

Of Hamsters and Zebrafish: Stress-Induced Neuronal & Phenotypic Plasticity

A Dissertation Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Jeffrey R. Kelly

May 2024

ACKNOWLEDGEMENTS

First, I have to thank my family and friends for always supporting me through the crazy ups and downs these last several years. Your patience and support through my PhD, career development, and personal growth is invaluable to me. It's been a wild ride, but I know that you have my back, and that's kept me going.

Years in this program have been complicated, and certainly not as planned, but some say our journey can be more important than the destination. And what a journey it's been. I understand many people played an important role this path, and I'd like to thank all I can.

Dissertation committee members and my advisor of course deserve ample thanks for their everlasting support and encouragement helping me reach this point to pursue my goals. A little encouragement went a long way to help me through. While I wasn't a typical inductee into their mentorship, I do appreciate how willing they were to help and support all my chaos. Finally, thanks to the Cooper's Cuties team for all their hard work in lab!

ABSTRACT

My dissertation investigates how stressful experiences induce neural and phenotypic plasticity in zebrafish and Syrian hamsters. Environmental stressors such as chemical pollution have been identified as the primary risk to health worldwide, and induce widespread changes in organism behavior, physiology, and neural systems. Reactivity to stressors differs between individuals and populations, suggesting that individuals vary in their stress coping strategies and their susceptibility to the negative consequences of stress. Social experience such as juvenile social play or social dominance status in a dominance hierarchy can modify how individuals respond to stressors and is associated with altered experience-dependent neural plasticity. This dissertation assessed differences in stress reactivity and the neural plasticity that may be driving these individual differences in two model species for toxicology and social stress research across four aims. The first aim asked how exposure to cadmium toxicity affects group decision-making and social behavior in zebrafish. The second aim categorized behavioral and physiological variation between two wild populations of zebrafish from environments with differing ecological factors. Aim three asked how juvenile social play in Syrian hamsters promotes resistance to social stressors and alters neural plasticity in the ventromedial prefrontal cortex and basolateral amygdala. The fourth aim investigated how social dominance encounters generate plasticity in a medial amygdala to bed nucleus of the stria terminalis pathway that may promote stress resistance. My findings highlight the myriad ways external stressors disrupt species-typical behavior. Further, I show that both juvenile social play experience and achieving a dominant social status in adulthood promotes stress resistance.

TABLE OF CONTENTS

CHAPTER ONE	1
OF HAMSTERS AND ZEBRAFISH: STRESS-INDUCED NEURONAL & PHENOTYPIC PLASTICITY	1
1. Overview	2
1.1. Stress and Homeostasis.....	2
1.2. Environmental Stressors Induce Individual Phenotypic Plasticity	5
1.3. Social Stress	7
1.4. Social Isolation Alters Prefrontal Cortex Plasticity	9
1.5. Social Dominance Status Modulates Stress Reactivity	11
1.6. Amygdala Plasticity Mediates Stress Reactivity	14
2. Over-Arching Questions	16
3. Specific Aims.....	16
3.1. Specific Aim 1: Identify behavioral plasticity within social networks induced by acute cadmium toxicity.	16
3.2. Specific Aim 2: Determine how environmental factors induce behavioral and morphological plasticity in wild zebrafish populations.	18
3.3. Specific Aim 3: Evaluate changes in perineuronal net expression in the mPFC, and BLA of play-deprived hamsters.	19
3.4. Specific Aim 4: Determine how activation of an MeA-BNST circuit is associated with social stress in socially dominant hamsters.	20
CHAPTER TWO A PAIR OF CADMIUM-EXPOSED ZEBRAFISH AFFECT SOCIAL BEHAVIOR OF THE UN-EXPOSED MAJORITY	55
Abstract	56
1. Introduction	56
2. Method	61
2.1. Subjects	61
2.2. Procedure	62
3. Results	68
3.1. Cd-exposed fish indirectly affect boldness and social behavior, but not cohesion and activity of the un-exposed majority.....	68
3.2. Cadmium leads to indirect effects on female zebrafish, but does not affect the connectivity of the social networks.....	70
3.3. Cadmium directly affects vision	71
4. Discussion	71
5. Conclusions	78
CHAPTER THREE	92
WILD ZEBRAFISH SENTINELS: BIOLOGICAL MONITORING OF SITE DIFFERENCES USING BEHAVIOR AND MORPHOLOGY	92
Abstract	93
1. Introduction.....	93
2. Materials and Methods.....	97
2.1. Study Location & Subjects	97

2.2. Ethics Statement.....	98
2.3. Data Collection & Analysis	98
3. Results.....	100
3.1. Fish Body Shape Is Distinct between Sites.....	100
3.2. Site 2 Fish Are More Cohesive Than Site 1 Fish.....	102
3.3. Site 1 Fish Are More Active Than Site 2 Fish.....	102
4. Discussion	103
5. Conclusions.....	108
CHAPTER FOUR.....	127
FROM PLAY DATE TO STRESS FATE: JUVENILE SOCIAL PLAY RESCUES ADULT STRESS REACTIVITY INDEPENDENT OF PERINEURONAL NET EXPRESSION IN SYRIAN HAMSTERS.....	127
1. Introduction.....	129
2. Method	135
2.1. Subjects	135
2.2. Experimental Design.....	135
2.3. Behavioral Testing.....	136
2.4. Tissue Processing.....	139
2.5. Statistical Analysis.....	141
3. Results.....	142
3.1. Play Behavior.....	142
3.2. Regrouping.....	143
3.3. Defeats	144
3.4. Conditioned Defeat	145
3.5. Social Avoidance	146
3.6. Perineuronal Nets.....	147
3.7. PNN Predictions of Behavior.....	148
4. Discussion	150
5. Conclusion	157
CHAPTER FIVE	172
DOMINANCE STATUS MODULATES ACTIVITY IN MEDIAL AMYGDALA CELLS WITH PROJECTIONS TO THE BED NUCLEUS OF THE STRIA TERMINALIS	172
Abstract	173
1. Introduction.....	174
2. Methods.....	178
2.1. Subjects	178
2.2. Experimental design.....	179
2.3. Dominance encounter	180
2.4. Stereotaxic surgery and microinjection	181
2.5. Social defeat.....	182
2.6. Histology and immunofluorescence microscopy	182
2.7. Data analysis	184

3. Results.....	185
3.1. Formation of dominance relationships.....	185
3.2. Study 1: MeA-BNST activity during dominance encounters.....	186
3.3. Study 2: MeA-BNST activity during social defeat stress.....	188
4. Discussion.....	191
5. Conclusions.....	198
CHAPTER SIX.....	213
DISCUSSION AND SYNTHESIS.....	213
1. Ecotoxicology & Anthropogenic Change: Synthesis of Specific Aims 1 & 2	214
1.1. Specific Aim 1: Identify behavioral plasticity within social networks induced by acute cadmium toxicity.....	214
1.2. Specific Aim 2: Determine how environmental factors induce behavioral and morphological plasticity in wild zebrafish populations.....	217
1.3. Synthesis	219
2. Social Experience Modifies Stress Vulnerability: Synthesis of Specific Aims 3 & 4	222
2.1. Specific Aim 3: Evaluate changes in perineuronal net (PNN) expression in the vmPFC and BLA of play-deprived hamsters.....	222
2.2. Specific Aim 4: Determine whether endogenous activity in a MeA-BNST circuit is associated with stress resilience in dominant hamsters.	226
2.3. Synthesis	228
3. Summary	233
APPENDIX.....	248
Chapter 2 Figures & Tables	248
Chapter 3 Figures & Tables	252
Chapter 4 Figures & Tables	258
Chapter 5 Figures & Tables	268
VITA.....	275

LIST OF FIGURES

Fig 2. 1	248
Fig 2. 2	249
Fig 2. 3	250
Fig 2. 4	251
Fig 3. 1	253
Fig 3. 2	254
Fig 3. 3	255
Fig 3. 4	256
Fig 3. 5	257
Fig 4. 1	258
Fig 4. 2	259
Fig 4. 3	260
Fig 4. 4	261
Fig 4. 5	262
Fig 4. 6	263
Fig 4. 7	264
Fig 4. 8	265
Fig 4. 9	266
Fig 4. 10	267
Fig 5. 1	268
Fig 5. 2	269
Fig 5. 3	270
Fig 5. 4	271
Fig 5. 5	272
Fig 5. 6	273
Fig 5. 7	274

CHAPTER ONE
OF HAMSTERS AND ZEBRAFISH: STRESS-INDUCED NEURONAL &
PHENOTYPIC PLASTICITY

1. Overview

My dissertation investigates how stressful experiences induce neural and phenotypic plasticity in both zebrafish (*Danio rerio*) and Syrian hamsters (*Mesocricetus auratus*). Myriad external challenges can constitute stressors to organisms, including ecological and toxicological variables in an individual's environment¹⁻⁵, and adverse social experience⁶⁻⁹. These environmental stressors induce plasticity in behavioral, physiological, and neural systems^{7,8,10-16}. Previous research has documented differences in stress reactivity between individuals and populations, which suggests individuals vary in their stress coping strategies and their susceptibility to the negative consequences of stress^{3,10,17-19}. To assess these individual and population-level differences in stress reactivity this dissertation evaluates both short-term and long-term responses to multiple stressors in zebrafish (*Danio rerio*) and Syrian hamsters (*Mesocricetus auratus*), two model species for toxicology and social stress research^{9,20-23}. The stressors investigated include chemical toxicity (Chapter 2), anthropogenic influence in natural environments (Chapter 3), social play deprivation (Chapter 3), and social defeat (Chapter 4).

1.1. Stress and Homeostasis

“Homeostasis”, as first defined in 1932, was a term used to describe the phenomena that physiological variables such as blood pressure and intracellular osmolarity have optimal set points, and that deviations from this set point induce physiological responses to return the body to baseline^{24,25}. In 1950 the term “stress” was first applied to biology and was defined as a non-specific physiological response to a noxious stimulus²⁶. The concepts of homeostasis and stress were linked to specify

“stressors” as a stimulus that threatens homeostasis, while a “stress response” was the body’s reaction to restore homeostasis ¹³. While stressors were initially viewed as a general challenge to homeostasis that evoked a corticosteroid response, further research found that different stressors induced different neuronal, neuroendocrine, and behavioral responses, indicating the existence of multiple physiological processes by which organisms respond to stressors ^{27–30}.

The term “allostasis” was established to describe an organism’s capacity to maintain homeostasis by adjusting physiological set points to stressors ^{30–33}. Allostasis is thus an adaptive process by which neuroendocrine and sympathetic nervous system activity works to maintain homeostasis ^{34,35}. These allostatic responses can include neuroendocrine, physiological, and behavioral actions that provide both short-term and long-term benefits to the organism by promoting adaptation and survival ^{35,36}. Individuals within populations vary in both their homeostatic set points and their allostatic responses when experiencing stress, which produces considerable variation in how individuals respond to stressors ¹⁷.

“Allostatic load” refers to the cumulative burden of chronic stress and highlights the negative physiological consequences of continuous allostatic responses ^{30,31,37}. A high allostatic load is characterized by neuroendocrine plasticity that produces ‘wear and tear’ on the body and impairs the ability to maintain homeostasis to future stressors. High allostatic load can be caused by exposure to chronic stress, lack of adaptation to stressors, continual expression of stress responses in the absence of a stressor, or allostatic responses that are insufficient to return the organism to homeostasis ^{36,38}.

Stressors vary widely, and can include myriad external environmental sources, including ecological stressors such as temperature changes, oxygen levels, and exposure to toxic pollutants ^{1,11,39–41}. Likewise, an individual's social environment can also function as a stressor, such as situations involving social isolation, social crowding, and aggression ^{6–8,42–45}. These stressors produce widespread changes in morphology, neuronal structure and function, and behavior. ^{6,8,9,12,14,15,23,46–50}. While some of these changes may represent adaptive allostatic responses, organisms are often unable to adapt to chronic and severe stressors and these stress-induced changes in physiology and behavior can have deleterious effects on health and fitness ^{1,18,51–53}.

Organisms employ myriad coping strategies to mitigate the deleterious effects of stress, and these strategies vary between individuals ^{19,54,55} and species. These coping strategies include changes in behavioral, physiological, and neuroendocrine responses such as risk aversion, learning, and up- or down-regulation of corticosteroids ^{3,10,19,54,55}. These behavioral and physiological responses are linked whereby stress coping behaviors alter physiological responses, and these physiological responses produce behavioral changes ^{3,6,17,23,56}. For example, engaging in proactive stress coping behaviors decreases basal cortisol levels in aging humans ⁵⁷. Conversely, Atlantic salmon with reactive stress coping behaviors showed higher post-stress plasma cortisol levels than proactive copers ⁵⁸. Emerging research suggests that individual differences in coping strategies can alter individuals' resilience and susceptibility to stressors ^{59–63}. For example, mice that show more escape behaviors on the first day of a chronic social defeat stress paradigm are more likely to be susceptible to stress ⁶¹. Further, a strain of zebrafish with reactive stress

coping strategies acquire fear memories faster and maintain them longer than a strain of zebrafish with proactive coping strategies ⁶². Such differences in stress resilience may be associated with experience-based neural plasticity. Overall, this dissertation characterizes behavioral, morphological, and neuronal responses to environmental stressors, and explores the mechanisms by which some individuals exhibit stress resilience.

1.2. Environmental Stressors Induce Individual Phenotypic Plasticity

Anthropogenic change poses a devastating and ubiquitous stressor across taxa by causing rapid and deleterious changes in natural environments ^{1,41,64,65}. These changes produce gradients of environmental challenges that impact organisms across a myriad of endpoints such as changes in physiology, neuroendocrine function, and behavior ^{4,40,52,66,67}. The complexity of anthropogenic change as a stressor is exemplified by the many types of environmental challenges that comprise the term. To focus on a specific type of anthropogenic change, this dissertation investigates responses to chemical pollutants and phenotypic changes between populations with differing environmental gradients.

Anthropogenic climate change is one of the most salient consequences of human impact and is projected to become the leading cause of biodiversity loss ^{68,69}. Increased temperatures in an organism's environment pose a considerable risk to organism fitness by downregulating gonadal steroid hormones ^{70–75} and increasing corticosteroid activity ^{76–78}, which thereby impairs neural activity and sexual behavior, and can alter sex ratios. Climate change is also driving rapid physiological changes in organisms ^{79–83}, including changes in body mass and growth rate ^{83–94}. These hormonal and physiological changes

also induce behavioral plasticity including alterations in motor activity^{95–99}, aggression^{95,97,100}, social behavior^{95,98,101–103}, and anxiety-like behavior^{96,104–107}.

Pollution has been identified as the greatest risk to human and environmental health and contributes to 16% of annual deaths worldwide¹. Despite these known risks, pollution rates continue to rise. Global production of human-engineered chemicals has increased from 1 million to 400 million tons per year between 1930 and 2000¹⁰⁸. About 75% of these anthropogenic chemicals have adverse environmental impacts, but most are not regulated^{108–111}. Further, over 140 million tons of petroleum-based polymers are produced annually to manufacture plastic products, but only 3.1% of these products are recovered and recycled^{112,113}. Consequently, chemical and plastic pollutants have become ubiquitous in natural environments, entering water and soil through wastewater, landfill, and agricultural activity^{114–120}. Chemical and plastic pollutants can function as endocrine disruptors that modify physical development^{121–131}, alter motor activity^{121,132,133}, increase anxiety-like behavior^{134–138}, impair learning & memory^{134,139}, and diminish affiliative social behaviors^{140–142} while increasing aggression^{143–145}. These changes are caused by the neurotoxic effects of these pollutants, which act via several mechanisms including acetylcholinesterase inhibition^{138,146–149}, perturbation of neuropeptide systems^{133,137,150,151}, activation of corticosteroid receptors^{134,137,152,153}, and estrogenic properties that disrupt androgen and estrogen synthesis^{123,154–157}. Some chemical contaminants such as cadmium have also been linked to disease formation^{158,159}, cognitive impairments¹⁵⁸, and increased aggression¹⁶⁰. Cadmium toxicity is

particularly concerning as the biological half-life of cadmium can reach decades, highlighting a critical need to assess the deleterious effects of cadmium toxicity ¹⁶¹.

1.3. Social Stress

While living in a social group can provide several benefits ^{162–165}, an organism's social environment can also function as a stressor. Social stress can come in many forms including competition for territory and limited access to reproductive partners and food resources ^{166,167}. When access to resources can be monopolized in group-living animals, dominance hierarchies are often established to dictate individual access to resources and promote social stability ^{168,169}. The social relationships between individuals within populations are often dependent on the context of social interactions and the individuals' social rank ¹⁶⁶. Differences in social rank and social context can influence health outcomes and stress susceptibility by producing alterations in neuroendocrine, physiological, and immune functions. In rodents, chronic social subordination impairs immune function by decreasing lymphocyte proliferation, granulocytosis, and lymphopenia, while increasing tumor induction and progression ¹⁷⁰. In both rodents and primates, subordinate individuals also tend to have higher basal corticosteroids and lower corticosteroid binding globulins than dominants, while dominants express greater circulatory androgens ¹⁶⁶. However, exceptions to these patterns have been observed depending on the types of social relationships within groups. For example, corticosteroids are elevated in primates that consistently receive high rates of aggression, and dominants have greater testosterone levels in groups with stable, but not unstable, dominance hierarchies ¹⁷¹. Similarly, subordinate mice show greater corticosteroids than dominants

only in groups with despotic hierarchies¹⁷². These neuroendocrine changes appear to drive behavioral outcomes associated with dominance status such that dominant individuals exhibit stress resilience and more aggression in future encounters, while subordinates are susceptible to stress and more submissive in future encounters^{166,173}. However, the extent to which dominance rank impacts stress vulnerability depends on the social context of the group.

Social defeat is a common laboratory approach in rodent models of social stress. In social defeat paradigms subjects are exposed to larger, aggressive individuals in which they receive attacks and submit to the aggressor^{174–176}. Rodent models of social defeat stress utilize a series of aggressive encounters and quantify changes in fear & anxiety behavior, and stress-associated biomarkers such as corticosteroids and neural activity in stress-sensitive brain regions^{166,175,176}. In both rodents and non-human primates, social defeat induces robust corticosteroid responses and causes long-term changes in fear and anxiety^{22,166,177}. Although humans do not experience social defeat in the same way as rodents, traumatic social stress comes in the form of bullying, sexual assault, physical trauma, and violence^{177–179}. Social experience, including social rank within dominance hierarchies, can modulate how individuals respond to social defeat stress, providing an opportunity to investigate experience-dependent changes in stress resilience and susceptibility^{9,23,44,180}.

Early life stressors have also been shown to affect how individuals respond to stressors later in life. While peer isolation and deprivation of social play during development does not appear to directly increase corticosteroids, play deprivation can

potentiate the effects of stress in adulthood^{181–183}. The increased stress response in adulthood is likely caused by impaired neuronal and social development that results from adolescent play deprivation^{183,184}. In addition to behavioral effects, social isolation and deprivation of social play during adolescence causes structural plasticity in many brain regions including the medial prefrontal cortex (mPFC)^{185,186}. Play deprivation diminishes neurogenesis while inducing cell apoptosis and changes in dendritic morphology compared to individuals with access to social play as juveniles^{8,183,187,188}. Play-deprived rats also exhibit impaired development of coping behaviors while showing increased plasma corticosteroids and adrenaline following social defeat stress in adulthood compared to controls¹⁸². Interestingly, even territorially aggressive, non-gregarious, animals such as Syrian hamsters exhibit social play in adolescence and are considered one of the most playful murine rodents¹⁸⁹. Hamsters deprived of play during adolescence exhibit increased submissive behavior and social avoidance following social defeat stress, indicating that social play deprivation increases stress susceptibility in hamsters¹⁸⁷. Social play deprivation also increases aggression in adult male hamsters compared to controls, while it decreases aggression in adult females, suggesting that social play is necessary for species-typical territorial aggression^{187,190}. This dissertation aimed to identify markers of mPFC plasticity that may drive play deprivation-induced deficits in stress resilience.

1.4. Social Isolation Alters Prefrontal Cortex Plasticity

The mPFC is a highly interconnected brain region containing distinct subregions including the prelimbic and infralimbic cortices¹⁹¹. In mammals the mPFC is divided

into six distinct layers (I-VI), with layer I being the most medial and VI the most lateral layer. Traditionally, layers II/III and V/VI contain pyramidal neurons with apical dendrites that project to layer I, while layer IV is considered a thalamic input layer^{192–195}. The mPFC also contains many different types of GABAergic interneurons that exert inhibitory control over pyramidal neurons and thus modulate projections to downstream targets^{180,196–199}. These efferent mPFC projections govern a myriad of functions including memory and decision-making²⁰⁰, emotional processing²⁰¹, reward²⁰², aggression^{203,204}, learned helplessness and depressive-like behaviors^{205–207}, and stress resilience and coping^{180,197,208,209}.

The mPFC governs these functions through several different projection pathways. In rodents, activation of mPFC neurons that project to the mediobasal and lateral hypothalamus increase the expression of aggressive behaviors including biting^{186,210,211}. The mPFC also exhibits inhibitory control over corticosteroid release via indirect projections to the paraventricular nucleus of the hypothalamus through the bed nucleus of the stria terminalis (BNST)^{209,212,213}. Similarly, mPFC projections to the basolateral amygdala (BLA) play a critical role in attenuating responses to fear and social stress^{180,197,214–217}. mPFC regulation of social stress responses is primarily governed by GABAergic interneuron microcircuits whereby somatostatin interneurons suppress firing of parvalbumin interneurons to disinhibit pyramidal neurons and increase mPFC output to downstream targets, including the BLA²¹⁸.

