. Novel animal models of affective disorders. *Semin Clin Neuropsychiatry.* 2001; 6:43–67. [PubMed: 11172532]
114. Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, Haggarty SJ, Kokel D, Rubin LL, Peterson RT, Schier AF. Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. *Science.* 2010; 327:348–351. [PubMed: 20075256]
115. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC. p53 activation by knockdown technologies. *PLoS Genet.* 2007; 3:e78. [PubMed: 17530925]
116. Ruiz-Gomez, MdL; Kittilsen, S.; Höglund, E.; Huntingford, FA.; Sørensen, C.; Pottinger, TG.; Bakken, M.; Winberg, S.; Korzan, WJ.; Overli, O. Behavioral plasticity in rainbow trout (*Oncorhynchus mykiss*) with divergent coping styles: when doves become hawks. *Horm Behav.* 2008; 54:534–538. [PubMed: 18632100]
117. Sapolsky RM, Krey LC, McEwen BS. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. *Endocr Rev.* 1986; 7:284–301. [PubMed: 3527687]
118. Schreck CB. Stress and fish reproduction: the roles of allostasis and hormesis. *General and Comparative Endocrinology.* 2010; 165:549–556. [PubMed: 19596332]
119. Scott AP, Ellis T. Measurement of fish steroids in water—a review. *General and Comparative Endocrinology.* 2007; 153:392–400. [PubMed: 17188270]
120. Scott GR, Sloman KA, Rouleau C, Wood CM. Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol.* 2003; 206:1779–1790. [PubMed: 12727999]
121. Sebire M, Katsiadaki I, Scott AP. Non-invasive measurement of 11-ketotestosterone, cortisol and androstenedione in male three-spined stickleback (*Gasterosteus aculeatus*). *General and Comparative Endocrinology.* 2007; 152:30–38. [PubMed: 17412342]
122. Serra EL, Medalha CC, Mattioli R. Natural preference of zebrafish (*Danio rerio*) for a dark environment. *Braz J Med Biol Res.* 1999; 32:1551–1553. [PubMed: 10585639]
123. Sihvola E, Rose RJ, Dick DM, Pulkkinen L, Marttunen M, Kaprio J. Early-onset depressive disorders predict the use of addictive substances in adolescence: a prospective study of adolescent Finnish twins. *Addiction.* 2008; 103:2045–2053. [PubMed: 18855807]
124. Sinha R. Chronic stress, drug use, and vulnerability to addiction. *Ann N Y Acad Sci.* 2008; 1141:105–130. [PubMed: 18991954]
125. Sivasubbu S, Balciunas D, Davidson AE, Pickart MA, Hermanson SB, Wangenstein KJ, Wolbrink DC, Ekker SC. Gene-breaking transposon mutagenesis reveals an essential role for histone H2afza in zebrafish larval development. *Mech Dev.* 2006; 123:513–529. [PubMed: 16859902]
126. Sloman KA, Metcalfe NB, Taylor AC, Gilmour KM. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. *Physiol Biochem Zool.* 2001; 74:383–389. [PubMed: 11331510]
127. Sora I, Li B, Igari M, Hall FS, Ikeda K. Transgenic mice in the study of drug addiction and the effects of psychostimulant drugs. *Ann N Y Acad Sci.* 2010; 1187:218–246. [PubMed: 20201856]