In rodents, stressful stimuli including social isolation induces myriad changes in mPFC connectivity and functionality including altered dendritic morphology¹⁸⁵, a

reduction in synaptic plasticity proteins ²¹⁹, decreased glial cell density ¹⁸⁶, altered excitatory glutamate responses to stressors ¹⁸⁵, and reduced pruning of pyramidal neuron apical dendrites ^{188,220–222}. Social deprivation during early life and adolescence contributes to dysregulated development of mPFC circuits that may underlie behavioral disruptions in adulthood. Male mice exposed to two weeks of post-weaning isolation show diminished neuronal activity in a mPFC to paraventricular thalamus pathway during social exposure in adulthood, which suggests that early-life social experience is critical for the development of neural pathways governing responses to social stimuli ²²³. In Syrian hamsters, deprivation of social play during adolescence increases the length and number of branch points of apical dendrites in mPFC pyramidal neurons, which suggests social play is required for typical dendritic pruning during adolescence ¹⁸⁸. Given the wider role of the mPFC in regulating agonistic and stress-related behaviors and the behavioral disruptions observed in socially isolated and play-deprived animals, it is likely that these morphological changes within the mPFC may be causal drivers of these behavioral disruptions.

1.5. Social Dominance Status Modulates Stress Reactivity

Individuals vary in their response to stressors whereby acute stressful experience produces long-lasting changes in stress-related behavior in some, but not all individuals. While 9 out of 10 people will experience a traumatic stressor during their lifetime, only 1 in 10 of these individuals will develop post-traumatic stress disorder, indicating individual differences in coping ability and stress resilience ²²⁴. Here we use resilience to refer to individuals that exhibit a rapid recovery from stress on one or more measures,

while we use susceptibility to refer to individuals that show sustained negative outcomes^{224–226}. Stress resilience is considered an active and adaptive process²²⁷ that is mediated by changes in experience-based neuronal plasticity, while susceptibility is indicative of a maladaptive response that is characterized by poor recovery from stress^{224,225}. While stress resilience refers to a rapid recovery from stress, stress resistance rather refers to individuals that do not develop negative effects from stress. Most rodent models of stress do not delineate the time course of stress recovery, which makes it difficult to distinguish between stress resilience and resistance. As such, this dissertation will use the term resilience for consistency and clarity.

Social experience is known to contribute to individual differences in stress reactivity. Social status within a dominance hierarchy often modulates stress reactivity whereby dominant individuals exhibit stress resilience and subordinate individuals exhibit stress susceptibility^{6,23,44,51}. These differences in stress reactivity have been associated with social status-dependent differences in basal corticosteroid and androgen levels. For example, subordinate wild male olive baboons typically exhibit higher basal cortisol and lower basal testosterone compared to their dominant counterparts²²⁸. However, the effects of dominance status on stress reactivity can vary broadly based on a variety of factors including resource access, maintenance of dominance, hierarchy stability, subordinate coping strategies, and species-specific breeding strategies⁵¹. As such, the precise mechanisms driving experience-dependent changes in stress reactivity are likely more complex than status-dependent corticosteroid and androgen levels and may instead be based on plasticity within select neural circuits. It is thus necessary to

characterize stress-induced changes in select neural ensembles that may mediate the effects of social experience on stress reactivity.

Syrian hamsters are an established model organism for investigating social stress and stress reactivity²²⁹. Male and female Syrian hamsters are highly territorial and aggressive animals that readily form dominance relationships⁴⁴. In the laboratory, hamsters form dyadic dominance relationships when two individuals are repeatedly paired in a resident-intruder format^{6,9,44,230}. Stress resilience and susceptibility can be measured by exposing hamsters to acute social defeat stress and quantifying defensive and avoidance behaviors towards a novel non-aggressive animal the following day^{9,44}. Resilient individuals do not exhibit stress-induced defensive behavior and social avoidance, but rather show social approach and territorial aggression toward novel hamsters the following day. Conversely, susceptible individuals exhibit large amounts of stress-induced avoidance and defensive behavior towards the conspecific, indicating heightened anxiety-like behavior after social defeat stress.

Social experience induces neuronal plasticity in dominant and subordinate hamsters that, at least in some cases, mediates their changes in stress reactivity. Dominant hamsters express greater c-Fos immunoreactivity in the medial amygdala (MeA) and mPFC compared to subordinate hamsters^{6,9,180,197,230}. Likewise, chemogenetic activation of mPFC cells projecting to the basolateral amygdala (BLA) induces a resilient response to social defeat stress in subordinate hamsters¹⁹⁷. However, very little is known about the types of cells in the MeA that underlie status-dependent changes in stress reactivity. This

dissertation aimed to delineate the role MeA neurons play in mediating status-dependent resilience to social stress.

1.6. Amygdala Plasticity Mediates Stress Reactivity

The BLA and central nuclei of the amygdala (CeA) are well known for their role in fear and stress-related anxiety²³¹. The BLA receives neuromodulatory inputs from the ventral tegmental area, dorsal raphe nucleus, locus coeruleus, and basal forebrain, as well as excitatory glutamatergic inputs from the associative cortex, thalamus, hippocampus, prefrontal cortex, and brainstem²³². The BLA sends excitatory glutamatergic projections to the CeA, entorhinal cortex, medial prefrontal cortex, hippocampus, nucleus accumbens, BNST, and MeA. The BLA is thus considered an important node in a network for processing conditioned fear responses as well as responses to other aversive cues in the environment^{197,217}.

In contrast, the MeA plays an important role in sexual behavior, aggression, and defensive behavior²³³. The MeA receives neuromodulatory inputs from the ventral tegmental area, dorsal raphe nucleus, locus coeruleus, and basal forebrain, and excitatory glutamatergic inputs from the BLA²³². The MeA projects GABA-ergic and glutamatergic outputs to the nucleus accumbens, hypothalamus, basal forebrain, and BNST. As a result, the MeA has been considered a key node in a social behavior neural network²³⁴.

Stress experience is known to induce synaptic plasticity in both the MeA and BLA. Chronic stress increases dendritic growth and spine density in the BLA, suggesting that stress promotes BLA synaptic plasticity^{50,231,235}. Conversely, chronic stress decreases dendritic spine density in the MeA²³⁵. These separate stress-induced

morphological changes in the MeA and BLA suggest that stress differentially modulates activity of these neural ensembles. To date, most studies investigating how experience modulates amygdala activity have primarily focused on prefrontal cortex projections to the amygdala. These studies have shown that mPFC projections inhibit BLA pyramidal cells and promote stress resilience, while decreased mPFC inhibition of the BLA promotes stress susceptibility^{197,236}. However, social experience also produces neuronal plasticity in the MeA, which can modulate responses to social stress. For example, androgen receptor expression in the MeA is positively correlated with stress resilience in male hamsters. Similarly, estrogen replacement in ovariectomized rats decreases anxiety and depression-like behavior in open field and forced-swim tests²³⁷. The expression of these gonadal steroid hormones and receptors can be dependent on social dominance status, whereby dominant hamsters express greater androgen and estrogen receptors than their subordinate counterparts^{6,230}. These findings suggest that changes in gonadal steroid hormone receptors in the MeA may also be a mechanism by which social experience modulates stress reactivity. However, currently little is known about the neural circuits in the MeA that underlie resilience to social stress.

The BNST is a sexually dimorphic structure that regulates stress responses, fear and anxiety-like behavior, and social behavior²³⁸. The BNST is also a stress-sensitive brain region that receives robust input from MeA neurons²³³. The MeA responds to social stimuli and thereby provides social information for the BNST to guide and sustain stress-related changes in behavior^{239,240}. Thus, the MeA-BNST pathway is well-positioned to drive individual differences in stress resilience and susceptibility. Previous

research has shown that activation of dopamine D1 receptors on GABAergic projections from the MeA to the BNST inhibits neuronal activation in the BNST and promotes social approach towards conspecifics in male mice ²⁴¹. Activation of MeA-BNST projecting neurons also promotes aggression priming in male mice, suggesting that this pathway plays a role in experience-dependent changes in aggressive behaviors ^{242,243}. While these findings indicate a MeA-BNST pathway mediates responses to social threats, it remains unknown whether MeA projections to the BNST underlie status-dependent changes in stress susceptibility.

2. Over-Arching Questions

This dissertation has two overarching questions. Specific aims 1 and 2 use a zebrafish model to ask whether changes in the physical environment creates phenotypic plasticity both within and between individuals in social groups. Specific aims 3 and 4 use a Syrian hamster model to ask whether social experience can generate neural plasticity in the mPFC and MeA and produce individual differences in stress susceptibility.

3. Specific Aims

3.1. Specific Aim 1: Identify behavioral plasticity within social networks induced by acute cadmium toxicity.

Prediction 1: We predicted that the presence of two acutely cadmium-exposed zebrafish would increase the aggressive behavior of the unexposed majority within adult mixed-sex shoals.

Prediction 2: We predicted that the presence of two cadmium-exposed fish would increase approach to a novel object in their home tank.

Prediction 3: We predicted that cadmium-exposed fish would show greater anxiety-like behaviors and decreased locomotor activity compared to unexposed zebrafish.

Prediction 4: We predicted that cadmium-exposed fish would show decreased visual perception in an optomotor response assay than unexposed fish.

Rationale: Social behaviors can be influenced by a wide range of biotic and abiotic factors. Among gregarious species there is evidence that a few individuals exhibit disproportionate influence over group behavior including group travel, decision-making, and learning ²⁴⁴. These individuals often have greater social centrality in social networks and may have leadership roles within social groups ^{245–249}. However, chemical contamination can induce behavioral changes in both group leadership and social dynamics ²⁵⁰.

Cadmium exposure in humans has been associated with diminished cognitive performance, antisocial behavior, and behavioral problems ¹⁵⁸. Aquatic organisms contaminated with cadmium exhibit increased aggression ¹⁶⁰, sensory deficits ^{251,252}, and altered species distribution ¹⁵⁹. Cadmium contamination also disrupts sensory systems, which impairs social recognition ²⁵³, shoaling ²⁵⁴, and communication ²⁵⁵. In zebrafish, vision informs shoaling and anti-predator strategies ²⁵⁶. The goal of this study was to determine how the presence of two cadmium-contaminated zebrafish can influence the behavior of the unexposed majority. Further, we assessed whether these behavioral changes were caused by visual impairment.

3.2. Specific Aim 2: Determine how environmental factors induce behavioral and morphological plasticity in wild zebrafish populations.

Prediction 1: We predicted that fish from an urban, more polluted environment would show decreased social, locomotor, and exploratory behavior than fish from a rural, less polluted environment.

Prediction 2: We predicted that urban, more polluted fish would show diminished size compared to rural, less polluted fish.

Rationale: Anthropogenic influence in natural environments is causing rapid and profound disruptions on a global scale ^{1,41,64,65,257}. These environmental challenges pose devastating risks to human and ecosystem health ^{4,40,52,66,258}. Current predictive models suggest that environmental disruptions caused by aquatic anthropogenic influence disproportionately affect aquatic environments ²⁵⁹. Environmental changes due to anthropogenic influence are known to induce behavioral and morphological plasticity and adaptations among aquatic organisms ^{11,14–16,260–266}. The complex interplay between biotic & abiotic factors in natural environments introduces a level of complexity and ecological validity that is often absent from laboratory studies ^{5,267,268}. By investigating behavioral and morphological changes to environmental perturbations in wild populations we can better understand ecologically-valid responses to chronic and developmental exposure to environmental challenges. To characterize responses to environmental challenge, we assayed behavioral and morphological differences between two populations of wild zebrafish in India living in different environments. The goal of the present study was to characterize phenotypic differences between two populations of wild zebrafish and

provide a method by which zebrafish can be used as sentinels to rapidly detect changes in water quality.

3.3. Specific Aim 3: Evaluate changes in perineuronal net expression in the mPFC, and BLA of play-deprived hamsters.

Prediction 1: We predicted that animals exposed to social play would show increased expression of PNNs on PV cells in the mPFC and BLA compared to animals deprived of juvenile social play.

Prediction 2: We predicted that animals with greater social avoidance would show lower expression of PNNs on PV cells than animals with less social avoidance.

Rationale: Social play is a crucial developmental experience across gregarious species and is critical for species-typical neural and behavioral development²⁶⁹. Adolescent social play deprivation decreases pruning of apical dendrites in the medial prefrontal cortex and increases stress susceptibility in Syrian hamsters^{187,188}. The mPFC plays a crucial role in attenuating stress reactivity^{180,197,208,209} while the BLA controls stress-related behaviors^{217,270,271}. This suggests that social play during adolescence may generate neural plasticity in these limbic regions that can influence social and stress-related behaviors in adulthood. PNNs are extracellular structures known to preferentially surround PV+ neurons and stabilize synapses by gating experience-dependent neuroplasticity²⁷². While the role of social play in PNN development remains unknown, some recent studies suggest that early life stress alters PNN expression^{273–276}.

In rodents, neonatal maternal separation diminishes mPFC PNN density at PND 40²⁷⁷, while chronic restraint stress in early life has been reported to both increase²⁷⁸ and

decrease²⁷⁹ mPFC PNNs. In the BLA maternal separation and limited bedding paradigms both increased PNN expression in adolescence, but only in males^{273,277}. Conversely, rats exposed to a scarcity-adversity model of stress showed diminished PNN intensity in the BLA²⁸⁰. Our goal was to assess how adolescent social play deprivation alters PNN and PV+ neuron co-expression and how these changes in neuronal plasticity are associated with stress susceptibility in a social defeat paradigm.

3.4. Specific Aim 4: Determine how activation of an MeA-BNST circuit is associated with social stress in socially dominant hamsters.

Prediction 1: We predicted that hamsters would show greater activation within a MeA-BNST pathway when maintaining a dominance status.

Prediction 2: We predicted that dominant hamsters would show greater activation within a MeA-BNST pathway than subordinates when experiencing social defeat stress.

Prediction 3: We predicted that the majority of MeA-BNST neurons would show androgen receptor expression.

Rationale: Exposure to acute traumatic stressors can produce long-term changes in stress reactivity^{6,23}. However, not all individuals who experience a traumatic stressor will develop exaggerated stress-related behavior and a dysregulated neuroendocrine stress response^{6,45,281,282}. Social status within dominance hierarchies modifies stress reactivity whereby socially dominant individuals exhibit stress resilience, while socially subordinate individuals exhibit stress susceptibility^{6,44,45}. Dominant individuals exposed to acute social defeat stress express greater c-Fos expression in MeA neurons than subordinate individuals, suggesting a potential role for the MeA in status-dependent

differences in stress reactivity⁹. Here we aimed to characterize the activity of MeA neurons that send projections to the BNST. Activation of a MeA-BNST pathway facilitates social approach, and may thus play a role in attenuating stress-related social anxiety²⁴¹. Our goal was to evaluate the contribution of a MeA-BNST pathway to status-dependent differences in stress susceptibility by assessing whether neural activity in this pathway occurs during the development of dominance relationships and/or during social defeat stress.

References

1. Landrigan, P. J. *et al.* The Lancet Commission on pollution and health. *The Lancet* **391**, 462–512 (2018).
2. Baum, A., Singer, J. E. & Baum, C. S. Stress and the Environment. *J. Soc. Issues* **37**, 4–35 (1981).
3. Mazza, V. *et al.* Coping with style: individual differences in responses to environmental variation. *Behav. Ecol. Sociobiol.* **73**, 142 (2019).
4. Sievers, M., Hale, R., Parris, K. M. & Swearer, S. E. Impacts of human-induced environmental change in wetlands on aquatic animals. *Biol. Rev.* **93**, 529–554 (2018).
5. Schoenfuss, H. L. *et al.* Complex mixtures, complex responses: Assessing pharmaceutical mixtures using field and laboratory approaches. *Environ. Toxicol. Chem.* **35**, 953–965 (2016).
6. Cooper, M. A., Clinard, C. T. & Morrison, K. E. Neurobiological mechanisms supporting experience-dependent resistance to social stress. *Neuroscience* **291**, 1–14 (2015).
7. Mumtaz, F., Khan, M. I., Zubair, M. & Dehpour, A. R. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. *Biomed. Pharmacother.* **105**, 1205–1222 (2018).
8. Patel, D., Kas, M. J., Chattarji, S. & Buwalda, B. Rodent models of social stress and neuronal plasticity: Relevance to depressive-like disorders. *Behav. Brain Res.* **369**, 111900 (2019).
9. Morrison, K. E., Curry, D. W. & Cooper, M. A. Social status alters defeat-induced neural activation in Syrian hamsters. *Neuroscience* **210**, 168–178 (2012).

10. Koolhaas, J. M. *et al.* Coping styles in animals: current status in behavior and stress-physiology. *Neurosci. Biobehav. Rev.* **23**, 925–935 (1999).
11. Claireaux, G., Webber, D. M., Kerr, S. R. & Boutilier, R. G. Physiology and Behaviour of Free-Swimming Atlantic COD (*Gadus Morhua*) Facing Fluctuating Salinity and Oxygenation Conditions. *J. Exp. Biol.* **198**, 61–69 (1995).
12. Egan, R. J. *et al.* Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* **205**, 38–44 (2009).
13. Chrousos, G. P. Stress and disorders of the stress system. *Nat. Rev. Endocrinol.* **5**, 374–381 (2009).
14. Haas, T. C., Blum, M. J. & Heins, D. C. Morphological responses of a stream fish to water impoundment. *Biol. Lett.* **6**, 803–806 (2010).
15. Sun, P. L., Hawkins, W. E., Overstreet, R. M. & Brown-Peterson, N. J. Morphological Deformities as Biomarkers in Fish from Contaminated Rivers in Taiwan. *Int. J. Environ. Res. Public Health* **6**, 2307–2331 (2009).
16. McCauley, S. J., Hammond, J. I. & Mabry, K. E. Simulated climate change increases larval mortality, alters phenology, and affects flight morphology of a dragonfly. *Ecosphere* **9**, e02151 (2018).
17. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neurosci. Biobehav. Rev.* **35**, 1291–1301 (2011).
18. Korte, S. M., Koolhaas, J. M., Wingfield, J. C. & McEwen, B. S. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci. Biobehav. Rev.* **29**, 3–38 (2005).

19. Matthews, G. & Campbell, S. E. Sustained performance under overload: personality and individual differences in stress and coping. *Theor. Issues Ergon. Sci.* **10**, 417–442 (2009).
20. Sprague, J. *et al.* The Zebrafish Information Network: the zebrafish model organism database. *Nucleic Acids Res.* **34**, D581–D585 (2006).
21. Dai, Y.-J. *et al.* Zebrafish as a model system to study toxicology. *Environ. Toxicol. Chem.* **33**, 11–17 (2014).
22. Wommack, J. C. & Delville, Y. Repeated social stress and the development of agonistic behavior: individual differences in coping responses in male golden hamsters. *Physiol. Behav.* **80**, 303–308 (2003).
23. Dulka, B. N. *et al.* Dominance relationships in Syrian hamsters modulate neuroendocrine and behavioral responses to social stress. *Stress Amst. Neth.* 1–6 (2018)
doi:10.1080/10253890.2018.1485646.
24. Cannon, W. B. *The Wisdom of the Body, 2nd Ed.* (Norton & Co., Oxford, England, 1939).
25. Selye, H. A Syndrome produced by Diverse Nocuous Agents. *Nature* **138**, 32–32 (1936).
26. Selye, H. Stress and the General Adaptation Syndrome. *Br. Med. J.* **1**, 1383–1392 (1950).
27. Levine, S. Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology* **30**, 939–946 (2005).
28. Romero, L. M., Dickens, M. J. & Cyr, N. E. The reactive scope model — A new model integrating homeostasis, allostasis, and stress. *Horm. Behav.* **55**, 375–389 (2009).
29. Day, T. A. Defining stress as a prelude to mapping its neurocircuitry: No help from allostasis. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **29**, 1195–1200 (2005).

30. McEWEN, B. S. Stress, Adaptation, and Disease: Allostasis and Allostatic Load. *Ann. N. Y. Acad. Sci.* **840**, 33–44 (1998).
31. McEwen, B. S. & Stellar, E. Stress and the Individual: Mechanisms Leading to Disease. *Arch. Intern. Med.* **153**, 2093–2101 (1993).
32. McEwen, B. S. & Wingfield, J. C. The concept of allostasis in biology and biomedicine. *Horm. Behav.* **43**, 2–15 (2003).
33. Sterling & Eyer. *Handbook of Life Stress, Cognition and Health*. (J. Wiley Ltd., 1988).
34. Stiedl, O., Tovote, P., Ögren, S. O. & Meyer, M. Behavioral and autonomic dynamics during contextual fear conditioning in mice. *Auton. Neurosci.* **115**, 15–27 (2004).
35. Koob, G. F. & Le Moal, M. Drug Addiction, Dysregulation of Reward, and Allostasis. *Neuropsychopharmacology* **24**, 97–129 (2001).
36. McEwen, B. S. Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur. J. Pharmacol.* **583**, 174–185 (2008).
37. Guidi, J., Lucente, M., Sonino, N. & Fava, G. A. Allostatic Load and Its Impact on Health: A Systematic Review. *Psychother. Psychosom.* **90**, 11–27 (2020).
38. McEwen, B. S. Protective and Damaging Effects of Stress Mediators. *N. Engl. J. Med.* **338**, 171–179 (1998).
39. Dingemanse, N. J., Both, C., Drent, P. J. & Tinbergen, J. M. Fitness consequences of avian personalities in a fluctuating environment. *Proc. R. Soc. Lond. B Biol. Sci.* **271**, 847–852 (2004).

40. Reid, G. K. *et al.* Climate change and aquaculture: considering biological response and resources. *Aquac. Environ. Interact.* **11**, 569–602 (2019).
41. Qu, S. *et al.* Distinguishing the impacts of climate change and anthropogenic factors on vegetation dynamics in the Yangtze River Basin, China. *Ecol. Indic.* **108**, 105724 (2020).
42. Newman, E. L. *et al.* Fighting Females: Neural and Behavioral Consequences of Social Defeat Stress in Female Mice. *Biol. Psychiatry* **86**, 657–668 (2019).
43. Verbitsky, A., Dopfel, D. & Zhang, N. Rodent models of post-traumatic stress disorder: behavioral assessment. *Transl. Psychiatry* **10**, 1–28 (2020).
44. Morrison, K. E. *et al.* Maintenance of dominance status is necessary for resistance to social defeat stress in Syrian hamsters. *Behav. Brain Res.* **270**, 277–286 (2014).
45. Wood, S. K., Walker, H. E., Valentino, R. J. & Bhatnagar, S. Individual differences in reactivity to social stress predict susceptibility and resilience to a depressive phenotype: role of corticotropin-releasing factor. *Endocrinology* **151**, 1795–1805 (2010).
46. Shukla, R. & Bhat, A. Morphological divergences and ecological correlates among wild populations of zebrafish (*Danio rerio*). *Environ. Biol. Fishes* **100**, 251–264 (2017).
47. Hallauer, J. *et al.* The Effect of Chronic Arsenic Exposure in Zebrafish. *Zebrafish* **13**, 405–412 (2016).
48. Tu, H. *et al.* Role of neurexin2a in lead-induced locomotor defect in developing zebrafish. *Aquat. Toxicol.* **194**, 167–175 (2018).
49. Mahan, A. L. & Ressler, K. J. Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. *Trends Neurosci.* **35**, 24–35 (2012).

50. Shekhar, A., Truitt, W., Rainnie, D. & Sajdyk, T. Role of stress, corticotrophin releasing factor (CRF) and amygdala plasticity in chronic anxiety. *Stress* **8**, 209–219 (2005).
51. Sapolsky, R. M. The Influence of Social Hierarchy on Primate Health. *Science* **308**, 648–652 (2005).
52. Tong, S. & Ebi, K. Preventing and mitigating health risks of climate change. *Environ. Res.* **174**, 9–13 (2019).
53. World Health Organization. Exposure to Arsenic: A Major Public Health Concern; WHO/CED/PHE/EPE/19.4.1. (2019).
54. Steptoe, A. Psychological coping, individual differences and physiological stress responses. in *Personality and stress: Individual differences in the stress process* 205–233 (John Wiley & Sons, Oxford, England, 1991).
55. Silva, P. I. M. *et al.* Individual differences in cortisol levels and behaviour of Senegalese sole (*Solea senegalensis*) juveniles: Evidence for coping styles. *Appl. Anim. Behav. Sci.* **124**, 75–81 (2010).
56. Carere, C., Caramaschi, D. & Fawcett, T. W. Covariation between personalities and individual differences in coping with stress: Converging evidence and hypotheses. *Curr. Zool.* **56**, 728–740 (2010).
57. O'Donnell, K., Badrick, E., Kumari, M. & Steptoe, A. Psychological coping styles and cortisol over the day in healthy older adults. *Psychoneuroendocrinology* **33**, 601–611 (2008).

58. Vindas, M. A. *et al.* How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish. *J. Exp. Biol.* **220**, 1524–1532 (2017).
59. Dumont, M. & Provost, M. A. Resilience in Adolescents: Protective Role of Social Support, Coping Strategies, Self-Esteem, and Social Activities on Experience of Stress and Depression. *J. Youth Adolesc.* **28**, 343–363 (1999).
60. Russo, S. J., Murrough, J. W., Han, M.-H., Charney, D. S. & Nestler, E. J. Neurobiology of resilience. *Nat. Neurosci.* **15**, 1475–1484 (2012).
61. Murra, D. *et al.* Characterizing the behavioral and neuroendocrine features of susceptibility and resilience to social stress. *Neurobiol. Stress* **17**, 100437 (2022).
62. Baker, M. R. & Wong, R. Y. Contextual fear learning and memory differ between stress coping styles in zebrafish. *Sci. Rep.* **9**, 9935 (2019).
63. Baker, M. R. & Wong, R. Y. Npas4a expression in the teleost forebrain is associated with stress coping style differences in fear learning. *Sci. Rep.* **11**, 12074 (2021).
64. Foley, J. A. *et al.* Global Consequences of Land Use. *Science* **309**, 570–574 (2005).
65. Kaniewski, D. *et al.* Recent anthropogenic climate change exceeds the rate and magnitude of natural Holocene variability on the Balearic Islands. *Anthropocene* **32**, 100268 (2020).
66. Ball, B. A. *et al.* Direct and Terrestrial Vegetation-mediated Effects of Environmental Change on Aquatic Ecosystem Processes. *BioScience* **60**, 590–601 (2010).
67. Bambrick, H., Dear, K., Woodruff, R., Hanigan, I. & McMichael, A. *The Impacts of Climate Change on Three Health Outcomes: Temperature-Related Mortality and*

- Hospitalisations, Salmonellosis and Other Bacterial Gastroenteritis, and Population at Risk from Dengue.* (Australian Government, Australian Government, Australia, 2008).
68. Urban, M. C. Accelerating extinction risk from climate change. *Science* **348**, 571–573 (2015).
 69. Trisos, C. H., Merow, C. & Pigot, A. L. The projected timing of abrupt ecological disruption from climate change. *Nature* **580**, 496–501 (2020).
 70. ZHENG, M., NAGAOKA, K. & WATANABE, G. Pre-pubertal exposure to high temperature impairs ovarian and adrenal gland function in female rats. *J. Vet. Med. Sci.* **81**, 279–286 (2019).
 71. Elisio, M., Chalde, T. & Miranda, L. A. Effects of short periods of warm water fluctuations on reproductive endocrine axis of the pejerrey (*Odontesthes bonariensis*) spawning. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **163**, 47–55 (2012).
 72. Lim, B.-S., Kagawa, H., Gen, K. & Okuzawa, K. Effects of water temperature on the gonadal development and expression of steroidogenic enzymes in the gonad of juvenile red seabream, *Pagrus major*. *Fish Physiol. Biochem.* **28**, 161–162 (2003).
 73. Watts, M., Pankhurst, N. W. & King, H. R. Maintenance of Atlantic salmon (*Salmo salar*) at elevated temperature inhibits cytochrome P450 aromatase activity in isolated ovarian follicles. *Gen. Comp. Endocrinol.* **135**, 381–390 (2004).
 74. Navarro-Martín, L. *et al.* DNA Methylation of the Gonadal Aromatase (*cyp19a*) Promoter Is Involved in Temperature-Dependent Sex Ratio Shifts in the European Sea Bass. *PLOS Genet.* **7**, e1002447 (2011).

75. Servili, A., Canario, A. V. M., Mouchel, O. & Muñoz-Cueto, J. A. Climate change impacts on fish reproduction are mediated at multiple levels of the brain-pituitary-gonad axis. *Gen. Comp. Endocrinol.* **291**, 113439 (2020).
76. Luo, Y., Yang, M., Guo, M., Zhong, X. & Hu, Y. Huang Qin Hua Shi decoction for high-temperature- and high-humidity-induced cognitive-behavioral disorder in rats is associated with deactivation of the hypothalamic–pituitary–adrenal axis. *J. Int. Med. Res.* **47**, 5752–5766 (2019).
77. Popovska-Perčinić, F. *et al.* A Moderate Increase in Ambient Temperature Influences The Structure and Hormonal Secretion of Adrenal Glands in Rats. *Cell J. Yakhteh* **22**, 415–424 (2021).
78. Lian, S. *et al.* Impact of prenatal cold stress on placental physiology, inflammatory response, and apoptosis in rats. *Oncotarget* **8**, 115304–115314 (2017).
79. Baudron, A. R., Needle, C. L., Rijnsdorp, A. D. & Tara Marshall, C. Warming temperatures and smaller body sizes: synchronous changes in growth of North Sea fishes. *Glob. Change Biol.* **20**, 1023–1031 (2014).
80. Cheung, W. *et al.* Shrinking of fishes exacerbates impacts of global ocean changes on marine ecosystems. *Nat. Clim. Change* **3**, (2012).
81. Lema, S. C., Bock, S. L., Malley, M. M. & Elkins, E. A. Warming waters beget smaller fish: evidence for reduced size and altered morphology in a desert fish following anthropogenic temperature change. *Biol. Lett.* **15**, 20190518 (2019).

82. Lema, S. C. & Nevitt, G. A. Testing an ecophysiological mechanism of morphological plasticity in pupfish and its relevance to conservation efforts for endangered Devils Hole pupfish. *J. Exp. Biol.* **209**, 3499–3509 (2006).
83. Forster, J., Hirst, A. G. & Atkinson, D. Warming-induced reductions in body size are greater in aquatic than terrestrial species. *Proc. Natl. Acad. Sci.* **109**, 19310–19314 (2012).
84. Yom-Tov, Y., Yom-Tov, S., Wright, J., Thorne, C. & Feu, R. Recent changes in body weight and wing length among some British passerine birds. *Oikos* **112**, 91–101 (2006).
85. Van Buskirk, J., Mulvihill, R. S. & Leberman, R. C. Declining body sizes in North American birds associated with climate change. *Oikos* **119**, 1047–1055 (2010).
86. Gardner, J., Peters, A., Kearney, M., Joseph, L. & Heinsohn, R. Declining body size: a third universal response to warming? *Trends Ecol. Evo* vol. 26 (2011).
87. Goodman, R. E., Lebuhn, G., Seavy, N. E., Gardali, T. & Bluso-Demers, J. D. Avian body size changes and climate change: warming or increasing variability? *Glob. Change Biol.* **18**, 63–73 (2012).
88. Barneche, D. R., Jahn, M. & Seebacher, F. Warming increases the cost of growth in a model vertebrate. *Funct. Ecol.* **33**, 1256–1266 (2019).
89. Ryding, S., Klaassen, M., Tattersall, G. J., Gardner, J. L. & Symonds, M. R. E. Shape-shifting: changing animal morphologies as a response to climatic warming. *Trends Ecol. Evol.* **36**, 1036–1048 (2021).
90. Villar, C. & Naya, D. Climate change and temporal trends in body size: The case of rodents. *Oikos* **127**, (2018).

91. Pergams, O. R. W. & Ashley, M. V. Rapid Morphological Change in Channel Island Deer Mice. *Evolution* **53**, 1573–1581 (1999).
92. Stumpp, R., Fuzessy, L. & Paglia, A. P. Environment Drivers Acting on Rodent Rapid Morphological Change. *J. Mamm. Evol.* **25**, 131–140 (2018).
93. Munday, P., Kingsford, M., O’Callaghan, M. & Donelson, J. Elevated temperature restricts growth potential of the coral reef fish *Acanthochromis polyacanthus*. *Coral Reefs* **27**, 927–931 (2008).
94. Del Rio, A. M., Davis, B. E., Fangué, N. A. & Todgham, A. E. Combined effects of warming and hypoxia on early life stage Chinook salmon physiology and development. *Conserv. Physiol.* **7**, (2019).
95. Biro, P. A., Beckmann, C. & Stamps, J. A. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. *Proc. R. Soc. B Biol. Sci.* **277**, 71–77 (2010).
96. Ruperto, E. F., Taraborelli, P. A., Menéndez, J. & Sassi, P. L. Developmental environment influences activity levels in a montane rodent, *Phyllotis xanthopygus*. *Zool. Jena Ger.* **142**, 125818 (2020).
97. Michel, V. *et al.* The relationship between locomotion and heat tolerance in heat exposed rats. *Behav. Brain Res.* **211**, 41–47 (2010).
98. Brandão, M. L. *et al.* Water temperature affects aggressive interactions in a Neotropical cichlid fish. *Neotropical Ichthyol.* **16**, e170081 (2018).
99. Beamish, F. W. H. Swimming performance and metabolic rate of three tropical fishes in relation to temperature. *Hydrobiologia* **83**, 245–254 (1981).

100. Greenberg, G. The effects of ambient temperature and population density on aggression in two inbred strains of mice, *Mus musculus*. *Behaviour* **42**, 119–130 (1972).
101. Wiles, S. C. *et al.* Long-Term Pharmaceutical Contamination and Temperature Stress Disrupt Fish Behavior. *Environ. Sci. Technol.* **54**, 8072–8082 (2020).
102. Bartolini, T., Butail, S. & Porfiri, M. Temperature influences sociality and activity of freshwater fish. *Environ. Biol. Fishes* **98**, 825–832 (2015).
103. Colchen, T., Teletchea, F., Fontaine, P. & Pasquet, A. Temperature modifies activity, inter-individual relationships and group structure in a fish. *Curr. Zool.* **63**, 175–183 (2017).
104. Toni, M. *et al.* Environmental temperature variation affects brain protein expression and cognitive abilities in adult zebrafish (*Danio rerio*): A proteomic and behavioural study. *J. Proteomics* **204**, 103396 (2019).
105. Gruene, T. M., Lipps, J., Rey, C. D., Bouck, A. & Shansky, R. M. Heat exposure in female rats elicits abnormal fear expression and cellular changes in prefrontal cortex and hippocampus. *Neurobiol. Learn. Mem.* **115**, 38–42 (2014).
106. Mete, F., Kilic, E., Somay, A. & Yilmaz, B. Effects of heat stress on endocrine functions & behaviour in the pre-pubertal rat. *Indian J. Med. Res.* **135**, 233–239 (2012).
107. Panakhova, E., Burešová, O. & Bureš, J. The effect of hypothermia on the rat's spatial memory in the water tank task. *Behav. Neural Biol.* **42**, 191–196 (1984).
108. Daughton, C. G. Non-regulated water contaminants: emerging research. *Environ. Impact Assess. Rev.* **24**, 711–732 (2004).

109. Larsson, D. G. J. Pollution from drug manufacturing: review and perspectives. *Philos. Trans. R. Soc. B Biol. Sci.* **369**, 20130571 (2014).
110. Cipullo, S., Prpich, G., Campo, P. & Coulon, F. Assessing bioavailability of complex chemical mixtures in contaminated soils: Progress made and research needs. *Sci. Total Environ.* **615**, 708–723 (2018).
111. Simmons, J. E. Chemical mixtures: challenge for toxicology and risk assessment. *Toxicology* **105**, 111–119 (1995).
112. Thushari, G. G. N. & Senevirathna, J. D. M. Plastic pollution in the marine environment. *Heliyon* **6**, e04709 (2020).
113. Li, P. *et al.* Characteristics of Plastic Pollution in the Environment: A Review. *Bull. Environ. Contam. Toxicol.* **107**, 577–584 (2021).
114. Posthuma, L. *et al.* Improved component-based methods for mixture risk assessment are key to characterize complex chemical pollution in surface waters. *Environ. Sci. Eur.* **31**, 70 (2019).
115. Diamond, M. L. *et al.* Exploring the planetary boundary for chemical pollution. *Environ. Int.* **78**, 8–15 (2015).
116. Schug, T. T., Janesick, A., Blumberg, B. & Heindel, J. J. Endocrine disrupting chemicals and disease susceptibility. *J. Steroid Biochem. Mol. Biol.* **127**, 204–215 (2011).
117. Diamanti-Kandarakis, E. *et al.* Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocr. Rev.* **30**, 293–342 (2009).
118. Clotfelter, E. D., Bell, A. M. & Levering, K. R. The role of animal behaviour in the study of endocrine-disrupting chemicals. *Anim. Behav.* **68**, 665–676 (2004).

119. Gavrilesco, M., Demnerová, K., Aamand, J., Agathos, S. & Fava, F. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. *New Biotechnol.* **32**, 147–156 (2015).
120. Patel, M. *et al.* Pharmaceuticals of Emerging Concern in Aquatic Systems: Chemistry, Occurrence, Effects, and Removal Methods. *Chem. Rev.* **119**, 3510–3673 (2019).
121. Wu, N. C. & Seebacher, F. Bisphenols alter thermal responses and performance in zebrafish (*Danio rerio*). *Conserv. Physiol.* **9**, coaa138 (2021).
122. Zhang, Y.-F. *et al.* Bisphenol A alternatives bisphenol S and bisphenol F interfere with thyroid hormone signaling pathway in vitro and in vivo. *Environ. Pollut. Barking Essex 1987* **237**, 1072–1079 (2018).
123. Rubin, B. S. Bisphenol A: an endocrine disruptor with widespread exposure and multiple effects. *J. Steroid Biochem. Mol. Biol.* **127**, 27–34 (2011).
124. Wassenaar, P. N. H., Trasande, L. & Legler, J. Systematic Review and Meta-Analysis of Early-Life Exposure to Bisphenol A and Obesity-Related Outcomes in Rodents. *Environ. Health Perspect.* **125**, 106001.
125. Wagner, V. A. *et al.* Bisphenol F Exposure in Adolescent Heterogeneous Stock Rats Affects Growth and Adiposity. *Toxicol. Sci. Off. J. Soc. Toxicol.* **181**, 246–261 (2021).
126. Yokota, H. *et al.* Effect of bisphenol A on early life stage in Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **19**, 1925–1930 (2000).
127. Gonzalez, H. O., Roling, J. A., Baldwin, W. S. & Bain, L. J. Physiological changes and differential gene expression in mummichogs (*Fundulus heteroclitus*) exposed to arsenic. *Aquat. Toxicol. Amst. Neth.* **77**, 43–52 (2006).

128. Li, D. *et al.* Developmental mechanisms of arsenite toxicity in zebrafish (*Danio rerio*) embryos. *Aquat. Toxicol. Amst. Neth.* **91**, 229–237 (2009).
129. Erickson, R. J., Mount, D. R., Highland, T. L., Russell Hockett, J. & Jenson, C. T. The relative importance of waterborne and dietborne arsenic exposure on survival and growth of juvenile rainbow trout. *Aquat. Toxicol. Amst. Neth.* **104**, 108–115 (2011).
130. Hodson, P., Dixon, D. G., Spry, D., Whittle, D. & Sprague, J. Effect of Growth Rate and Size of Fish on Rate of Intoxication by Waterborne Lead. *Can. J. Fish. Aquat. Sci.* **39**, 1243–1251 (2011).
131. Yu, L. *et al.* Dietary *Lactobacillus plantarum* supplementation enhances growth performance and alleviates aluminum toxicity in tilapia. *Ecotoxicol. Environ. Saf.* **143**, 307–314 (2017).
132. Goundadkar, B. B. & Katti, P. Environmental estrogen(s) induced swimming behavioural alterations in adult zebrafish (*Danio rerio*). *Environ. Toxicol. Pharmacol.* **54**, 146–154 (2017).
133. Moreno Ávila, C. L., Limón-Pacheco, J. H., Giordano, M. & Rodríguez, V. M. Chronic Exposure to Arsenic in Drinking Water Causes Alterations in Locomotor Activity and Decreases Striatal mRNA for the D2 Dopamine Receptor in CD1 Male Mice. *J. Toxicol.* **2016**, 4763434 (2016).
134. Poimenova, A., Markaki, E., Rahiotis, C. & Kittraki, E. Corticosterone-regulated actions in the rat brain are affected by perinatal exposure to low dose of bisphenol A. *Neuroscience* **167**, 741–749 (2010).

135. Chen, F., Zhou, L., Bai, Y., Zhou, R. & Chen, L. Hypothalamic-pituitary-adrenal axis hyperactivity accounts for anxiety- and depression-like behaviors in rats perinatally exposed to bisphenol A. *J. Biomed. Res.* **29**, 250–258 (2015).
136. Tyler, C. R., Solomon, B. R., Ulibarri, A. L. & Allan, A. M. Fluoxetine treatment ameliorates depression induced by perinatal arsenic exposure via a neurogenic mechanism. *Neurotoxicology* **44**, 98–109 (2014).
137. Martinez, E. J., Kolb, B. L., Bell, A., Savage, D. D. & Allan, A. M. Moderate perinatal arsenic exposure alters neuroendocrine markers associated with depression and increases depressive-like behaviors in adult mouse offspring. *Neurotoxicology* **29**, 647–655 (2008).
138. Chtourou, Y., Slima, A. B., Gdoura, R. & Fetoui, H. Naringenin Mitigates Iron-Induced Anxiety-Like Behavioral Impairment, Mitochondrial Dysfunctions, Ectonucleotidases and Acetylcholinesterase Alteration Activities in Rat Hippocampus. *Neurochem. Res.* **40**, 1563–1575 (2015).
139. Xu, X. *et al.* Perinatal exposure to bisphenol-A inhibits synaptogenesis and affects the synaptic morphological development in offspring male mice. *Chemosphere* **91**, 1073–1081 (2013).
140. Salahinejad, A. *et al.* Effects of chronic exposure to bisphenol-S on social behaviors in adult zebrafish: Disruption of the neuropeptide signaling pathways in the brain. *Environ. Pollut.* **262**, 113992 (2020).
141. Gao, T. *et al.* The effects of pubertal exposure to bisphenol-A on social behavior in male mice. *Chemosphere* **244**, 125494 (2020).

142. Wang, J. *et al.* Chronic bisphenol A exposure alters behaviors of zebrafish (*Danio rerio*). *Environ. Pollut. Barking Essex 1987* **206**, 275–281 (2015).
143. Sloman, K. A., Baker, D. W., Ho, C. G., McDonald, D. G. & Wood, C. M. The effects of trace metal exposure on agonistic encounters in juvenile rainbow trout, *Oncorhynchus mykiss*. *Aquat. Toxicol. Amst. Neth.* **63**, 187–196 (2003).
144. Henry, M. G. & Atchison, G. J. Behavioral Changes in Social Groups of Bluegills Exposed to Copper. *Trans. Am. Fish. Soc.* **115**, 590–595 (1986).
145. Henry, M. G. & Atchison, G. J. Influence of social rank on the behaviour of bluegill, *Lepomis macrochirus* Rafinesque exposed to sublethal concentrations of cadmium and zinc*. *J. Fish Biol.* **15**, 309–315 (1979).
146. Pal, A. *et al.* Biochemical, histological, and memory impairment effects of chronic copper toxicity: a model for non-Wilsonian brain copper toxicosis in Wistar rat. *Biol. Trace Elem. Res.* **153**, 257–268 (2013).
147. Reddy, G. R. *et al.* Lead induced effects on acetylcholinesterase activity in cerebellum and hippocampus of developing rat. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* **21**, 347–352 (2003).
148. Namgyal, D. *et al.* Curcumin Ameliorates the Cd-Induced Anxiety-like Behavior in Mice by Regulating Oxidative Stress and Neuro-Inflammatory Proteins in the Prefrontal Cortex Region of the Brain. *Antioxid. Basel Switz.* **10**, 1710 (2021).
149. Czarnecki, L. A. *et al.* Functional rehabilitation of cadmium-induced neurotoxicity despite persistent peripheral pathophysiology in the olfactory system. *Toxicol. Sci. Off. J. Soc. Toxicol.* **126**, 534–544 (2012).