128. Speedie N, Gerlai R. Alarm substance induced behavioral responses in zebrafish (*Danio rerio*). Behavioural Brain Research. 2008; 188:168–177. [PubMed: 18054804]
129. Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, *Danio rerio*. Biological reviews of the Cambridge Philosophical Society. 2008; 83:13–34. [PubMed: 18093234]
130. Sterling, P.; Eyer, J. Allostasis: A new paradigm to explain arousal pathology. In: Fisher, S.; Reason, J., editors. Handbook of Life Stress, Cognition and Health. New York: John Wiley & Sons; 1988. p. 629–649.
131. Sullivan PF, Neale MC, Kendler KS. Genetic epidemiology of major depression: review and meta-analysis. The American Journal of Psychiatry. 2000; 157:1552–1562. [PubMed: 11007705]
132. Swain HA, Sigstad C, Scalzo FM. Effects of dizocilpine (MK-801) on circling behavior, swimming activity, and place preference in zebrafish (*Danio rerio*). Neurotoxicology and teratology. 2004; 26:725–729. [PubMed: 15451036]
133. Szisch V, Papandroulakis N, Fanouraki E, Pavlidis M. Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. General and Comparative Endocrinology. 2005; 142:186–192. [PubMed: 15862562]
134. Thirumalai V, Cline HT. Endogenous dopamine suppresses initiation of swimming in prefeeding zebrafish larvae. J Neurophysiol. 2008; 100:1635–1648. [PubMed: 18562547]
135. Tiefenbach J, Moll PR, Nelson MR, Hu C, Baev L, Kislinger T, Krause HM. A live zebrafish-based screening system for human nuclear receptor ligand and cofactor discovery. PLoS ONE. 2010; 5:e9797. [PubMed: 20339547]
136. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci. 2009; 10:397–409. [PubMed: 19469025]
137. von Krogh K, Sørensen C, Nilsson G, Overli O. Forebrain cell proliferation, behavior, and physiology of zebrafish, *Danio rerio*, kept in enriched or barren environments. Physiology & behavior. 2010
138. Waldman B. Quantitative and developmental analyses of the alarm reaction in the zebra danio, *Brachydanio rerio*. Copeia. 1982; 1982:1–9.
139. Wang D, Jao L-E, Zheng N, Dolan K, Ivey J, Zonies S, Wu X, Wu K, Yang H, Meng Q, Zhu Z, Zhang B, Lin S, Burgess SM. Efficient genome-wide mutagenesis of zebrafish genes by retroviral insertions. Proc Natl Acad Sci USA. 2007; 104:12428–12433. [PubMed: 17640903]
140. Wellcome Trust Genome Campus. Zebrafish Mutation Resource. 2010. from http://www.sanger.ac.uk/Projects/D_rerio/mutres/
141. Wendelaar Bonga SE. The stress response in fish. Physiol Rev. 1997; 77:591–625. [PubMed: 9234959]
142. Wienholds E, van Eeden F, Kusters M, Mudde J, Plasterk RHA, Cuppen E. Efficient target-selected mutagenesis in zebrafish. Genome Res. 2003; 13:2700–2707. [PubMed: 14613981]
143. Wittchen H-U, Kessler RC, Beesdo K, Krause P, Höfler M, Hoyer J. Generalized anxiety and depression in primary care: prevalence, recognition, and management. J Clin Psychiatry. 2002; 63 (Suppl 8):24–34. [PubMed: 12044105]
144. Wolf OT. HPA axis and memory. Best Pract Res Clin Endocrinol Metab. 2003; 17:287–299. [PubMed: 12787553]
145. Wong CCY, Schumann G. Review. Genetics of addictions: strategies for addressing heterogeneity and polygenicity of substance use disorders. Philos Trans R Soc Lond, B, Biol Sci. 2008; 363:3213–3222. [PubMed: 18640915]
146. World Health Organization. The global burden of disease: 2004 update. Geneva, Switzerland: WHO Press; 2008. p. 1–160.
147. Wright D, Butlin RK, Carlborg O. Epistatic regulation of behavioural and morphological traits in the zebrafish (*Danio rerio*). Behav Genet. 2006; 36:914–922. [PubMed: 16752096]
148. Wright D, Nakamichi R, Krause J, Butlin RK. QTL analysis of behavioral and morphological differentiation between wild and laboratory zebrafish (*Danio rerio*). Behav Genet. 2006; 36:271–284. [PubMed: 16408248]

149. Wu SM, Shih M-J, Ho Y-C. Toxicological stress response and cadmium distribution in hybrid tilapia (*Oreochromis sp.*) upon cadmium exposure. *Comp Biochem Physiol C Toxicol Pharmacol.* 2007; 145:218–226. [PubMed: 17251063]
150. Zebrafish Tilling Consortium. Zebrafish TILLING Project. 2010. from <http://webapps.fhcrc.org/science/tilling/index.php>