150. Mejía, J. J., Díaz-Barriga, F., Calderón, J., Ríos, C. & Jiménez-Capdeville, M. E. Effects of lead-arsenic combined exposure on central monoaminergic systems. *Neurotoxicol. Teratol.* **19**, 489–497 (1997).
151. Bardullas, U. *et al.* Chronic low-level arsenic exposure causes gender-specific alterations in locomotor activity, dopaminergic systems, and thioredoxin expression in mice. *Toxicol. Appl. Pharmacol.* **239**, 169–177 (2009).
152. Aluru, N., Leatherland, J. F. & Vijayan, M. M. Bisphenol A in Oocytes Leads to Growth Suppression and Altered Stress Performance in Juvenile Rainbow Trout. *PLOS ONE* **5**, e10741 (2010).
153. Prasanth, G. K., Divya, L. M. & Sadasivan, C. Bisphenol-A can bind to human glucocorticoid receptor as an agonist: an in silico study. *J. Appl. Toxicol. JAT* **30**, 769–774 (2010).
154. Amereh, F., Babaei, M., Eslami, A., Fazelipour, S. & Rafiee, M. The emerging risk of exposure to nano(micro)plastics on endocrine disturbance and reproductive toxicity: From a hypothetical scenario to a global public health challenge. *Environ. Pollut. Barking Essex 1987* **261**, 114158 (2020).
155. Aloisi, A. M., Della Seta, D., Ceccarelli, I. & Farabollini, F. Bisphenol-A differently affects estrogen receptors-alpha in estrous-cycling and lactating female rats. *Neurosci. Lett.* **310**, 49–52 (2001).
156. Chang, H. *et al.* Perinatal exposure to low-dose bisphenol A disrupts learning/memory and DNA methylation of estrogen receptor alpha in the hippocampus. *Toxicol. Res.* **5**, 828–835 (2016).

157. Li, X. *et al.* Zebrafish neurobehavioral phenomics applied as the behavioral warning methods for fingerprinting endocrine disrupting effect by lead exposure at environmentally relevant level. *Chemosphere* **231**, 315–325 (2019).
158. Satarug, S. *et al.* A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* **137**, 65–83 (2003).
159. Larison, J. R., Likens, G. E., Fitzpatrick, J. W. & Crock, J. G. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. *Nature* **406**, 181–183 (2000).
160. Terçariol, S. G., Almeida, A. A. & Godinho, A. F. Cadmium and exposure to stress increase aggressive behavior. *Environ. Toxicol. Pharmacol.* **32**, 40–45 (2011).
161. Satarug, S., Garrett, S. H., Sens, M. A. & Sens, D. A. Cadmium, Environmental Exposure, and Health Outcomes. *Environ. Health Perspect.* **118**, 182–190 (2010).
162. Ebensperger, L. A. A review of the evolutionary causes of rodent group-living. *Acta Theriol. (Warsz.)* **46**, 115–144 (2001).
163. Markham, A. C. & Gesquiere, L. R. Costs and benefits of group living in primates: an energetic perspective. *Philos. Trans. R. Soc. B Biol. Sci.* **372**, 20160239 (2017).
164. Gardner, M. G., Pearson, S. K., Johnston, G. R. & Schwarz, M. P. Group living in squamate reptiles: a review of evidence for stable aggregations. *Biol. Rev.* **91**, 925–936 (2016).
165. Ebensperger, L. A. & Hayes, L. D. Causes and evolution of group-living. in *Sociobiology of Caviomorph Rodents* 173–200 (John Wiley & Sons, Ltd, 2016).
doi:10.1002/9781118846506.ch7.

166. Tamashiro, K. L. K., Nguyen, M. M. N. & Sakai, R. R. Social stress: From rodents to primates. *Front. Neuroendocrinol.* **26**, 27–40 (2005).
167. Lederbogen, F. *et al.* City living and urban upbringing affect neural social stress processing in humans. *Nature* **474**, 498–501 (2011).
168. Tibbetts, E. A., Pardo-Sanchez, J. & Weise, C. The establishment and maintenance of dominance hierarchies. *Philos. Trans. R. Soc. B Biol. Sci.* **377**, 20200450 (2022).
169. Sapolsky, R. M. The physiology of dominance in stable versus unstable social hierarchies. in *Primate social conflict* 171–204 (State University of New York Press, Albany, NY, US, 1993).
170. Bartolomucci, A. Social stress, immune functions and disease in rodents. *Front. Neuroendocrinol.* **28**, 28–49 (2007).
171. Abbott, D. H. *et al.* Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. *Horm. Behav.* **43**, 67–82 (2003).
172. Williamson, C. M., Lee, W., Romeo, R. D. & Curley, J. P. Social context-dependent relationships between mouse dominance rank and plasma hormone levels. *Physiol. Behav.* **171**, 110–119 (2017).
173. LeClair, K. B. *et al.* Individual history of winning and hierarchy landscape influence stress susceptibility in mice. *eLife* **10**, e71401 (2021).
174. Björkqvist, K. Social defeat as a stressor in humans. *Physiol. Behav.* **73**, 435–442 (2001).
175. Martinez, M., Calvo-Torrent, A. & Pico-Alfonso, M. A. Social defeat and subordination as models of social stress in laboratory rodents: A review. *Aggress. Behav.* **24**, 241–256 (1998).

176. Toyoda, A. Social defeat models in animal science: What we have learned from rodent models. *Anim. Sci. J.* **88**, 944–952 (2017).
177. Allen, A. P., Kennedy, P. J., Cryan, J. F., Dinan, T. G. & Clarke, G. Biological and psychological markers of stress in humans: Focus on the Trier Social Stress Test. *Neurosci. Biobehav. Rev.* **38**, 94–124 (2014).
178. Takahashi, A., Flanigan, M. E., McEwen, B. S. & Russo, S. J. Aggression, Social Stress, and the Immune System in Humans and Animal Models. *Front. Behav. Neurosci.* **12**, (2018).
179. Choi, G.-Y. Organizational Impacts on the Secondary Traumatic Stress of Social Workers Assisting Family Violence or Sexual Assault Survivors. *Adm. Soc. Work* **35**, 225–242 (2011).
180. Dulka, B. N., Bress, K. S., Grizzell, J. A. & Cooper, M. A. Social Dominance Modulates Stress-induced Neural Activity in Medial Prefrontal Cortex Projections to the Basolateral Amygdala. *Neuroscience* **388**, 274–283 (2018).
181. Wickham, S., Taylor, P., Shevlin, M. & Bentall, R. P. The Impact of Social Deprivation on Paranoia, Hallucinations, Mania and Depression: The Role of Discrimination Social Support, Stress and Trust. *PLOS ONE* **9**, e105140 (2014).
182. van den Berg, C. L. *et al.* Play is indispensable for an adequate development of coping with social challenges in the rat. *Dev. Psychobiol.* **34**, 129–138 (1999).
183. Pellis, S. M. & Pellis, V. C. Rough-and-Tumble Play and the Development of the Social Brain. *Curr. Dir. Psychol. Sci.* **16**, 95–98 (2007).

184. Vanderschuren, L. J. M. J. & Trezza, V. What the Laboratory Rat has Taught us About Social Play Behavior: Role in Behavioral Development and Neural Mechanisms. in *The Neurobiology of Childhood* (eds. Andersen, S. L. & Pine, D. S.) 189–212 (Springer, Berlin, Heidelberg, 2014). doi:10.1007/7854_2013_268.
185. Luczynski, P., Moquin, L. & Gratton, A. Chronic stress alters the dendritic morphology of callosal neurons and the acute glutamate stress response in the rat medial prefrontal cortex. *Stress* **18**, 654–667 (2015).
186. Biro, L. *et al.* Structural and functional alterations in the prefrontal cortex after post-weaning social isolation: relationship with species-typical and deviant aggression. *Brain Struct. Funct.* **222**, 1861–1875 (2017).
187. Kyle, S. C., Burghardt, G. M. & Cooper, M. A. Development of social play in hamsters: Sex differences and their possible functions. *Brain Res.* **1712**, 217–223 (2019).
188. Burleson, C. A. *et al.* Social Play in Juvenile Hamsters Alters Dendritic Morphology in the Medial Prefrontal Cortex and Attenuates Effects of Social Stress in Adulthood. *Behav. Neurosci.* **130**, 437–447 (2016).
189. Burghardt, G. M. & Pellis, S. M. New directions in studying the evolution of play. in *The Cambridge handbook of play: Developmental and disciplinary perspectives* 11–29 (Cambridge University Press, New York, NY, US, 2019).
190. Onyekwere, D. I. & Ramírez, J. M. Play fighting versus serious fighting in golden hamsters (*Mesocricetus auratus*). *Bull. Psychon. Soc.* **31**, 503–506 (1993).

191. Heidbreder, C. A. & Groenewegen, H. J. The medial prefrontal cortex in the rat: evidence for a dorso-ventral distinction based upon functional and anatomical characteristics. *Neurosci. Biobehav. Rev.* **27**, 555–579 (2003).
192. Miller, K. D., Pinto, D. J. & Simons, D. J. Processing in layer 4 of the neocortical circuit: new insights from visual and somatosensory cortex. *Curr. Opin. Neurobiol.* **11**, 488–497 (2001).
193. Yang, C. R., Seamans, J. K. & Gorelova, N. Electrophysiological and morphological properties of layers V–VI principal pyramidal cells in rat prefrontal cortex in vitro. *J. Neurosci.* **16**, 1904–1921 (1996).
194. Wang, Y. *et al.* Heterogeneity in the pyramidal network of the medial prefrontal cortex. *Nat. Neurosci.* **9**, 534–542 (2006).
195. Marek, R., Strobel, C., Bredy, T. W. & Sah, P. The amygdala and medial prefrontal cortex: partners in the fear circuit. *J. Physiol.* **591**, 2381–2391 (2013).
196. Van De Werd, H. J. J. M., Rajkowska, G., Evers, P. & Uylings, H. B. M. Cytoarchitectonic and chemoarchitectonic characterization of the prefrontal cortical areas in the mouse. *Brain Struct. Funct.* **214**, 339–353 (2010).
197. Dulka, B. N. *et al.* Chemogenetic activation of an infralimbic cortex to basolateral amygdala projection promotes resistance to acute social defeat stress. *Sci. Rep.* **10**, 6884 (2020).
198. Radley, J. J., Gosselink, K. L. & Sawchenko, P. E. A Discrete GABAergic Relay Mediates Medial Prefrontal Cortical Inhibition of the Neuroendocrine Stress Response. *J. Neurosci.* **29**, 7330–7340 (2009).

199. Delli Pizzi, S. *et al.* GABA content within medial prefrontal cortex predicts the variability of fronto-limbic effective connectivity. *Brain Struct. Funct.* **222**, 3217–3229 (2017).
200. Euston, D. R., Gruber, A. J. & McNaughton, B. L. The Role of Medial Prefrontal Cortex in Memory and Decision Making. *Neuron* **76**, 1057–1070 (2012).
201. Etkin, A., Egner, T. & Kalisch, R. Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends Cogn. Sci.* **15**, 85–93 (2011).
202. Tzschentke, T. M. The medial prefrontal cortex as a part of the brain reward system. *Amino Acids* **19**, 211–219 (2000).
203. Takahashi, A., Nagayasu, K., Nishitani, N., Kaneko, S. & Koide, T. Control of Intermale Aggression by Medial Prefrontal Cortex Activation in the Mouse. *PLOS ONE* **9**, e94657 (2014).
204. Lotze, M., Veit, R., Anders, S. & Birbaumer, N. Evidence for a different role of the ventral and dorsal medial prefrontal cortex for social reactive aggression: An interactive fMRI study. *NeuroImage* **34**, 470–478 (2007).
205. Lemogne, C., Delaveau, P., Freton, M., Guionnet, S. & Fossati, P. Medial prefrontal cortex and the self in major depression. *J. Affect. Disord.* **136**, e1–e11 (2012).
206. Maier, S. F. & Watkins, L. R. Stressor controllability and learned helplessness: The roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. *Neurosci. Biobehav. Rev.* **29**, 829–841 (2005).

207. Marques, D. B., Ruggiero, R. N., Bueno-Junior, L. S., Rossignoli, M. T. & Leite, J. P. Prediction of Learned Resistance or Helplessness by Hippocampal-Prefrontal Cortical Network Activity during Stress. *J. Neurosci.* **42**, 81–96 (2022).
208. Wang, M., Perova, Z., Arenkiel, B. R. & Li, B. Synaptic Modifications in the Medial Prefrontal Cortex in Susceptibility and Resilience to Stress. *J. Neurosci.* **34**, 7485–7492 (2014).
209. Johnson, S. B. *et al.* Prefrontal–Bed Nucleus Circuit Modulation of a Passive Coping Response Set. *J. Neurosci.* **39**, 1405–1419 (2019).
210. Biro, L. *et al.* Task Division within the Prefrontal Cortex: Distinct Neuron Populations Selectively Control Different Aspects of Aggressive Behavior via the Hypothalamus. *J. Neurosci. Off. J. Soc. Neurosci.* **38**, 4065–4075 (2018).
211. Cheng, S.-Y., Taravosh-Lahn, K. & Delville, Y. Neural circuitry of play fighting in golden hamsters. *Neuroscience* **156**, 247–256 (2008).
212. Weinberg, M. S., Johnson, D. C., Bhatt, A. P. & Spencer, R. L. Medial prefrontal cortex activity can disrupt the expression of stress response habituation. *Neuroscience* **168**, 744–756 (2010).
213. Radley, J. J., Anderson, R. M., Hamilton, B. A., Alcock, J. A. & Romig-Martin, S. A. Chronic Stress-Induced Alterations of Dendritic Spine Subtypes Predict Functional Decrements in an Hypothalamo–Pituitary–Adrenal-Inhibitory Prefrontal Circuit. *J. Neurosci.* **33**, 14379–14391 (2013).
214. Cho, J.-H., Deisseroth, K. & Bolshakov, V. Y. Synaptic Encoding of Fear Extinction in mPFC-amygdala Circuits. *Neuron* **80**, 1491–1507 (2013).

215. Gutzeit, V. A. *et al.* Optogenetic reactivation of prefrontal social neural ensembles mimics social buffering of fear. *Neuropsychopharmacology* **45**, 1068–1077 (2020).
216. Likhtik, E., Stujenske, J. M., A Topiwala, M., Harris, A. Z. & Gordon, J. A. Prefrontal entrainment of amygdala activity signals safety in learned fear and innate anxiety. *Nat. Neurosci.* **17**, 106–113 (2014).
217. Vouimba, R.-M. & Maroun, M. Learning-Induced Changes in mPFC–BLA Connections After Fear Conditioning, Extinction, and Reinstatement of Fear. *Neuropsychopharmacology* **36**, 2276–2285 (2011).
218. Xu, H. *et al.* A Disinhibitory Microcircuit Mediates Conditioned Social Fear in the Prefrontal Cortex. *Neuron* **102**, 668–682.e5 (2019).
219. Leussis, M. P. & Andersen, S. L. Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse* **62**, 22–30 (2008).
220. Bell, H. C., Pellis, S. M. & Kolb, B. Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. *Behav. Brain Res.* **207**, 7–13 (2010).
221. Pellis, S. M., Pellis, V. C., Ham, J. R. & Stark, R. A. Play fighting and the development of the social brain: The rat’s tale. *Neurosci. Biobehav. Rev.* **145**, 105037 (2023).
222. Himmler, B. T. *et al.* Juvenile social experience and differential age-related changes in the dendritic morphologies of subareas of the prefrontal cortex in rats. *Synapse* **72**, e22022 (2018).

223. Yamamuro, K. *et al.* A prefrontal–paraventricular thalamus circuit requires juvenile social experience to regulate adult sociability in mice. *Nat. Neurosci.* **23**, 1240–1252 (2020).
224. Southwick, S. M. & Charney, D. S. *Resilience: The Science of Mastering Life's Greatest Challenges*. (Cambridge University Press, 2018).
225. Franklin, T. B., Saab, B. J. & Mansuy, I. M. Neural Mechanisms of Stress Resilience and Vulnerability. *Neuron* **75**, 747–761 (2012).
226. Bhatnagar, S. Rethinking stress resilience. *Trends Neurosci.* **44**, 936–945 (2021).
227. Charney, D. S. Psychobiological mechanisms of resilience and vulnerability: implications for successful adaptation to extreme stress. *Am. J. Psychiatry* **161**, 195–216 (2004).
228. Virgin Jr., C. E. & Sapolsky, R. M. Styles of male social behavior and their endocrine correlates among low-ranking baboons. *Am. J. Primatol.* **42**, 25–39 (1997).
229. Huhman, K. L. Social conflict models: can they inform us about human psychopathology? *Horm. Behav.* **50**, 640–646 (2006).
230. Cooper, M. A. *et al.* Gonadal steroid hormone receptors in the medial amygdala contribute to experience-dependent changes in stress vulnerability. *Psychoneuroendocrinology* **129**, 105249 (2021).
231. Roozendaal, B., McEwen, B. S. & Chattarji, S. Stress, memory and the amygdala. *Nat. Rev. Neurosci.* **10**, 423–433 (2009).
232. Zhang, W.-H., Zhang, J.-Y., Holmes, A. & Pan, B.-X. Amygdala Circuit Substrates for Stress Adaptation and Adversity. *Biol. Psychiatry* **89**, 847–856 (2021).

233. Raam, T. & Hong, W. Organization of neural circuits underlying social behavior: A consideration of the medial amygdala. *Curr. Opin. Neurobiol.* **68**, 124–136 (2021).
234. O’Connell, L. A. & Hofmann, H. A. Evolution of a Vertebrate Social Decision-Making Network. *Science* **336**, 1154–1157 (2012).
235. Bennur, S. *et al.* Stress-induced spine loss in the medial amygdala is mediated by tissue-plasminogen activator. *Neuroscience* **144**, 8–16 (2007).
236. Chen, F. *et al.* Increased Inhibition of the Amygdala by the mPFC may Reflect a Resilience Factor in Post-traumatic Stress Disorder: A Resting-State fMRI Granger Causality Analysis. *Front. Psychiatry* **9**, (2018).
237. Estrada, C. M. *et al.* Estrogen signaling in the medial amygdala decreases emotional stress responses and obesity in ovariectomized rats. *Horm. Behav.* **98**, 33–44 (2018).
238. Lebow, M. A. & Chen, A. Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatry* **21**, 450–463 (2016).
239. Davis, M., Walker, D. L., Miles, L. & Grillon, C. Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. *Neuropsychopharmacology* **35**, 105–135 (2010).
240. Blanchard, R. J., Shepherd, J. K., John Rodgers, R., Magee, L. & Caroline Blanchard, D. Attenuation of antipredator defensive behavior in rats following chronic treatment with imipramine. *Psychopharmacology (Berl.)* **110**, 245–253 (1993).
241. Miller, S. M., Marcotulli, D., Shen, A. & Zweifel, L. S. Divergent medial amygdala projections regulate approach-avoidance conflict behavior. *Nat. Neurosci.* **22**, 565–575 (2019).

242. Nordman, J., Ma, X. & Li, Z. Traumatic Stress Induces Prolonged Aggression Increase through Synaptic Potentiation in the Medial Amygdala Circuits. *eNeuro* **7**, ENEURO.0147-20.2020 (2020).
243. Nordman, J. C. *et al.* Potentiation of Divergent Medial Amygdala Pathways Drives Experience-Dependent Aggression Escalation. *J. Neurosci.* **40**, 4858–4880 (2020).
244. Vital, C. & Martins, E. P. Socially-Central Zebrafish Influence Group Behavior More than Those on the Social Periphery. *PLOS ONE* **8**, e55503 (2013).
245. Clutton-brock, T. H. & Parker, G. A. Sexual coercion in animal societies. *Anim. Behav.* **49**, 1345–1365 (1995).
246. Conradt, L. & Roper, T. J. Group decision-making in animals. *Nature* **421**, 155–158 (2003).
247. Ioannou, C., Couzin, I., James, R., Croft, D. & Krause, J. Social Organisation and Information Transfer in Schooling Fish. in 217–239 (2011).
doi:10.1002/9781444342536.ch10.
248. Laland, K. N. & Williams, K. Shoaling generates social learning of foraging information in guppies. *Anim. Behav.* **53**, 1161–1169 (1997).
249. Lewis, M. A. *et al.* Learning and Animal Movement. *Front. Ecol. Evol.* **9**, (2021).
250. Killen, S. S. *et al.* The Potential for Physiological Performance Curves to Shape Environmental Effects on Social Behavior. *Front. Physiol.* **12**, (2021).
251. Yang, F. *et al.* Cadmium exposure disrupts the olfactory sensitivity of fire ants to semiochemicals. *Environ. Pollut.* **287**, 117359 (2021).

252. LeFauve, M. K. & Connaughton, V. P. Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish. *Curr. Zool.* **63**, 221–227 (2017).
253. Ward, A. J. W., Duff, A. J., Horsfall, J. S. & Currie, S. Scents and scents-ability: pollution disrupts chemical social recognition and shoaling in fish. *Proc. R. Soc. B Biol. Sci.* **275**, 101–105 (2008).
254. McElroy, K. N., Beakes, M. P. & Merz, J. E. Hide and seek: Turbidity, cover, and ontogeny influence aggregation behavior in juvenile salmon. *Ecosphere* **9**, e02175 (2018).
255. van der Sluijs, I. *et al.* Communication in troubled waters: responses of fish communication systems to changing environments. *Evol. Ecol.* **25**, 623–640 (2011).
256. Fleisch, V. C. & Neuhauss, S. C. F. Visual behavior in zebrafish. *Zebrafish* **3**, 191–201 (2006).
257. van Oldenborgh, G. J. *et al.* Attribution of the Australian bushfire risk to anthropogenic climate change. *Nat. Hazards Earth Syst. Sci.* **21**, 941–960 (2021).
258. Williamson, C. E., Dodds, W., Kratz, T. K. & Palmer, M. A. Lakes and streams as sentinels of environmental change in terrestrial and atmospheric processes. *Front. Ecol. Environ.* **6**, 247–254 (2008).
259. Burrows, M. T. *et al.* The Pace of Shifting Climate in Marine and Terrestrial Ecosystems. *Science* **334**, 652–655 (2011).
260. Guerin, G. R., Wen, H. & Lowe, A. J. Leaf morphology shift linked to climate change. *Biol. Lett.* **8**, 882–886 (2012).

261. Cureton, J. C. & Broughton, R. E. Rapid morphological divergence of a stream fish in response to changes in water flow. *Biol. Lett.* **10**, 20140352 (2014).
262. Kruitwagen, G., Hecht, T., Pratap, H. B. & Wendelaar Bonga, S. E. Changes in morphology and growth of the mudskipper (*Periophthalmus argentilineatus*) associated with coastal pollution. *Mar. Biol.* **149**, 201–211 (2006).
263. Jacquin, L., Petitjean, Q., Côte, J., Laffaille, P. & Jean, S. Effects of Pollution on Fish Behavior, Personality, and Cognition: Some Research Perspectives. *Front. Ecol. Evol.* **8**, (2020).
264. Beever, E. A. *et al.* Behavioral flexibility as a mechanism for coping with climate change. *Front. Ecol. Environ.* **15**, 299–308 (2017).
265. Seebacher, F. & Post, E. Climate change impacts on animal migration. *Clim. Change Responses* **2**, 5 (2015).
266. Zhang, H. *et al.* Hypoxia-driven changes in the behavior and spatial distribution of pelagic fish and mesozooplankton in the northern Gulf of Mexico. *J. Exp. Mar. Biol. Ecol.* **381**, S80–S91 (2009).
267. Inderjit & Weston, L. A. Are Laboratory Bioassays for Allelopathy Suitable for Prediction of Field Responses? *J. Chem. Ecol.* **26**, 2111–2118 (2000).
268. Underwood, A. J. Toxicological testing in laboratories is not ecological testing of toxicology. *Hum. Ecol. Risk Assess. Int. J.* **1**, 178–182 (1995).
269. Kellman, J. & Radwan, K. Towards an expanded neuroscientific understanding of social play. *Neurosci. Biobehav. Rev.* **132**, 884–891 (2022).
270. Sah, P. Fear, Anxiety, and the Amygdala. *Neuron* **96**, 1–2 (2017).

271. Tovote, P., Fadok, J. P. & Lüthi, A. Neuronal circuits for fear and anxiety. *Nat. Rev. Neurosci.* **16**, 317–331 (2015).
272. Fawcett, J. W., Oohashi, T. & Pizzorusso, T. The roles of perineuronal nets and the perinodal extracellular matrix in neuronal function. *Nat. Rev. Neurosci.* **20**, 451–465 (2019).
273. Guadagno, A., Belliveau, C., Mechawar, N. & Walker, C.-D. Effects of Early Life Stress on the Developing Basolateral Amygdala-Prefrontal Cortex Circuit: The Emerging Role of Local Inhibition and Perineuronal Nets. *Front. Hum. Neurosci.* **15**, (2021).
274. Guadagno, A., Verlezza, S., Long, H., Wong, T. P. & Walker, C.-D. It Is All in the Right Amygdala: Increased Synaptic Plasticity and Perineuronal Nets in Male, But Not Female, Juvenile Rat Pups after Exposure to Early-Life Stress. *J. Neurosci.* **40**, 8276–8291 (2020).
275. Catale, C. *et al.* Early-life social stress induces permanent alterations in plasticity and perineuronal nets in the mouse anterior cingulate cortex. *Eur. J. Neurosci.* **56**, 5763–5783 (2022).
276. Jakovljevic, A. *et al.* The impact of early life maternal deprivation on the perineuronal nets in the prefrontal cortex and hippocampus of young adult rats. *Front. Cell Dev. Biol.* **10**, (2022).
277. Gildawie, K. R., Honeycutt, J. A. & Brenhouse, H. C. Region-specific Effects of Maternal Separation on Perineuronal Net and Parvalbumin-expressing Interneuron Formation in Male and Female Rats. *Neuroscience* **428**, 23–37 (2020).

278. Pesarico, A. P. *et al.* Chronic Stress Modulates Interneuronal Plasticity: Effects on PSA-NCAM and Perineuronal Nets in Cortical and Extracortical Regions. *Front. Cell. Neurosci.* **13**, (2019).
279. Li, X. *et al.* Perineuronal Nets Alterations Contribute to Stress-Induced Anxiety-Like Behavior. *Mol. Neurobiol.* (2023) doi:10.1007/s12035-023-03596-1.
280. Santiago, A. N., Lim, K. Y., Opendak, M., Sullivan, R. M. & Aoki, C. Early life trauma increases threat response of peri-weaning rats, reduction of axo-somatic synapses formed by parvalbumin cells and perineuronal net in the basolateral nucleus of amygdala. *J. Comp. Neurol.* **526**, 2647–2664 (2018).
281. Wyrofsky, R. R. *et al.* Endocannabinoids, stress signaling, and the locus coeruleus-norepinephrine system. *Neurobiol. Stress* **11**, 100176 (2019).
282. Grafe, L. A. *et al.* Passive Coping Strategies During Repeated Social Defeat Are Associated With Long-Lasting Changes in Sleep in Rats. *Front. Syst. Neurosci.* **14**, (2020).

CHAPTER TWO
A PAIR OF CADMIUM-EXPOSED ZEBRAFISH AFFECT SOCIAL BEHAVIOR
OF THE UN-EXPOSED MAJORITY

Abstract

To account for global contamination events, we must identify direct and indirect pollutant effects. Although pollutants can have direct effects on individuals, it is unknown how a few contaminated individuals affect groups, a widespread social organization. We show environmentally relevant levels of cadmium (Cd) can have indirect social effects revealed in the social context of a larger group. Cd-contaminated individuals had poor vision and more aggressive responses, but no other behavioral effects. The presence of experienced Cd-exposed pairs in the groups had an indirect effect on the un-exposed individual's social interactions leading to the shoal becoming bolder and moving closer to a novel object than control groups. Because a few directly affected individuals could indirectly affect social behavior of the un-exposed majority, we believe that such acute but potentially important heavy metal toxicity could inform reliable predictions about the consequences of their use in a changing world.

1. Introduction

Pollution is one of the greatest challenges to the Anthropocene Epoch, threatening the stability of ecosystems and human societies ^{1,2}. To protect ecosystems and human health, we must identify the direct and indirect effects of pollution. Modified behavior represents a suite of physiological and epigenetic changes and links individual-, group-, and ecosystem-level processes, thereby representing a sensitive marker for assessing impacts of contaminants ^{3,4}. Recent studies implicate sublethal effects of pollution in

behavioral deficits, sensory impairments, learning disabilities, and achievement gaps, and ultimately, negative food web effects and ecological cascades ⁵⁻⁸. Although pollutants can have multi-level sublethal effects, it is unknown how a few contaminated individuals affect groups, social organizations that are pervasive among human and non-human animals ⁹. Here we ask, can a few directly contaminated individuals indirectly influence the behavior of an un-exposed majority.

Cadmium (Cd) threatens wildlife and human health. Epidemiological studies link some non-communicable diseases and ecological effects to metals such as Cd ^{8,10,11}. In addition to documented industrial Cd exposure ¹¹, Cd toxicity is associated with contaminated food, water, and air ⁸, and is of significant exposure concern for tobacco-smoking populations ¹¹. Once accumulated, Cd has a biological half-life that can reach decades ^{7,11}. Wildlife are exposed to Cd through similar exposure routes as humans ¹⁰. For aquatic organisms, water-borne exposures are the most common route of exposure with adverse effects expressed at lower concentration than in humans. Wildlife exposed to Cd show heightened aggression ¹², sensory deficits ^{13,14}, and altered species distribution ¹⁰. Elevated Cd exposure in humans is linked to a variety of multi-level sublethal effects such as: individual reading difficulties, behavioral problems, reduced attention, lower psychomotor speed, memory deficits, antisocial behavior, and altered demographics ^{8,11}. Therefore, it is important to understand how Cd exposure influences interactions between exposed and un-exposed individuals.

Organisms exhibit a wide range of tolerance to toxicants with some individuals exhibiting adverse effects, but others showing no observable effects^{15–18}. Individual and systemic susceptibility is a key risk factor in phenotypic alterations induced by contamination events^{8,11,16,19–21}. For example, first-time breeding female birds are more susceptible to pollutants than are other birds²², and dominant fish accumulate more Cd in their gill tissue than do subordinates²³. Similarly, in humans, women show more adverse effects of Cd than men^{7,8}. Thus, it is important to understand the effects of Cd on male and female individuals. For social animals, direct pollutant effects on less tolerant individuals may have indirect impacts by altering the behavior of the group as a whole.

Social interactions can be influenced by a range of biotic and abiotic factors. In animal groups, there is evidence that a few individuals can influence disease spread, group travel, decision-making, and learning^{24,25}. Thus, these individuals can exert disproportionate influence over social networks within groups. Influential individuals may have physiological advantages²⁶, more experience²⁴, greater social centrality in the social network²⁵ or higher cognitive competency²⁷. These influential individuals may have leadership roles that lead to coercion²⁸, information transfer^{29,30}, conformity³¹, and social learning³² in group members. Abiotic factors such as contaminants may also affect leadership and social dynamics²⁶. For example, turbid environments increase risk perception and limit cohesion during collective foraging in stickleback³³.

Sensory systems are a gateway for social interactions. They guide coordination and synchronization in collective movement³⁴, influence the propagation of information

³⁵, inform mate choice ³⁶, and help to communicate social status ³⁷. In coordinated and synchronized animal groups, vision is important for assessing the behavioral movements of their neighbors ³⁸, determining spacing patterns ³⁹, and informing collective antipredator defense ⁴⁰. Visual cues are also important for determining shoaling preferences, as fish prefer others with similar striping patterns ⁴¹ and color ⁴². In a contamination event, sensory systems are prime targets, as they are in direct contact with the external environment. When a sensory deficit is induced by a pollutant, it can disrupt social recognition ⁴³, reduce shoaling ⁴⁴, decrease charging ⁴⁵, and alter communication ⁴⁶. Contaminants can also block channels of communication by altering the ability of an animal to receive the signal due to shifts in receptor sensitivity. For example, increased turbidity in lakes was linked to changes in signaling colors, and to shifts in visual physiology ⁴⁷. Alternatively, groups can adapt to these changes in sensory ability by modifying their behavior. For example, fish in turbid environments shift their reliance from vision to olfactory cues ⁴⁵, reduce cohesion, and have a longer latency to forage in novel environments ³³. These behavioral shifts may be due to differences in perception of risk and employing more effective strategies under altered conditions. For example, in clear environments dilution effects may lead to more cohesive shoals as sighted predators can easily hunt prey, but in turbid or low light environments the vision of sighted predators is obscured so the prey's strategy changes to be more dispersed and spend more time in refuge ³³.

Impairments in sensory systems may also alter interactions with familiar stimuli in the environment. The change in behavior may occur through alteration in risk-assessment, impeding recognition ¹³ or disruption of learning and memory ^{5,6,48}. For example, freshwater fishes exposed to pollutants that interfered with olfactory systems showed atypical anti-predatory responses, moving more quickly towards threatening stimuli ⁴⁹, and decreased sensitivity to conspecifics that previously elicited aggressive responses ⁵⁰. Pollutant exposure can also lead animals to change their approach-avoidance responses ⁵¹. For example, natural populations of fish exposed to an ecotoxicological agent were bolder and more active than were un-exposed fish, approaching food sources more often and avoiding social interactions more than did un-treated fish ⁵². In the present study, we assessed if contaminated individuals could influence a larger group's response to novel stimuli and social interactions.

Here we tested whether fish directly exposed to Cd have indirect effects on group behavior of an un-exposed majority. We also asked whether the effects of a few contaminated individuals could be caused by changes in vision. Documenting direct and indirect effects of Cd in an important biomedical, developmental, and toxicological model such as the zebrafish (*Danio rerio*) expands our ability to identify mechanisms of pollutant action and translate them to humans and other species. Zebrafish possesses well-documented social behavioral repertoires and diverse sensory systems that reveal contaminant effects, including sensory impairments, neurotoxicity, oxidative stress, endocrine disruption, and epigenetic effects ^{53,54}, some of which are also associated with

Cd exposure¹¹. Vision, a prime target of Cd is a key sensory modality for zebrafish, as it informs shoaling behavior, neighbor preferences, anti-predatory strategies, and general approach-avoidance responses⁵⁵. To test for the presence of direct and indirect effects of Cd on zebrafish behavior, we formed groups of familiar fish and then selected a pair of fish from each group, to acutely expose to an ecologically relevant concentration of Cd or control conditions. We then tested the Cd-exposed and control pairs' response to a novel stimulus. We then returned the pairs to the groups and quantified differences between pairs and groups with control and Cd-treated fish in terms of their responses to novel stimuli (or boldness), aggressive social interactions (i.e., advances, chases, mouth contacts), social networks, activity, and cohesion. This experimental design allowed us to test if experienced contaminated pairs could influence the behavior of un-experienced group members. These behavior patterns have ecological and evolutionary importance and may be predictive of how affected individuals can influence group behavior and how groups may in turn respond abnormally to altered environments^{18,56,57}. To assess the direct action of Cd through the visual system, we also examined the optomotor response of Cd-exposed and control fish.

2. Method

2.1. Subjects

We used a wild-type, outbred, Scientific Hatcheries zebrafish line that was bred by Aquatica Biotech (Florida, USA). Several recent studies have used this zebrafish strain^{58–61}. We housed the fish under standard conditions: 38 L (10 gallon) aquaria, 28o

C, 10:14 h light/dark cycle, and ad libitum flake food. The water quality characteristics and assessment method for the system water was as follows: Cd was below the detection limit of $< 0.1 \mu\text{g/L}$ (US EPA Method 200.8), total Chlorine: 0.07 mg/L (SM 4500-Cl G), Free Chlorine: 0.00 mg/L (SM 4500-Cl G), Calcium: 34 mg/L , Hardness as CaCO_3 : 40 mg/L (SM 2340 C), Ammonia: 0.04 mg/L (SM 45000 – NH_3), pH: 6.8 (API Freshwater Test Kit), Iron: $< 0.01 \text{ mg/L}$ (SM 3111 B), Manganese: $< 0.01 \text{ mg/L}$ (SM 3111 B). These water quality parameters are recommended for maintaining typical behavior and health of zebrafish⁶² and are below the US EPA specific maximum contaminant level of $1.8 \mu\text{g/L}$ Cd for acute (24 h) exposures for aquatic life (US EPA 820-R-16–002) and $5 \mu\text{g/L}$ Cd for drinking water⁶³.

2.2. Procedure

Direct and indirect effects of cadmium on boldness, activity, cohesion, and social behavior

We formed 36 groups of adult zebrafish (6 fish per group) and allowed them to become familiar with each other over one week. We then tested each group twice in an experimental arena consisting of an 18.9 L ($43 \times 23 \text{ cm}$) aquarium with gravel and a buried filter with an overhead Logitech c525 HD webcam. To construct a novel stimulus, we concealed a 100 ml Eppendorf conical plastic tube (diameter of opening = 57 mm, height = 76 mm, length of = 76 mm) in the gravel on one side of the arena. The tube was attached to clear fishing line so that it could be pulled swiftly from the gravel bottom. To present the stimulus, we pulled the fishing line, gently raising and shaking the attached

tube for 30 s, before removing it from the tank. Similar novel stimuli presentations have been used previously ^{64,65}. From video recordings, we scored the number of fish that approached or stayed away from this novel stimulus.

We measured and marked each fish via fin clipping for individual identification prior to testing. We confirmed that fin clips did not alter the movement of experimental and control fish. We then selected two fish of the same size (a male and a female) from each group for treatment. We exposed half of these pairs to an environmentally relevant concentration of Cd (1 µg/L) by placing each pair in a 1 L beaker of Cd-water overnight for a total of 17 h. These Cd-treated fish are referred to as the directly Cd-exposed fish. Concentrations of Cd in our study were within the range of levels found in the freshwaters of India, a native habitat of zebrafish ^{66,67}. As a control, we placed the remaining pairs in 1 L beakers of water for 17 h. The following morning, we rinsed each of the 36 pairs of (Cd-exposed and water-exposed) fish in freshwater to remove any unbound Cd and control for handling effects. To rinse the fish, we gently netted them, allowed the excess water to drip from the net and then placed them in a new beaker of freshwater. This process was done twice with a new net and beaker. After the rinsing, we placed them into their respective experimental arenas for a 1 h acclimation period. Following the acclimation period, we tested the direct impact of the Cd exposure by measuring the response of each treated pair to the novel stimulus. We then reburied the stimulus in the gravel and added the remaining four fish to re-form each group of six. The four fish added to the tank with Cd-treated fish are referred to as indirectly Cd-exposed

fish, and the fish added to the tank with the control pairs are termed indirectly Control-exposed fish. On the following day (after 24 h), we tested the indirect effect of the Cd exposure by repeating the assay, gently lifting the novel stimulus to test the behavioral response of each full group of six fish. We recorded each pair or group of six fish from above.

We first counted the number of “advances”, “chases”, “mouth contacts”, and lines crossed by any fish in the shoal during the 30 s immediately prior to the presentation of the novel stimulus from video recordings. We defined an “advance” as any event in which a fish rapidly approached a stationary fish, “chases” were any episode in which one fish accelerated towards a fleeing fish, and “mouth contacts” were instances where there was direct mouth-to-body contact between two fish. All three behaviors are likely forms of aggression or socially investigative behavior, with the intensity of the interaction increasing from “advances” to “chases” and “mouth contacts”. To measure responses to the novel stimulus, we counted the number of fish on each side of the arena (same or opposite side from the stimulus) at 10 s after the stimulus presentation and assessed the nearest-neighbor distance (minimum distance between any two individuals in the group) and the shoal diameter (maximum distance between any two individuals in the group) at that single instance in time. We also measured activity of the fish in the pair and group condition by dividing the tank into a grid and assessing the average number of lines crossed by each fish per second. Each square in the grid measured about 1 fish length. We then used independent sample’s t-test (two-tailed) to compare groups of fish

containing Cd- and water-treated individuals in terms of the number of fish on the stimulus side of the aquarium, nearest-neighbor distance, shoal diameter, and line crosses. We conducted all statistical analyses in R with the “base”, “blme4”, “lme4”, and “emmeans” packages ^{68–71}. We used the ‘dispersion_glmer’ function from the “blme4” package to ensure that the residual variance was below a ratio of 1.5 in order to determine that the data fit a negative binomial distribution ⁷². We then used a repeated measures generalized linear mixed models fit to a negative binomial distribution with Treatment (Cd or Control) and Group Size (Pair or Group) as independent variables and tank ID as a random factor to assess if Cd pairs had a disproportionate effect on group advances, chases and mouth contacts, when compared to control pairs and groups. For significant interactions, we used the ‘emmeans’ function with a Tukey adjustment to do post hoc pairwise comparisons. Due to technology failures, we dropped two videos, one each in the pair (n = 17 Cd, 18 control pairs) and group (n = 17 Cd, 18 control groups) condition.

Multilayer networks of zebrafish groups

To determine who was the initiator of interactions in the shoals, we created directed social networks of each shoal by identifying the initiator of the “chase” during a 120 s sample period three days after the initial Cd exposure. We compared the number of chases initiated across levels of sex (♀, ♂), chemical (Cd, water), and exposure (directly, indirectly). We defined fish as directly exposed if they were placed in Cd solutions or water beakers for 17 h overnight. We classified fish as indirectly exposed if they were left

unexposed, and placed them in groups with the directly exposed pairs. Therefore, indirectly Cd exposed fish were unexposed fish placed in groups with Cd-exposed pairs, and indirectly exposed control fish were unexposed fish placed in groups with water-exposed pairs. Ten tanks of 6 fish from each chemical condition were analyzed. We selected 2 of the 4 indirectly exposed fish in each tank at random for analysis to maintain equal sample sizes in analysis, totaling 40 fish per chemical condition. To determine if the data fit a negative binomial distribution, we used the ‘dispersion_glmmer’ to ensure that the residual variance was below a ratio of 1.5⁷². We then used a generalized linear mixed models fit to a negative binomial distribution using tank ID as a random factor to test if the “chase” networks of control and Cd groups differed. For significant interactions, we used the ‘emmeans’ function with a Tukey adjustment to do post hoc pairwise comparisons. To test whether Cd shoals were more interactive than controls, we compared the network density or the ratio of the number of observed ties to the possible number of ties in the network between the Cd and controls using Welch's two sample t-test.

Direct effects of cadmium on vision

To determine whether our Cd treatment had an impact by influencing vision or visual response behavior, we treated a second set of 76 fish and tested their response in an optomotor assay. As described above, we first placed 38 pairs of fish in 1 L beakers containing either Cd (1 µg/L) or water for 17 – 20 h overnight (n = 38 fish each condition). Next, we rinsed the Cd-exposed and control pairs with system water and

placed each fish individually into a small plastic container (7.7 cm diameter) with shallow system water (3 cm water height). The fish were acclimated for at least 10 mins prior to suspending their container in the center of a rotating visual stimulus. The stimulus was a repeating pattern of black and white vertical bars printed on paper and affixed to a circular drum that rotated at speeds powered and controlled by a motor (Bodine 24 v). We tested each fish at 7 different spatial frequencies (0.1, 0.13, 0.17, 0.2, 0.23, 0.26, 0.29 cycles/deg), created by changing the speed of the rotating drum. We showed individual fish each of the 7 frequencies in a random order during a series of consecutive 2 min trials. We lit the arena with a bright white led light, which was 5600 lumens when measured at the top of the holding cup located in the testing arena.