Figure 1. Activity Rates in zebrafish larvae at 4 days post-fertilization (dpf) with hyperosmotic shock

Larval movements were recorded on 4 dpf zebrafish larvae with embryo medium (Control) or embryo medium with NaCl that would increase the NaCl concentration by 31.6mM, 100mM, or 316mM. Embryos were transferred to 48-well microtiter plates at 2 dpf and kept on a 14/10 hour light/dark cycle at 28° C. After acclimation to the recording cabinet, the larvae were recorded for 5 minutes before media addition. Analyze™ software (Mayo Clinic) was used to perform frame subtractions to detect movement of each individual embryo every second. Activity rate represents the percent movement averaged in a two-minute bin (+/- 1 minute). Error bars represent mean variance of 4 treatment groups per condition. 24 individuals were used for each treatment group.

Figure 2. Behavioral Screening Larvae from Gene-Break Transposon Mutagenized Lines
Co-injecting a Tol2 gene-break transposon and Tol2 mRNA into a fertilized embryo at 1 cell makes gene-break mutants. The raised embryos or P0 generation are mosaic, out crossing yields completely transgenic F1 founders. Using the F1 founders or any future generation, clutches of embryos (F2+) can be produced following an out cross to wild-type fish (dominant heterozygous screen). These embryos are placed in individual wells of two microtiter plates. One plate is subjected to the chosen stressor while the other to the control or unstressed stimulus. The behavior is recorded, and subsequently the embryos are scored for fluorescence that is indicative of the transposon-tagged (Tpn-Tagged) carrier. The data can then be processed to compare carrier versus non-carrier under stressed versus unstressed environments. For clarity, fish shown expressing RFP are also designated with a 'R' on their dorsal fin (adult) or within their yolk sac (larval).

Figure 3. Fluorescent-Based Screening of Non-Tagged, Cloned Alleles

The Zebrafish International Resource Center (ZIRC) and other stock centers are being populated with cloned mutants, most of which are not fluorescently tagged. Behavioral screens benefit from being able to quickly identify carriers versus non-carriers in larval zebrafish. To achieve these non-labeled lines, mutant sperm (or mutants) can be used to fertilize oocytes from fluorescently-tagged lines with the marker on the same linkage group as the cloned mutant (example is linkage group one, $LG1$). In the first generation fluorescent, mutant carriers are identified by PCR, sequencing, or other genotyping method. These fish can be used in dominant heterozygous screening by crossing to a wild-type partner or in recessive screens by crossing siblings. The embryos are placed into microtiter plates. One plate is treated with control stimuli, and the other with the desired stressor. The behavior is recorded and carriers are identified. In the dominant screen non-fluorescent larvae are heterozygous carriers. In recessive screens, non-fluorescent larvae are the homozygous mutant larvae. The data can then be processed to compare carrier versus non-carrier under unstressed and stressed environments. For clarity, fish shown expressing GFP are also designated with a 'G' on their dorsal fin (adult) or within their yolk sac (larval).

Figure 4. Longitudinal Studies of the Stress Response

Larval fish can be tested for behaviors in response to a stressor and compared to exposure to a control. Within these groups mutant carrier sibling can be distinguished from non-carriers (e.g. by fluorescence). The larval fish can then be raised as the "stressed" (S) or "unstressed" (U) population. As adults these fish can be split into two groups and again treated to a stressor (S) or control (U). This would yield four groups of adult fish. Those that were exposed to a stressor as a larvae and control as an adult (SU), stressor/ stressor (SS), control/ control (UU), and control/stressor (US). Within each group the mutant carriers can be identified. This screen permits the identification of larval phenotypes, adult phenotypes, and epigenetic or longitudinal phenotypes. For clarity, fish shown expressing RFP are also designated with a 'R' on their dorsal fin.

Table 1

A comprehensive list of published papers that have measured cortisol in zebrafish listing age group of specimens, the stressor(s), and experimental variables tested.