During the optomotor assay, a human observer scored the responses dichotomously as either (0) no response or (1) a positive response. A positive response was recorded if the fish made 2 full circular turns within 20 s in the direction of the stimulus in the 2 min trial, and other behaviors were labeled no response. We used Repeated-Measures Logistic regressions to test whether Cd- and un-treated fish differed in their visual-motor response. To fit the repeated-measures logistic regression, we used the ‘glmer’ function of the “lme4” package ⁷⁰.

3. Results

3.1. Cd-exposed fish indirectly affect boldness and social behavior, but not cohesion and activity of the un-exposed majority

Cadmium exposure had indirect effects on group boldness (or responses to novel stimuli; Fig. 2.1B), **all figures and tables presented in appendices**) and social interaction (Fig. 2.2 A, 2B, 2 C), but not on cohesion or activity. At 10 s after the novel stimulus was removed from the arena, we found that about 5 of the six fish ($M = 4.71$ fish, $SE = 0.33$) in groups containing Cd-treated fish were on the stimulus side of the arena, whereas the number of fish on the stimulus side of the arena in control groups hovered around chance levels ($M = 2.61$, $SE = 0.46$). That is, groups containing a pair of Cd-treated fish remained near the area of the novel stimulus presentation, whereas control groups did not (Fig. 2.1B; $t(30.47) = 3.71$, $p < 0.01$). The treated pair responses to the novel stimulus showed no difference from the control animals (Fig. 2.1A; $t(32.85) = -0.44$, $p = 0.66$), but an indirect behavior effect on a larger un-treated group was observed when the treated pair was present (Fig. 2.1B).

The social interactions within larger groups were limited by the presence of a Cd-exposed pair (Fig. 2 A, 2B, 2 C). Indirect effects of Cd included differences in advances and mouth contacts (Fig. 2 A, 2 C). We observed larger differences in the Cd and control treatments in the pairs than in the groups for advances (Fig. 2 A). In the group condition, the number of advances was disproportionate for both the groups with the Cd-exposed pairs and the control pairs but in opposite directions. In the groups with the directly Cd-

exposed pairs, the number of chases decreased disproportionately and increased disproportionately for groups with control pairs. This difference led to a significant Treatment $\times$ Group Size interaction for the number of advances ($\chi^2(df=1) = 6.28$, $p = 0.01$). The number of advances was higher in the larger groups than in the pair condition. This difference led to a significant main effect of Group Size for advances ($\chi^2(df=1) = 14.74$, $p < 0.001$). Pairs of directly Cd-exposed and groups with directly Cd-exposed pairs showed more advances than control pairs and groups ($\chi^2(df=1) = 9.27$, $p < 0.01$). The large difference in advances between the pairs of directly Cd-exposed fish and the directly water-exposed (or control) fish likely drove the main effect ($p = 0.01$, *Tukey*). Control pairs showed fewer advances than groups with control pairs ($p < 0.001$, *Tukey*) and groups with directly Cd-exposed pairs ($p < 0.001$, *Tukey*).

In the group condition, the number of mouth contacts scaled with group size for controls with approximately three times as many mouth contacts in the control group (6 fish) as in the pair of directly water-exposed fish (2 fish; Fig. 2 C). The number of mouth contacts was disproportionate for groups with Cd-exposed fish as the groups with directly Cd-exposed fish showed less than 1.5 times as many mouth contacts as the directly Cd-exposed fish. This difference led to a significant main effect of Treatment for the number of mouth contacts ($\chi^2(df=1) = 10.62$, $p < 0.01$). All group sizes with directly Cd-exposed fish showed more mouth contacts than pair and group controls. This led to a significant main effect of Group Size for mouth contacts ($\chi^2(df=1) = 11.21$, $p < 0.001$). There was a significant difference between the control pairs and Cd pairs ($p < 0.01$, *Tukey*), control

groups ($p < 0.01$, *Tukey*), and groups with Cd-treated pairs ($p < 0.001$, *Tukey*). The Treatment $\times$ Group Size interaction for mouth contacts did not reach significance ($\chi^2(df=1) = 2.90$, $p = 0.08$). For chases, there were no significant differences between treatments and group sizes ($\chi^2(df=1) = 0.76$, $p = 0.76$). Cd-treated pairs were more socially investigative than the control pairs and suppressed the social behavior of the larger groups more than groups without a Cd-treated pair.

3.2. Cadmium leads to indirect effects on female zebrafish, but does not affect the connectivity of the social networks

To identify the initiator of chases in the social networks, we created multilayer networks for the Cd and control shoals (Fig. 3A). We found that indirectly and directly Cd exposed female fish initiated more chases than male zebrafish in the same shoal and female control zebrafish (Fig. 3B). Female zebrafish indirectly exposed to Cd ($M = 12.11$, $SE = 2.65$) initiated more chases than other fish, as they initiated approximately two to six times as many chases as the directly exposed Cd female fish and indirectly exposed control female zebrafish ($M = 2.30$, $SE = 1.36$), respectively. When comparing the female zebrafish indirectly exposed to Cd, we found that they initiated two and four times as many chases as the directly exposed male Cd zebrafish ($M = 1.75$, $SE = 1.62$) and directly exposed control male zebrafish, respectively. This difference led to a significant three-way interaction between chemical exposure and sex ($\chi^2(df=1, N = 80) = 5.37$, $p=0.02$). The difference between indirectly exposed Cd female zebrafish and indirectly exposed control female zebrafish ($p<0.001$, *Tukey*). The difference between

the indirectly exposed control female fish and the directly exposed Cd male and female zebrafish was significant ($p < 0.01$ and $p = 0.05$, Tukey, respectively). We found no evidence that Cd affected the network connectivity as Cd ($M = 0.49$, $SE = 0.04$) and control shoals ($M = 0.45$, $SE = 0.05$) had similar network densities ($t(16.61) = 0.54$, $p = 0.59$).

3.3. Cadmium directly affects vision

To determine whether Cd impacted visual motor responses, we used an optomotor response assay (OMR; $n = 38$ fish/treatment; Fig. 4A). A positive response was scored for fish that made 2 full circular turns within 20 s in the direction of the stimulus, in the 2 min trial, and a negative response for those that did not. Cd-treated fish had a lower OMR (Fig. 4B; main effect of Treatment: slope estimate = $1.75 + 0.71$, $z = 2.47$, $p = 0.01$). Logistic regression confirmed that zebrafish responded significantly to the OMR stimulus (intercept estimate = $-4.59 + 0.61$, $z = -7.46$, $p < 0.0001$) with more robust responses at lower spatial frequencies (main effect of frequency: slope estimate = $1.27 + 0.20$, $z = 6.33$, $p < 0.0001$). There was no significant interaction between Treatment and the OMR frequency (Cd x frequency: slope estimate = $-0.15 + 0.25$, $z = -0.69$, $p = 0.54$).

4. Discussion

Zebrafish shoals with a few Cd-treated fish were bolder or approached a novel object more readily than did shoals with un-treated fish, pairs of Cd-exposed and pairs of

un-exposed fish. Pairs of Cd-treated fish influenced the group's social interactions in a sex-dependent manner. Here, we demonstrated that the behavior of an un-treated shoal can be perturbed by the metal-affected behavior of only a few individuals. Such phenotypes are easily missed unless the query is expanded to a larger panel of group behavior including aggression and response to novel stimuli. It is reasonable to expect that some of the group behavior phenotypes detected here could affect the fitness of an affected population or, depending on the ubiquity of the exposure, even the ecology of the affected species (e.g., Figs. 1, 2, 3). These results provide greater insight into the direct and indirect effects of pollutants, especially those not captured with traditional toxicology studies where direct effects and single individuals are often the subject of manipulation and analysis.

Such indirect effects may arise when the direct action of pollutants on sensory systems disrupt social recognition ⁴³, learning ⁷³, aggression ⁷⁴ and communication ⁴⁶ or handicap a few individuals, thereby diluting the risk for the healthy members, perhaps, emboldening uncontaminated individuals (the majority) to engage in more risky behavior or approach novel stimuli ⁷⁵, leading to population and ecosystem-level consequences ^{5,6,48}. Such dilutions of risk occur when male guppies engage in vigorous courtship behavior in the presence of a predator when there are more vigilant females inspecting the predator ⁴⁸, and female mosquitofish forage more efficiently when harassment by solitary males is reduced by aggregating with more females ⁷⁵. The presence of the Cd-treated fish altered the social dynamics of the majority with groups with contaminated

fish being more investigative and aggressive than controls. Our multilayer networks revealed that Cd-induced changes in networks were mediated by female fish. In Cd shoals, female fish initiated more chases with indirectly Cd-exposed female zebrafish showing the greatest number of chases. These sex-dependent effects are consistent with other literature showing that female organisms are more adversely affected by elevated Cd exposure ^{7,8}. Future studies should leverage social network analyses to better understand the sex effects of Cd on social architecture of groups with a few contaminated individuals ^{18,76}.

Our study adds groups as a level of organization for indirect pollutant effects, which already includes transgenerational transfer of toxic effects ⁵³, pesticides triggering colony collapse disorder across populations ⁷⁷, oil exposure of a single individual affecting the cohesion of the unexposed majority ⁷⁸, and ecological cascades through trophic transfer of microplastics ⁷⁹. The enhanced group novel object exploration induced by Cd pairs is important for gauging the effects of anthropogenic change, because animals will come into contact with novel objects and how they interact with them may have consequences for their survival (e.g., “evolutionary trap”) ^{42,80,81}. At a group-level, the reinforcing nature of contaminated individuals in the group due to dilution of risk for the majority could lead to an evolutionary stable strategy where susceptible individuals are maintained in the population ⁸⁰, or a “social trap” where the initial positive impact of contaminated individuals, leads to negative consequences for the group ⁸¹. Identifying

indirect effects at multiple levels of organization helps with assessing and predicting the impact of pollutants.

Cadmium may have acted directly, in part, by altering the visual system of exposed fish (Fig. 4). Adult zebrafish responded to optomotor assay with the response increasing in robustness at lower spatial frequencies. The responsive adult zebrafish moved in the same direction as the moving stimulus typical of a positive optomotor response, which contrasts with other reports that argue zebrafish switch from showing a positive to a negative optomotor response early in life ⁸². Cd-treated zebrafish showed a reduced optomotor response in comparison to uncontaminated fish. Thus, the pollutant likely affected un-treated fish through influencing the interactions of contaminated and control fish, through altering the visual system. In other species, environmental stimuli have affected social interactions through influencing sensory systems. For example, animals that normally aggregate in coordinated clumps, disperse when light levels are low ⁸³, or their ability to see is impaired by debris ⁴⁴. Animals learn how to interact with some stimuli via social interactions ⁸⁴, but those experiences may be disrupted by sensory impairments ¹⁶, and thus interfere with an organism's ability to mount an appropriate behavioral response ^{85,86}. For example, rats rendered anosmic fail to learn about novel food sources from demonstrator rats ⁷³. Here, we found that Cd affects the visual system of the zebrafish, and consequently affected social interactions and group responses. We found that contaminated fish in larger uncontaminated groups influenced the interaction of the group with novel stimuli. Perhaps, the presence of the Cd-treated fish influenced

the tendency of the larger social group to investigate objects in their environment. In other species, pollutant exposure has been shown to influence exploratory behavior and personality traits. For example, the offspring of gestating-mother mice that consumed a pesticide had an increased propensity to explore novel environments⁸⁷ and deficient diets have been shown to affect boldness, with birds having micronutrient deficiencies being less bold⁸⁸. The pollutant may have also interfered with the behavioral repertoire of the fish thereby inducing an abnormal behavioral interaction with the novel stimulus. In other species pollutants have been shown to induce aberrant behaviors in contaminated individuals⁸⁵ with cascading adverse effects on the group^{56,78}. Thus, there are several avenues by which Cd exposure may have influenced the response of the zebrafish to the novel stimulus, and future studies should uncover the mechanisms of action.

Our study corroborates previous findings that low concentrations of Cd affect visually-guided behavior in zebrafish^{14,89} and extends the adverse effects to a shorter exposure period in adult fish. For example, Shankar et al.⁸⁹ demonstrated that larval zebrafish exposed for 5 days post-fertilization (dpf) to 0.183 µg/L of Cd displayed visual deficits and LeFauve et al.,¹⁴ showed that larval zebrafish exposed at 7 dpf showed OMR deficits after 66 h of exposure to 0.5 µg/L in comparison to controls. The concentrations used in these studies are nearly four and 1.5 times lower than the US EPA aquatic water quality criterion for chronic exposures, respectively, which is 0.72 µg/L for Cd exposures lasting four or more days.

Although the ambient water quality criteria for Cd concentrations for acute exposures were recently lowered from 2.0 µg/L in 2001 to 1.8 µg/L in 2016 and raised from 0.25 µg/L to 0.72 µg/L for chronic exposures, these criteria may be insufficient for protecting the behavioral health of fishes. Water quality can alter the effects of Cd through augmenting bioavailability and enhancing toxicity. Historically, toxicology studies have linked water hardness to Cd toxicity with fish in softer water having a lower LC50 than harder water^{90,91}. More recently, there has been more emphasis on the sublethal effects of Cd, which can be observed at lower concentrations of Cd^{14,89}. In comparison to US EPA water hardness for Cd assessments for aquatic organisms (100 mg/L as CaCO₃), the water used in the present study is soft (40 mg/L). Thus, the softer water quality may have enhanced the potency of Cd. Perhaps, the ambient water quality criterion should be revisited to consider the effects on behavioral health.

Vision is a sensory modality that guides the interactions of all sighted organisms. In studies of fish collective movement, vision is important for the maintenance of shoal cohesion³⁴ and decision making. Shoal cohesion is modified when abiotic factors affect vision. In some instances, the fish respond to these changes by dispersing and then regrouping when the abiotic stimulus is removed. For example, fish disperse at night and then regroup during daylight⁹². Under longer-term exposures to low light conditions, fish may evolve a reliance on other sensory modalities to form cohesive shoals^{83,93,94}. In the present study, Cd's effect on vision did not disrupt shoal cohesion as pairs and groups with control and Cd-treated fish were equally cohesive. The visual deficits induced in the

Cd-exposed fish likely altered the group's perception of risk. The presence of the Cd pairs may have reduced the perception of risk in the larger group, because the Cd contaminated fish may have served as handicaps for the un-exposed majority. That is, during a predation event the impaired Cd-exposed fish might be the first targets, thereby reducing the risk for the un-contaminated fish. The dilution effect is usually applied to group size, where individuals in larger groups have a lower individual risk of harm than individuals in smaller groups and thus are bolder, however. There is increasing evidence that individual behavior (e.g., boldness) is influenced by group composition^{95,96}. Here, we showed that boldness did not change with group size (i.e., pair and group control fish showed similar responses, Fig. 1), but did change with group composition. Thus, contamination events that affect group composition may have a stronger impact than group size on risk taking behavior. Others find that zebrafish use social learning to assess risk when presented with a moving stimulus⁶⁴, which may also be occurring in the shoals with the Cd-treated fish. In the present study, the indirectly Cd-exposed zebrafish may also be using social learning strategies to respond to the novel stimulus. That is, the indirectly Cd-exposed zebrafish from the experienced Cd-treated fish to approach the moving stimulus. The social strategies or other mechanisms by which the groups with Cd-treated fish influence the response of their shoal members to novel stimuli should be explored further.

Our results demonstrate that a few Cd-exposed individuals can radically alter group responses, providing support for a growing body of literature⁵⁶. The present work

is linked with the keystone species concept, where some species have an inordinately large effect on the functioning of the group, which has been extended to encompass diverse interactions at multiple levels of organization, from individual to ecosystem^{18,56}. In other ecologically and evolutionary important contexts keystone or influential individuals include superspreaders in epidemiology, leaders in collective decisions, superclones in populations, the portfolio effect in fisheries, and other influential individual variants in invasions and species persistence^{56,97,98}. Recent studies suggest that contaminant exposure can directly and indirectly influence disease dynamics and pollinator-plant interactions with consequences for plant fitness^{99,100}. The identification of influential individuals will help direct containment plans through targeted interventions to mitigate the behavioral effects of pollutants.

5. Conclusions

Here, we integrate aspects of anthropogenic change and social roles to highlight the profound effect a few contaminated individuals can have on social behavior. We found that a few contaminated individuals influenced the boldness and aggression of an un-exposed majority, which was possibly driven by altered visual systems. We emphasize sensory and social mechanisms, but other studies should rule out other nonsocial mechanisms that may have led to differences between Cd-exposed and control fish. Understanding the impact of pollution on social dynamics is critical to the development of reliable predictions about the consequences of metal exposure in a changing world.

Ethics

All procedures were conducted in accordance with the Indiana University Institutional Animal Care and Use Committee guidelines (protocols 12–042 and 15–017–9).

Authors Statement

DSS, ZMD, AK, DJS, and EPM designed the experiment. SG and JS helped with cadmium exposure, DSS, ZMD, AK, and DJS ran the experiments, DEPS, PB, JRK, MB, HA, ML, and HP assisted with behavioral analysis, and DSS wrote the manuscript with substantial input from EPM and feedback on earlier versions from ZMD, AK, PSS, JS, SG, HA. DSS and EPM acquired funding for the project.

Funding

This material is based on work supported by the National Science Foundation (NSF) through Grant IOS-1257562 to EPM, Graduate Research Fellowship to DSS, internship awards to DSS on an NSF Integrative Graduate Education and Research Traineeship in “Dynamics of Brain–Body–Environment Systems in Behavior and Cognition” (DGE 0903495) to DSS, NSF Postdoctoral Fellowship (1611616), NIH NIEHS R00ES030398 to DSS, travel support from the Oregon Hatchery Research Center to DSS, and a Research Experiences for Undergraduates site in “Animal Behavior” (DBI 0851607) to ZMD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Jeffrey Alberts, Laura Hurley, Meredith West, David Noakes, Carol Carter, Meital Shachaf, Jesualdo Fuentes-G, Johanel Caceres, Jens Krause, Dennis Higgs, Michael Simonich, Alison Ossip-Drahos, Stephanie Campos, Jay Goldberg, Jaime Zúñiga-Vega, and Monserrat Suárez Rodríguez for helpful discussions and comments on earlier versions of this article. We thank reviewer Christos Ioannou and an anonymous reviewer whose comments helped to improve the manuscript. Authors declare no competing interests.

References

1. Fuller, R. et al. Pollution and health: a progress update. *Lancet Planet. Health* 6, e535–e547 (2022).
2. Landrigan, P. J. et al. The Lancet Commission on pollution and health. *The Lancet* 391, 462–512 (2018).
3. Landecker, H. & Panofsky, A. From Social Structure to Gene Regulation, and Back: A Critical Introduction to Environmental Epigenetics for Sociology. *Annu. Rev. Sociol.* **39**, 333–357 (2013).
4. Ledón-Rettig, C. C., Richards, C. L. & Martin, L. B. Epigenetics for behavioral ecologists. *Behav. Ecol.* **24**, 311–324 (2013).
5. Fleeger, J. W., Carman, K. R. & Nisbet, R. M. Indirect effects of contaminants in aquatic ecosystems. *Sci. Total Environ.* **317**, 207–233 (2003).
6. Köhler, H.-R. & Triebskorn, R. Wildlife Ecotoxicology of Pesticides: Can We Track Effects to the Population Level and Beyond? *Science* **341**, 759–765 (2013).
7. Satarug, S., Garrett, S. H., Sens, M. A. & Sens, D. A. Cadmium, Environmental Exposure, and Health Outcomes. *Environ. Health Perspect.* **118**, 182–190 (2010).
8. Satarug, S. et al. A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* **137**, 65–83 (2003).
9. Ruxton, G. D. *Living in Groups*. (Oxford University Press, 2002).
10. Larison, J. R., Likens, G. E., Fitzpatrick, J. W. & Crock, J. G. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. *Nature* **406**, 181–183 (2000).

11. Nordberg, G. F., Nogawa, K., Nordberg, M. & Friberg, L. *Cadmium. Chapter 23. Handbook on the Toxicology of Metals*. (Academic Press, San Diego, 2015).
12. Terçariol, S. G., Almeida, A. A. & Godinho, A. F. Cadmium and exposure to stress increase aggressive behavior. *Environ. Toxicol. Pharmacol.* **32**, 40–45 (2011).
13. Yang, F. *et al.* Cadmium exposure disrupts the olfactory sensitivity of fire ants to semiochemicals. *Environ. Pollut.* **287**, 117359 (2021).
14. LeFauve, M. K. & Connaughton, V. P. Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish. *Curr. Zool.* **63**, 221–227 (2017).
15. Bridges, C. M. & Semlitsch, R. D. Variation in Pesticide Tolerance of Tadpoles among and within Species of Ranidae and Patterns of Amphibian Decline. *Conserv. Biol.* **14**, 1490–1499 (2000).
16. Saaristo, M. *et al.* Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. *Proc. R. Soc. B Biol. Sci.* **285**, 20181297 (2018).
17. Nikinmaa, M. & Anttila, K. Individual variation in aquatic toxicology: Not only unwanted noise. *Aquat. Toxicol.* **207**, 29–33 (2019).
18. Fisher, D. N. *et al.* Anticipated effects of abiotic environmental change on intraspecific social interactions. *Biol. Rev.* **96**, 2661–2693 (2021).
19. Beldomenico, P. M. & Begon, M. Disease spread, susceptibility and infection intensity: vicious circles? *Trends Ecol. Evol.* **25**, 21–27 (2010).
20. Perera, F. P. Environment and Cancer: Who Are Susceptible? *Science* **278**, 1068–1073 (1997).

21. O'Neill, M. S. *et al.* Health, wealth, and air pollution: advancing theory and methods. *Environ. Health Perspect.* **111**, 1861–1870 (2003).
22. Brasso, R. L. & Cristol, D. A. Effects of mercury exposure on the reproductive success of tree swallows (*Tachycineta bicolor*). *Ecotoxicology* **17**, 133–141 (2008).
23. Sloman, K. A. Effects of trace metals on salmonid fish: The role of social hierarchies. *Appl. Anim. Behav. Sci.* **104**, 326–345 (2007).
24. Vital, C. & Martins, E. P. Strain differences in zebrafish (*Danio rerio*) social roles and their impact on group task performance. *J. Comp. Psychol.* **125**, 278–285 (2011).
25. Vital, C. & Martins, E. P. Socially-Central Zebrafish Influence Group Behavior More than Those on the Social Periphery. *PLOS ONE* **8**, e55503 (2013).
26. Killen, S. S. *et al.* The Potential for Physiological Performance Curves to Shape Environmental Effects on Social Behavior. *Front. Physiol.* **12**, (2021).
27. Wascher, C. A. F., Kulahci, I. G., Langley, E. J. G. & Shaw, R. C. How does cognition shape social relationships? *Philos. Trans. R. Soc. B Biol. Sci.* **373**, 20170293 (2018).
28. Clutton-brock, T. H. & Parker, G. A. Sexual coercion in animal societies. *Anim. Behav.* **49**, 1345–1365 (1995).
29. Conradt, L. & Roper, T. J. Group decision-making in animals. *Nature* **421**, 155–158 (2003).
30. Ioannou, C., Couzin, I., James, R., Croft, D. & Krause, J. Social Organisation and Information Transfer in Schooling Fish. in 217–239 (2011).
doi:10.1002/9781444342536.ch10.

31. Laland, K. N. & Williams, K. Shoaling generates social learning of foraging information in guppies. *Anim. Behav.* **53**, 1161–1169 (1997).
32. Lewis, M. A. *et al.* Learning and Animal Movement. *Front. Ecol. Evol.* **9**, (2021).
33. Chamberlain, A. C. & Ioannou, C. C. Turbidity increases risk perception but constrains collective behaviour during foraging by fish shoals. *Anim. Behav.* **156**, 129–138 (2019).
34. Partridge, B. L. The effect of school size on the structure and dynamics of minnow schools. *Anim. Behav.* **28**, 68-IN3 (1980).
35. Strandburg-Peshkin, A. *et al.* Visual sensory networks and effective information transfer in animal groups. *Curr. Biol.* **23**, R709–R711 (2013).
36. Henshaw, J. M., Fromhage, L. & Jones, A. G. The evolution of mating preferences for genetic attractiveness and quality in the presence of sensory bias. *Proc. Natl. Acad. Sci.* **119**, e2206262119 (2022).
37. Milewski, T. M., Lee, W., Champagne, F. A. & Curley, J. P. Behavioural and physiological plasticity in social hierarchies. *Philos. Trans. R. Soc. B Biol. Sci.* **377**, 20200443 (2022).
38. Rosenthal, S. B., Twomey, C. R., Hartnett, A. T., Wu, H. S. & Couzin, I. D. Revealing the hidden networks of interaction in mobile animal groups allows prediction of complex behavioral contagion. *Proc. Natl. Acad. Sci.* **112**, 4690–4695 (2015).
39. Gerlai, R. Social behavior of zebrafish: From synthetic images to biological mechanisms of shoaling. *J. Neurosci. Methods* **234**, 59–65 (2014).

40. Kim, J.-W. *et al.* Combined effects of chemical and visual information in eliciting antipredator behaviour in juvenile Atlantic salmon *Salmo salar*. *J. Fish Biol.* **74**, 1280–1290 (2009).
41. Rosenthal, G. G. & Ryan, M. J. Assortative preferences for stripes in danios. *Anim. Behav.* **70**, 1063–1066 (2005).
42. Frey, J. M. (Brinegar), Robertson, M. W. & Wilcoxon, T. E. Color assortative shoaling in zebrafish, *Danio rerio* (Cypriniformes: Cyprinidae). *Bios* **93**, 97–103 (2022).
43. Ward, A. J. W., Duff, A. J., Horsfall, J. S. & Currie, S. Scents and scents-ability: pollution disrupts chemical social recognition and shoaling in fish. *Proc. R. Soc. B Biol. Sci.* **275**, 101–105 (2008).
44. McElroy, K. N., Beakes, M. P. & Merz, J. E. Hide and seek: Turbidity, cover, and ontogeny influence aggregation behavior in juvenile salmon. *Ecosphere* **9**, e02175 (2018).
45. Suriyampola, P. S. *et al.* Water flow impacts group behavior in zebrafish (*Danio rerio*). *Behav. Ecol.* **28**, 94–100 (2017).
46. van der Sluijs, I. *et al.* Communication in troubled waters: responses of fish communication systems to changing environments. *Evol. Ecol.* **25**, 623–640 (2011).
47. Seehausen, O. *et al.* Speciation through sensory drive in cichlid fish. *Nature* **455**, 620–626 (2008).
48. Candolin, U. & Wong, B. B. M. Mate choice in a polluted world: consequences for individuals, populations and communities. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20180055 (2019).

49. Martin, J. M. *et al.* The psychoactive pollutant fluoxetine compromises antipredator behaviour in fish. *Environ. Pollut.* **222**, 592–599 (2017).
50. Eisenreich, B. R., Greene, S. & Szalda-Petree, A. Of fish and mirrors: Fluoxetine disrupts aggression and learning for social rewards. *Physiol. Behav.* **173**, 258–262 (2017).
51. Tierney, K. B. *et al.* Olfactory toxicity in fishes. *Aquat. Toxicol.* **96**, 2–26 (2010).
52. Brodin, T., Fick, J., Jonsson, M. & Klaminder, J. Dilute Concentrations of a Psychiatric Drug Alter Behavior of Fish from Natural Populations. *Science* **339**, 814–815 (2013).
53. Cavalieri, V. & Spinelli, G. Environmental epigenetics in zebrafish. *Epigenetics Chromatin* **10**, 46 (2017).
54. Dai, Y.-J. *et al.* Zebrafish as a model system to study toxicology. *Environ. Toxicol. Chem.* **33**, 11–17 (2014).
55. Fleisch, V. C. & Neuhauss, S. C. F. Visual behavior in zebrafish. *Zebrafish* **3**, 191–201 (2006).
56. Michelangeli, M. *et al.* Predicting the impacts of chemical pollutants on animal groups. *Trends Ecol. Evol.* **37**, 789–802 (2022).
57. Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R. & Kalueff, A. V. Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci.* **37**, 264–278 (2014).
58. Suriyampola, P. S., Cacéres, J. & Martins, E. P. Effects of short-term turbidity on sensory preference and behaviour of adult fish. *Anim. Behav.* **146**, 105–111 (2018).

59. Shelton, D. S., Price, B. C., Ocasio, K. M. & Martins, E. P. Density and group size influence shoal cohesion, but not coordination in zebrafish (*Danio rerio*). *J. Comp. Psychol.* **129**, 72–77 (2015).
60. Suriyampola, P. S. *et al.* Zebrafish Social Behavior in the Wild. *Zebrafish* **13**, 1–8 (2016).
61. Suriyampola, P. S. *et al.* Small increases in group size improve small shoals' response to water flow in zebrafish. *J. Zool.* **316**, 271–281 (2022).
62. Avdesh, A. *et al.* Regular Care and Maintenance of a Zebrafish (*Danio rerio*) Laboratory: An Introduction. *JoVE J. Vis. Exp.* e4196 (2012) doi:10.3791/4196.
63. US Environmental Protection Agency E. 2016 Aquatic Life Ambient Water Quality Criteria Cadmium (CAS # 7440–43-9). (EPA 820-R-16–002,).
64. Zala, S. M., Määttänen, I. & Penn, D. J. Different social-learning strategies in wild and domesticated zebrafish, *Danio rerio*. *Anim. Behav.* **83**, 1519–1525 (2012).
65. Magyary, I. Floating novel object recognition in adult zebrafish: a pilot study. *Cogn. Process.* **20**, 359–362 (2019).
66. Singh, V. K., Singh, K. P. & Mohan, D. Status of heavy metals in water and bed sediments of river Gomti--a tributary of the Ganga River, India. *Environ. Monit. Assess.* **105**, 43–67 (2005).
67. Srikanth, R., Rao, A. M., Kumar, CH. S. & Khanum, A. Lead, cadmium, nickel, and zinc contamination of ground water around Hussain Sagar lake, Hyderabad, India. *Bull. Environ. Contam. Toxicol.* **50**, 138–143 (1993).
68. R Core Team. R: A language and environment for statistical computing. (2022).

69. Korner-Nievergelt, F. *et al.* *Bayesian Data Analysis in Ecology Using Linear Models with R*. (BUGS, and Stan, Academic Press, 2015).
70. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *ArXiv E-Prints* **arXiv:1406**, (2014).
71. Lenth, R., Singmann, H., Love, J., Buerkner, P. & Herve, M. Emmeans: estimated marginal means, aka least-squares means. (2018).
72. Payne, E. H., Gebregziabher, M., Hardin, J. W., Ramakrishnan, V. & Egede, L. E. An empirical approach to determine a threshold for assessing overdispersion in Poisson and negative binomial models for count data. *Commun. Stat. - Simul. Comput.* **47**, 1722–1738 (2018).
73. Galef, B. G. & Wigmore, S. W. Transfer of information concerning distant foods: A laboratory investigation of the ‘information-centre’ hypothesis. *Anim. Behav.* **31**, 748–758 (1983).
74. Scott, G. R. & Sloman, K. A. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* **68**, 369–392 (2004).
75. Pilastro, A., Benetton, S. & Bisazza, A. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Anim. Behav.* **65**, 1161–1167 (2003).
76. Croft, D. P., Madden, J. R., Franks, D. W. & James, R. Hypothesis testing in animal social networks. *Trends Ecol. Evol.* **26**, 502–507 (2011).

77. Gill, R. J., Ramos-Rodriguez, O. & Raine, N. E. Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature* **491**, 105–108 (2012).
78. Armstrong, T. *et al.* Oil exposure alters social group cohesion in fish. *Sci. Rep.* **9**, 13520 (2019).
79. Miller, M. E., Hamann, M. & Kroon, F. J. Bioaccumulation and biomagnification of microplastics in marine organisms: A review and meta-analysis of current data. *PLOS ONE* **15**, e0240792 (2020).
80. Perc, M., Gómez-Gardeñes, J., Szolnoki, A., Floría, L. M. & Moreno, Y. Evolutionary dynamics of group interactions on structured populations: a review. *J. R. Soc. Interface* **10**, 20120997 (2013).
81. Platt, J. Social traps. *Am. Psychol.* **28**, 641–651 (1973).
82. Bak-Coleman, J., Smith, D. & Coombs, S. Going with, then against the flow: evidence against the optomotor hypothesis of fish rheotaxis. *Anim. Behav.* **107**, 7–17 (2015).
83. Kowalko, J. E. *et al.* Loss of Schooling Behavior in Cavefish through Sight-Dependent and Sight-Independent Mechanisms. *Curr. Biol.* **23**, 1874–1883 (2013).
84. Zentall, T. R. & Galef, B. G. *Social Learning: Psychological and Biological Perspectives*. (Psychology Press, 2013).
85. Zala, S. M. & Penn, D. J. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Anim. Behav.* **68**, 649–664 (2004).
86. Green, A. J. & Planchart, A. The neurological toxicity of heavy metals: A fish perspective. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **208**, 12–19 (2018).

87. Palanza, P., Morellini, F., Parmigiani, S. & vom Saal, F. S. Ethological methods to study the effects of maternal exposure to estrogenic endocrine disrupters: A study with methoxychlor. *Neurotoxicol. Teratol.* **24**, 55–69 (2002).
88. Noguera, J. C., Metcalfe, N. B., Surai, P. F. & Monaghan, P. Are you what you eat? Micronutritional deficiencies during development influence adult personality-related traits. *Anim. Behav.* **101**, 129–140 (2015).
89. Shankar, P. *et al.* Developmental toxicity in zebrafish (*Danio rerio*) exposed to uranium: A comparison with lead, cadmium, and iron. *Environ. Pollut.* **269**, 116097 (2021).
90. Calamari, D., Marchetti, R. & Vailati, G. Influence of water hardness on cadmium toxicity to *Salmo gairdneri* rich. *Water Res.* **14**, 1421–1426 (1980).
91. Pascoe, D., Evans, S. A. & Woodworth, J. Heavy metal toxicity to fish and the influence of water hardness. *Arch. Environ. Contam. Toxicol.* **15**, 481–487 (1986).
92. Ginnaw, G. M. *et al.* Effects of multiple stressors on fish shoal collective motion are independent and vary with shoaling metric. *Anim. Behav.* **168**, 7–17 (2020).
93. Pitcher, T. J., Partridge, B. L. & Wardle, C. S. A Blind Fish Can School. *Science* **194**, 963–965 (1976).
94. Patch, A. *et al.* Kinematic analysis of social interactions deconstructs the evolved loss of schooling behavior in cavefish. *PLOS ONE* **17**, e0265894 (2022).
95. Jolles, J. W., King, A. J. & Killen, S. S. The Role of Individual Heterogeneity in Collective Animal Behaviour. *Trends Ecol. Evol.* **35**, 278–291 (2020).
96. Magnhagen, C. & Staffan, F. Is boldness affected by group composition in young-of-the-year perch (*Perca fluviatilis*)? *Behav. Ecol. Sociobiol.* **57**, 295–303 (2005).

97. Wilson, M. W. *et al.* Ecological impacts of human-induced animal behaviour change. *Ecol. Lett.* **23**, 1522–1536 (2020).
98. Manfredini, F., Arbetman, M. & Toth, A. L. A Potential Role for Phenotypic Plasticity in Invasions and Declines of Social Insects. *Front. Ecol. Evol.* **7**, (2019).
99. Xun, E., Zhang, Y., Zhao, J. & Guo, J. Heavy metals in nectar modify behaviors of pollinators and nectar robbers: Consequences for plant fitness. *Environ. Pollut.* **242**, 1166–1175 (2018).
100. Neff, E. & Dharmarajan, G. The direct and indirect effects of copper on vector-borne disease dynamics. *Environ. Pollut.* **269**, 116213 (2021).

CHAPTER THREE
WILD ZEBRAFISH SENTINELS: BIOLOGICAL MONITORING OF SITE
DIFFERENCES USING BEHAVIOR AND MORPHOLOGY

Abstract

Environmental change poses a devastating risk to human and environmental health. Rapid assessment of water conditions is necessary for monitoring, evaluating, and addressing this global health danger. Sentinels or biological monitors can be deployed in the field using minimal resources to detect water quality changes in real time, quickly and cheaply. Zebrafish (*Danio rerio*) are ideal sentinels for detecting environmental changes due to their biomedical tool kit, widespread geographic distribution, and well-characterized phenotypic responses to environmental disturbances. Here, we demonstrate the utility of zebrafish sentinels by characterizing phenotypic differences in wild zebrafish between two field sites in India. Site 1 was a rural environment with flowing water, low-hypoxic conditions, minimal human-made debris, and high iron and lead concentrations. Site 2 was an urban environment with still water, hypoxic conditions, plastic pollution, and high arsenic, iron, and chromium concentrations. We found that zebrafish from Site 2 were smaller, more cohesive, and less active than Site 1 fish. We also found sexually dimorphic body shapes within the Site 2, but not the Site 1, population. Advancing zebrafish sentinel research and development will enable rapid detection, evaluation, and response to emerging global health threats.

1. Introduction

Environmental change is rapidly accelerating, producing profound changes in natural environments^{1–5}. Aquatic ecosystems are changing at alarming rates due to anthropogenic influences including temperature, carbonization, acidification, flow, and pollution^{6–11}.

These changes produce devastating effects on ecosystems^{12–14} and human health^{15–17}. Marginalized communities are disproportionately impacted by environmental change^{18–20}. The severity and rapid change in aquatic ecosystems requires real-time assessments in the field using sentinels to characterize site conditions²¹. Zebrafish (*Danio rerio*) can serve as effective biological monitors for site differences because of their widespread distribution²² and technical innovation²³.

Ecosystems are differentially impacted by environmental change due to the diversity of environmental conditions among regions^{24–26}. Current methods for assessing environmental change include measuring water parameters, adaptation and evolution among local organisms, and species abundance across temporal scales^{27–31}. Although these metrics provide insights into the impact of environmental change on the locale or site, they often necessitate meticulously trained personnel, expensive techniques and time-consuming laboratory procedures. Such requirements lead to exclusive technologies that are impractical for detecting rapid, emerging changes in environments, and, consequently, exacerbate environmental health disparities^{32–35}. It is necessary to characterize a sentinel species that will provide powerful and quick assessments of environmental conditions that are accessible to non-specialists and individuals seeking alternative monitoring methods.

Many aspects of environmental change including temperature, oxygenation, water flow, and pollution alter morphology and behavior in multiple taxa^{36–46}. Quantification of these morphological and behavioral endpoints in the lab and in the field can thus be utilized to expeditiously detect differences in environmental conditions across sites using a sentinel species. Analysis of organism morphology and behavior can be accomplished quickly and

inexpensively by both specialists and non-specialists alike to characterize site differences in real-time. Zebrafish morphology and behavior vary across these endpoints and can be used as a proxy to rapidly detect these changes in environmental conditions ^{47–52}. Zebrafish are an ideal sentinel species for detecting site differences across these endpoints because they eliminate the need for cost-prohibitive laboratory technology and assays, provide a unique opportunity for use in lower socio-economic regions, and serve as alternatives for communities seeking disruptive technologies ³⁴.

Zebrafish are powerful model organisms utilized in multiple disciplines due to their vast biomedical toolkit and well-characterised phenotypic and genotypic changes in response to environmental change ^{49,53–56}. The use of zebrafish as laboratory-based sentinels for toxicity has been embraced internationally through the development of the International Organization for Standardization Zebrafish Toxicity Test ⁵⁷, the British standard BS/EN/ISO 7346-3-1998, the German standard DIN/EN/ISO 7346-3-1998, and the Chinese standard GB/T13267-91 ^{53,58}. Zebrafish are found in rivers, streams, canals, and rice paddies of India, Pakistan, Bangladesh, Nepal, Myanmar, and Bhutan ⁵⁹. Feral zebrafish distribution includes Brazil, Colombia, Malaysia, Sri Lanka, Thailand, and the United States ^{60–66}. Domestic zebrafish are found in over 100 countries in more than 3000 institutions ²², and are a popular aquarium fish. Through escape, release, and reproductive fervor, zebrafish may have an even larger distribution. Due to their wide distribution across rural and urban environments, zebrafish are present in regions with drastically different environmental conditions and can be leveraged in these environments to characterize site differences. Environmental change disproportionately affects marginalized people, so

deploying accessible biological monitors is needed ^{18–20}. The use of wild zebrafish as sentinels for environmental change will thus be a crucial step in improving the health of marginalized communities, leading to global health improvements for all.

Here, we present simple morphometric and behavioral measures that can be conducted at various field sites by both specialists and non-specialists to detect local site differences using zebrafish. All analyses can be performed rapidly in real-time using free and open-source software, allowing for expeditious assessment and minimal financial burden on investigators. Our goal is that these assays be used for community scientists living where zebrafish are found to facilitate the evaluation of environments and to guide decisions to improve health outcomes. To highlight the utility of wild zebrafish as sentinels using these proposed metrics, we characterized phenotypic differences in zebrafish sampled from two Indian populations that differed in environmental conditions. One population (Site 1) was obtained from a rural environment with flowing water with low-hypoxic conditions that had minimal human-made debris and iron and lead concentrations that exceeded acceptable limits, and had low predation pressure. The other population (Site 2) was from an urban environment with still water with hypoxic conditions, plastic pollution, and arsenic, iron, and chromium concentrations that exceeded acceptable WHO and BIS arsenic limits, and had greater predation pressure ^{27,67–69}. To assess phenotypic differences between these populations, we used three classes of endpoints: body shape, social behavior, and activity. We propose that these three endpoint classes monitored in wild zebrafish can be used as indicators to detect site differences to rapidly characterize the impacts of environmental change.

2. Materials and Methods

2.1. Study Location & Subjects

We conducted a field study at two field sites in India during the post-monsoon season in November 2017. Wild zebrafish were previously studied at these field sites^{50,70–72}. The sites differed in documented pollution, human population density, oxygenation, and water flow (**Table 1**).

We sampled zebrafish from a rural site (Site 1), a fast-moving river in West Bengal, northeast India (a tributary of the Torsa River in Cooch Behar) with low normoxic conditions. Site 1 had arsenic concentrations below (0.01 mg/L), iron (0.3 mg/L) and lead (10 µg/L) concentrations above the WHO and BIS acceptable limits, and was situated in a location with a low human population density (~700 persons/km²)^{27,73}. The other population of zebrafish was sampled at an urban site (Site 2), a canal with stagnant water in the Nadia district of West Bengal, a densely populated human settlement (~1250 persons/km²) with hypoxic conditions, and arsenic, iron, and chromium (0.05 mg/L) concentrations that exceeded the WHO and Indian Standard acceptable limits^{27,52,74–78}. It should be noted that BIS, but not the WHO, has limits on iron in drinking water. At all sites, the fish were collected by local fishermen in the morning. The fish were photographed for morphometric analysis and placed in a holding container with local water for at least an hour prior to the commencement of preparation for behavioral trials. A total of 144 zebrafish were sampled, with 72 fish from each field site, a larger sample size than in other recent zebrafish field studies^{52,71}. A random collection of the fish led to sampling 62 male fish and 9 female fish from Site 1, and 49 male fish and 23 female fish from Site

2. All fish were visually sexed prior to analysis⁶⁰. One Site 1 fish was omitted from morphometric analysis due to a missing photograph, decreasing the total sample size for morphometric analysis to $n = 143$.

2.2. Ethics Statement

All procedures involving animals were approved by Arizona State University's Institutional Animal Care Use Committees under protocol 17-1596R on 22 June 2017, and complied with the existing rules and guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, the Institutional Animal Ethics Committee (IAEC), and the Indian Institute of Science Education and Research (IISER) Kolkata.

2.3. Data Collection & Analysis

Following a post-capture acclimation period of at least 1 h, we divided zebrafish into equal groups of 6 fish, creating 12 groups per field site. Before behavioral testing, we photographed all fish from above. All photos were taken at the same height and with a scale within the frame to allow for morphological analysis. We then sequentially placed zebrafish into a white plastic tank (dimensions 30.8 cm $\times$ 34 cm) filled to a height of 5 cm with habitat water, where they acclimated for an additional 5 min before behavioral assessments, giving a total acclimation time of 65 min. The tanks were recorded from above using a GoPro Hero 5 Session camera. The acclimation period appeared sufficient as the fish did not freeze and displayed exploratory behavior consistent with an absence of stress responses^{79,80}.