Age	Stressor	Variables	REF
Larval	Physical- Stirring	Larval Age	[4]
Larval	Hyperosmotic	Larval Age	[1]
Adult	Physical- Transport, Net Handling	Tank Acclimation Time	[105]
Adult	Crowding	Tank Size, Feeding, Duration	[111]
Adult	Predator	Presence or Visualization of Predator	[10]
Adult	Novel Tank	Fluoxetine (Anxiolytic)	[43]
Adult	Crowding, Net Handling, Simulated Transport	Infection, Cortisol Feeding	[112]
Adult	Chronic (21 day) Waterborne Copper	Cu concentration	[33]
Adult	Novel Tank Following Drug Withdrawal	Diazepam, Ethanol, Morphine, and Caffeine	[24]
Adult	Physical- Turbulent Water (heavily bubbled)	Enriched vs. Barren Environment	[137]

Table 2
Anxiety/Boldness Assays in juvenile and adult zebrafish

Listed are data end-points, experimental variables, and references of assays used to examine anxiety and boldness in adult zebrafish.

Anxiety/Boldness Assays	
<u>Recorded Data End Point</u>	<u>Experimental Variables</u>
Novel Tank	
<ul style="list-style-type: none"> • Time in Top vs Bottom [14,15,24,43,57,100,128] • Vertical Transitions [24,43,100] • Erratic Movements/Jumping [24,43,57,100,127] • Freezing/Creeping [24,43,100,128] • Latency to Top Half [24,43] • Latency to Feed [137] • Turns [137] • Distance [43,108,132] • Average Velocity [14,15,43] • Shoaling Distance [57,128] • Latency to Response [17] • Time Dashing [17] • Escape Response [122] • Circling Perimeter [132] 	<ul style="list-style-type: none"> • Tank Richness [137] • Drug Withdrawal [24] • Fear: Alarm Pheromone [17,43,128] • Fear: Hypoxanthine 3-N-Oxide [100] • Fear: Predator [128] • Ethanol [43,57] • Fluoxetine [43] • Caffeine [43] • Nicotine [14] • Nicotinic Antagonists [14] • Cocaine [122] • Home vs Novel Tank [15] • Anxiolytics [15] • Latent Cadmium Toxicity [17] • Light Intensity [122] • NMDA antagonist MK-801 [132]
Novel Object	
<ul style="list-style-type: none"> • Latency to Approach [147,148] • Time Near Object [147,148] • Entries into Object Zones [147,148] 	<ul style="list-style-type: none"> • Fish Strain [147,148]

Table 3
Selected larval zebrafish behavioral assays

Listed are data end points, experimental variables, and references of behavioral assays that could be adapted to look at stress responses in larval zebrafish.

Larval Behavioral Screens	
<u>Recorded Data End Point</u>	<u>Experimental Variables</u>
Movement	
<ul style="list-style-type: none"> Extended Profile 4–7 DPF [109, 114] <ul style="list-style-type: none"> number/duration of rest bouts first rest bout after light transition average wakeful activity average total activity Distance Traveled [19,86,107,134] Path of Travel [134] Individual Activity [110] Population Activity [103] 	<ul style="list-style-type: none"> Time of Day [86] Acoustic/Vibratory Stimuli [134] Light/Dark Adaptation and Lighting [86] Heat and Cold Shock [103,110] Transgene: Heat-shock hcr [109] Mutant Trp1a, Trp1b [110] Small Molecule Screen [114] Dopamine, NMDA, bupropion, L741, 626 [134] Irritants: Mustard Oil, Cinnamon Oil [103, 110] Irritants: Acrolein, DADS, 4-HNE [134] Acute Toxicology: MPTP [19] Toxicology: Silver [107] Nicotine [103] Ethanol [86]
Place Preference	
<ul style="list-style-type: none"> Phototaxis (light/dark) [21] Gradient Chamber [20] 	<ul style="list-style-type: none"> Timing/Presentation of Lit Chamber [21] Morphine [20]