We extracted behavioral data from video recordings using ToxTrac video tracking software in a 3 min sample period ^{81,82}. We identified fish within the tank by adjusting contrast and size thresholds for detection. A minimum threshold tracking accuracy of 80% was required to consider video tracking reliable. This threshold was determined based on the minimum Kappa statistic of inter-rater reliability for data to be deemed reliable ⁸³. We analyzed all data using RStudio ⁸⁴.

We categorized video data as either Social measures or Activity measures. Social measures analyzed were inter-individual distance (average distance from each individual to all other group members), time in proximity (number of seconds spent within two body-lengths of a group member), and nearest-neighbour distance (average distance to the closest group member). As the threshold for size detection for video tracking was most reliable between 400–500 pixels for both groups, we standardized the distance used for the two-body-length metric as 1000 pixels. Pixel data were converted to millimeters for statistical analysis. Activity measures analyzed were distance traveled (total number of mm traversed), swim speed (the number of mm traversed divided by the sample duration), tank exploration (mm² of the tank visited), and number of seconds in motion. We collected data for each individual and collapsed data into tank means for analysis between levels of Population to account for the independence of observations, leaving a total of 12 observations per field site. As residuals were normally distributed, we analyzed behavioral data with Welch's two-sample *t*-tests between populations.

We collected morphometric and geomorphometric data using the TPS series software ⁸⁵. We placed seven landmarks on each photograph of subjects to capture the shape of each

individual using tpsUtil⁸⁶ and tpsDIG⁸⁷ (**Figure 3.1**). We based our selection of landmarks on previous studies that characterized zebrafish body length, depth, and shape in the field and in toxicology studies^{52,71,88,89}. We reduced the number of landmarks to make the endpoint amenable to high-throughput assessments. We computed partial warps and consensus shapes for all individuals using the seven digitized landmarks with tpsRelw^{85,90}. We analyzed the effects of independent variables on fish shape using a multivariate analysis of variance (MANOVA). These shape variances were explored by comparing the relative size ratios between these regions across levels of Population and Sex with a two-way analysis of variance (ANOVA). As residuals did not meet the assumption of normality, total posterior, and anterior body region size was contrasted across all classes of individuals using Wilcoxon Signed Rank paired *t*-tests. Finally, we calculated standard length as the distance between landmarks 1 and 4 (length from the anterior to posterior points of the body) and analyzed length data with a Kruskal Wallis test. All morphometric analyses were conducted using the geomorph⁹¹ and lme4⁹² packages.

3. Results

3.1. Fish Body Shape Is Distinct between Sites

We found distinct fish shape differences between the two populations. We analyzed partial warps of fish using PCA and found that the first two axes explained 74.69% of the total variance in body shape (**Figure 3.2**). Morphometric differences between Population and Sex levels were plotted using composite plots of consensus shape (**Figure 3.2**). We then compared these morphometric differences across levels of Population and Sex using MANOVA to find statistical differences in consensus shape. We found a significant main

effect of Population on consensus shape ($F(1140) = 3.44$, $p = 0.001$), as well as a non-significant trend for an interaction between Population and Sex ($F(1140) = 1.137$, $p = 0.07$). Consensus shape differed between sexes, but did not reach statistical significance, $F(1140) = 0.872$, $p = 0.19$. To characterize these differences in morphology between populations, we then analyzed body size ratio and standard length.

We compared the relative area of the posterior body region (LM 2, 3, 4, 5) to the anterior body region (LM 1, 5, 6, 7) (**Figure 3.3**). The posterior region of all fish was significantly larger than their anterior regions (Site 1 Female Anterior $M = 97.3 \text{ mm}^2 \pm 14.31$, Posterior $M = 153.4 \text{ mm}^2 \pm 22.86 \text{ SEM}$; Site 1 Male Anterior $M = 429.7 \text{ mm}^2 \pm 59.69 \text{ SEM}$, Posterior $M = 676.9 \text{ mm}^2 \pm 96.83 \text{ SEM}$; Site 2 Female Anterior $M = 55.2 \text{ mm}^2 \pm 3.14 \text{ SEM}$, Posterior $M = 83.5 \text{ mm}^2 \pm 5.45 \text{ SEM}$; Site 2 Male Anterior $M = 77.1 \text{ mm}^2 \pm 8.176 \text{ SEM}$, Posterior $M = 96.7 \text{ mm}^2 \pm 10.62 \text{ SEM}$; all $p < 0.0001$). Further analysis showed that the ratio between posterior and anterior body regions of Site 2 male fish ($M = 1.3$, $\text{SEM} = 0.02$) was 22% smaller than Site 1 female fish ($M = 1.6$, $\text{SEM} = 0.08$), 21% smaller than Site 1 male fish ($M = 1.6$, $\text{SEM} = 0.03$), and 17% smaller than Site 2 female fish ($M = 1.5$, $\text{SEM} = 0.03$) (**Figure 3.3**). This difference led to a significant two-way interaction between levels of Population and Sex on body size ratio, $F(1, 140) = 7.79$, $p = 0.01$. When analyzed with Tukey's post-hoc test, we found that all fish had relatively larger posterior regions than Site 2 male fish (Site 1 Female vs. Site 2 Male $p < 0.0001$, Site 1 Male vs. Site 2 Male $p < 0.0001$, Site 2 Female vs. Site 2 Male $p < 0.0001$). This shows that Site 1 fish and Site 2 female fish have a significantly more torpedo shape with elongated posterior ends than male fish from Site 2⁹³.

Male fish from Site 1 were the longest fish ($M = 2.2$ cm, $SEM = 0.18$), measuring more than twice as long as both male ($M = 1.0$ cm, $SEM = 0.05$) and female ($M = 0.9$ cm, $SEM = 0.03$) fish from Site 2, and nearly twice as long as female fish from Site 1 ($M = 1.3$ cm, $SEM = 0.10$). These differences in standard length led to a significant main effect of Population on standard length, $H(78) = 101.15$, $p = 0.040$ (**Figure 3.3**).

3.2. Site 2 Fish Are More Cohesive Than Site 1 Fish

Overall, we found that groups of fish from Site 2 were more cohesive than groups of fish from Site 1. Fish from Site 2 spent approximately 20% more time within 2 body-lengths of tankmates than fish from Site 1. This difference between sites led to a significant difference in time within two body-lengths, $t(22) = -2.69$, $p = 0.014$ (Site 2 $M = 219.4$ ms ± 12.45 SEM, Site 1 $M = 174.2$ ms ± 11.26 SEM) (**Figure 3.4**). Fish from Site 2 also maintained approximately one-quarter shorter inter-individual distances than fish from Site 1, $t(22) = 2.384$, $p = 0.028$ (Site 2 $M = 121.1$ mm ± 10.09 SEM, Site 1 $M = 165.1$ mm ± 15.49 SEM) (**Figure 3.4**). We found a similar pattern of increased cohesion in Site 2 using the Nearest Neighbor Distance metric, though this finding did not reach statistical significance, $t(22) = 1.42$, $p = 0.175$ (Site 1 $M = 165.1$ mm ± 15.49 SEM, Site 2 $M = 121.1$ mm ± 10.09 SEM) (**Figure 3.4**).

3.3. Site 1 Fish Are More Active Than Site 2 Fish

We also found that fish from Site 1 were more active than fish from Site 2. Fish from Site 2 swam at two-thirds the speed as fish from Site 1, $t(22) = 2.42$, $p = 0.027$ (Site 1 $M = 13.9$ mm/s ± 1.71 SEM, Site 2 $M = 9.3$ mm/s ± 0.95 SEM) (**Figure 3.5**). Similarly, fish from Site 1 swam nearly twice as far on average than fish from Site 2, $t(22) = 3.40$, $p =$

0.003 (Site 1 M = 4316.9 mm $\pm$ 372.09 SEM, Site 2 M = 2547.1 mm $\pm$ 363.56 SEM) (**Figure 3.5**). We found that fish from both populations spent a similar amount of time in motion ($t(1,22) = -0.82$, $p = 0.423$, Site 1 M = 159.3 s $\pm$ 4.74 SEM, Site 2 M = 164.5 s $\pm$ 4.29 SEM) (**Figure 3.5**), with no observed instances of any fish freezing in the tanks. Exploration was not affected by swimming speed or distance traveled, as both populations explored the majority of the testing arena and did not differ significantly in the area of the tank explored ($t(22) = -0.79$, $p = 0.44$, Site 2 = 69.7% explored $\pm$ 3.96 SEM, Site 1 = 65.9% explored $\pm$ 2.59 SEM) (**Figure 3.5**).

As there were disproportionately more male than female fish sampled, we investigated whether behavior across these levels differed between single-sex and mixed-sex groups. We found that fish in mixed-sex groups from both populations swam nearly half the distance as single-sex groups (Mixed-Sex M = 2648.0 mm $\pm$ 278.22 SEM, Single-Sex M = 4738.7 mm $\pm$ 439.51 SEM, $F(1,20) = 7.06$, $p = 0.02$). Mixed- and single-sex groups did not significantly differ across other metrics. However, the Site 2 sample had 11 mixed-sex tanks and 1 single-sex tank while the Site 1 sample had 4 mixed-sex tanks and 8 single-sex tanks. Thus, the difference in distance travelled may reflect population, but not group composition-dependent activity.

4. Discussion

We propose three endpoints that can be used in wild zebrafish to detect site differences. Morphometric, Social, and Activity endpoints aid in characterizing phenotypic differences between wild populations of zebrafish from sites with differing environmental conditions. Zebrafish showed between-population and sex differences in size, with longer Site 1 fish

than Site 2 fish (**Figure 3.3**). We found sexual dimorphism for body size ratio in Site 2; Site 2 male fish had more similar posterior and anterior body regions, leading to a smaller body size ratio, whereas Site 2 female fish had larger anterior regions and smaller posterior regions resulting in a larger body size ratio. The Site 1 fish had posterior body regions that dwarfed the anterior body regions (**Figure 3.3**). There was a visible sexual dimorphism for length within Site 1; however, this did not reach statistical significance, likely because of the low number of female fish and high male size variability (**Figure 3.3**). We found that fish from Site 2 were more cohesive than Site 1 fish, spending more time within two body-lengths and maintaining closer inter-individual distances between group members. Fish from Site 1 were more active, swimming faster and further distances than Site 2, more polluted fish. Our results indicate that these measurements can be used to identify site differences. Given that there is a relationship between reported site-specific environmental conditions and wild zebrafish morphology and behavior, an intensive research program is needed to identify the causal relationship between the phenotypic differences and pollutants, and other abiotic and biotic factors.

Phenotypic variation across these endpoints in zebrafish has been shown in laboratory settings previously. Zebrafish exposed to plastic pollution ^{94,95}, arsenic ⁹⁶, lead ⁹⁷, antibiotics ⁹⁸, polycyclic aromatic hydrocarbons ⁹⁹, organic pollutants ¹⁰⁰, elevated temperatures ¹⁰¹, or emerging pollutants ¹⁰² may exhibit altered body mass, size, and swimming behavior. Similarly, hypoxia ^{52,103} and water flow ^{49,52,70,71} are associated with changes in zebrafish morphological and behavioural phenotypes. Other studies documented larger fish with deeper bodies and caudal peduncles in flowing water, and

relatively dwarfed regions in fish from stagnant water, similar to the present study (**Figure 3.2** and **Figure 3.3**); sites sampled in the previous studies overlapped with the present study^{52,71}. Hypoxic conditions¹⁰⁴, sex-specific predation pressure¹⁰⁵, or endocrine disruptors¹⁰⁶ and other pollutants¹⁰⁷ might have also led to the male-biased sex ratio in these populations. Our findings show these measurements can be used in the field to assess population differences. The observed phenotypic variation across a range of environmental factors demonstrates the utility of zebrafish biological monitors. The zebrafish's broad rangeland coupled with its significance in biomedical research leverages its candidacy as a potential flagship umbrella species, or through their socio-ecological capital and conservation, zebrafish can extend protection to others^{108,109}. To effectively develop zebrafish as sentinels there is a need for tight integration of lab and field studies.

Using wild zebrafish as sentinels for site differences provides unique benefits, including rapid assessment, interrogation of diverse, novel chemical profiles, and a cost-effective approach. Laboratory analysis of environmental conditions can take extensive periods to complete, and is frequently limited to assessing a small number of factors which decreases external validity^{110–112}. We suggest zebrafish as sentinels that can harmonize sampling and analysis of environmental conditions. Zebrafish's small size, adaptability, and presence near the surface in clear water permits the positioning of accessible, inconspicuous monitoring stations in areas of interest, such as near residential areas or regions with factory or agricultural activity^{53,70}. Zebrafish's biomedical toolkit, rapid generation time, well-characterized behavioral repertoire, evolutionarily conserved traits and genetic homology across vertebrates permit advances in mechanistic, life span, and

predictive toxicology and conservation ^{113–117}. Implementing zebrafish as biological monitors increases the potential to rapidly detect, evaluate, and respond to environmental change, thus facilitating preservation, interventions, bioremediation, and improvement of health outcomes.

The next steps required for implementing zebrafish sentinels are comprehensive and predictive characterization of phenotypic variation in response to different classes and combinations of biotic and abiotic factors. This could be accomplished by analyzing zebrafish phenotypes at multiple field sites, supplemented with identifying and quantifying factors associated with environmental change tested in a laboratory, and predictive modeling ¹¹⁸. Due to the increased complexity of field versus laboratory environments, it is imperative to validate that observed phenotypic variation results from hypothesized sources of variation rather than external variables such as water flow, predation, environmental complexity and stability, genetics, and social context ^{49,52,70–72,119,120}. Optimization and implementation of zebrafish biological monitors in the field could rely on partially submerged slotted tanks or cages with passages for water flow but not fish and with grated lids to prevent predation. Using laboratory strains of zebrafish and common garden or transplant designs will increase monitoring sensitivity by minimizing potential influences of developmental or evolutionary adaptation to water conditions ^{121–123}. Studies should house fish in same-sex groups, or utilize sterile or triploid fish to prevent disruption of natural ecosystems through the breeding of domestic and wild fish ^{124,125}. Genetic or physiological identification techniques such as fluorescent tagging can also ensure monitoring fidelity and experimental integrity. Many of these technologies exist in

disparate fields and have the potential to be realized through Collaborative, Entrepreneurial, Translational, and Innovative (CETI) research and development.

Additional Thoughts on Using Wild Zebrafish as Sentinels

Just, Equitable, Diverse, and Inclusive (J.E.D.I.) research practices are required to effectively implement biological sentinels ^{126–129}. Just sentinel programs require developers to center environmental justice and racism at the forefront by recognizing environmental change risks are unequally distributed by race and class ^{32,33,130}. Monitoring systems and procedures should be accessible and Equitable, so that members of local communities with varying financial, natural, produced, and human capital can obtain sampling systems to quickly and actively monitor their environments ¹³¹. For example, people and communities whose capital is devoid of zebrafish (i.e., zebrafish are absent), knowledge of fish, or physical environments conducive to using the monitoring device (e.g., concrete city landscapes), equitable technologies, training, and surrogate species should be implemented. Diverse endpoints, at multiple timepoints in several populations in the lab and field, can aid detection, assessment, and causal inference, thereby providing a more holistic view of environmental change. Simplifying assessments will permit endpoints that rely on single axes that accurately distinguish male vs. female ¹³², and exposed vs. unexposed ⁸⁸ zebrafish at different developmental exposure periods ¹³³, thereby dramatically enhancing screening throughput. Using a breadth of biomarkers at multiple time points is essential for detecting adverse effects because some deleterious perturbations are revealed at some endpoint, but not others ¹³⁴. Inclusive sentinel programs involve local people and communities as contributors and decision-makers in developing the biological

monitors. Including users as decision makers and developing technology that addresses their pains is common in startups prioritizing customers ¹³⁵.

Startups are engines of ingenuity that can help introduce zebrafish sentinel devices into the market as commercial tools ¹³⁶. Entrepreneurial thinking may accelerate zebrafish sentinel monitors into disruptive technologies, or technologies that are cheaper, simpler, and more convenient than the dominant technology, thereby leading to an upheaval in the existing market structure ³⁴. Prioritizing sentinels to help people combat environmental racism may provide a competitive advantage, because it taps a market that has cost the USD 16 trillion in revenue over the last 20 years ¹³⁷. Evaluating the implementation of zebrafish sentinels and competing technologies is needed to determine their impact and ability to enhance human and environmental health. Some dimensions of evaluation may include changes in health disparities, environmental engagement, stewardship and community resilience ^{138,139}. Zebrafish sentinels can be technological innovations that may lead to socio-economic-environmental-political changes in society.

5. Conclusions

Environmental change poses a considerable threat to natural environments and global health ¹⁻⁵. Using wild zebrafish as sentinels is an efficient and economical approach to monitoring, assessing, and addressing global health risks. Developing zebrafish as sentinels in the field fills the need for rapid water quality analysis not addressed by laboratory-based methods and presents a unique opportunity for implementation in underserved regions and communities looking for alternative monitoring systems. Utilizing social, activity, and morphometric endpoints in zebrafish highlighted here can thus provide

a crucial step in assessing site differences. Coupling JEDI biological monitoring practices with CETI research and development will lead to technology that transforms environmental monitoring and improves health outcomes worldwide.

Author Contributions

Conceptualization, D.S.S. and E.P.M.; Methodology, D.S.S. and J.R.K.; Validation, R.M., D.K.D., J.R.K. and D.S.S.; Formal Analysis, J.R.K.; Investigation, R.M., D.K.D., S.G.S., F.N., J.R.K., H.A., M.E.B., G.I. and G.M.; Resources, E.P.M.; Data Curation, D.S.S. and J.R.K.; Writing–Original Draft Preparation, J.R.K. and D.S.S.; Writing–Review & Editing, R.M., D.K.D., S.G.S., F.N., H.A., M.E.B., G.I., G.M., A.B. and E.P.M.; Visualization, J.R.K., G.M., M.E.B. and D.S.S.; Supervision, E.P.M. and A.B.; Project Administration, E.P.M., A.B. and D.S.S.; Funding Acquisition, E.P.M. and D.S.S. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the National Science Foundation NSF Postdoc Fellowship 1611616 and NIH NIEHS K99 1ES030398 01A1 (to D.S.S.) and through Grant IOS-1257562 (to E.P.M.).

Institutional Review Board Statement

All procedures involving animals were approved by Arizona State University's Institutional Animal Care Use Committees under protocol 17-1596R on 22 June 2017 and complied with the existing rules and guidelines outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India,

the Institutional Animal Ethics Committee's (IAEC) and the Indian Institute of Science Education and Research (IISER) Kolkata.

Data Availability Statement

Data from this publication have been archived in the Mendeley Data Repository (<https://data.mendeley.com/datasets/7gbxfg7jy5/2>, accessed on 9 March 2021).

Acknowledgments

We thank Aditya Ghoshal, Tamal Roy, Bappi, and several local fishermen for their hospitality and field assistance. The authors also thank Piyumika Suriyampola, Monserrat Suárez-Rodríguez, Lani C. O'Neill, Jens Krause, David Noakes, Robyn Tanguay and members of the Martins and Tanguay laboratories for helpful discussions on planning the field expedition and for comments on early versions of this article. This work was supported by the National Science Foundation NSF Postdoc Fellowship 1611616 and NIH NIEHS K99 1ES030398 01A1 (to D.S.S.) and through Grant IOS-1257562 (to E.P.M.).

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Foley, J. A. *et al.* Global Consequences of Land Use. *Science* **309**, 570–574 (2005).
2. Landrigan, P. J. *et al.* The Lancet Commission on pollution and health. *The Lancet* **391**, 462–512 (2018).
3. van Oldenborgh, G. J. *et al.* Attribution of the Australian bushfire risk to anthropogenic climate change. *Nat. Hazards Earth Syst. Sci.* **21**, 941–960 (2021).
4. Kaniewski, D. *et al.* Recent anthropogenic climate change exceeds the rate and magnitude of natural Holocene variability on the Balearic Islands. *Anthropocene* **32**, 100268 (2020).
5. Qu, S. *et al.* Distinguishing the impacts of climate change and anthropogenic factors on vegetation dynamics in the Yangtze River Basin, China. *Ecol. Indic.* **108**, 105724 (2020).
6. Vörösmarty, C. J., Pahl-Wostl, C., Bunn, S. E. & Lawford, R. Global water, the anthropocene and the transformation of a science. *Curr. Opin. Environ. Sustain.* **5**, 539–550 (2013).
7. FN, C. & Malik, M. F. Factors Affecting Water Pollution: A Review. *J. Ecosyst. Ecography* **07**, (2017).
8. Häder, D.-P. *et al.* Anthropogenic pollution of aquatic ecosystems: Emerging problems with global implications. *Sci. Total Environ.* **713**, 136586 (2020).
9. Malhi, Y. *et al.* Climate change and ecosystems: threats, opportunities and solutions. *Philos. Trans. R. Soc. B Biol. Sci.* **375**, 20190104 (2020).

10. Liu, S. *et al.* Global river water warming due to climate change and anthropogenic heat emission. *Glob. Planet. Change* **193**, 103289 (2020).
11. Stewart, I. T., Rogers, J. & Graham, A. Water security under severe drought and climate change: Disparate impacts of the recent severe drought on environmental flows and water supplies in Central California. *J. Hydrol. X* **7**, 100054 (2020).
12. Sievers, M., Hale, R., Parris, K. M. & Swearer, S. E. Impacts of human-induced environmental change in wetlands on aquatic animals. *Biol. Rev.* **93**, 529–554 (2018).
13. Ball, B. A. *et al.* Direct and Terrestrial Vegetation-mediated Effects of Environmental Change on Aquatic Ecosystem Processes. *BioScience* **60**, 590–601 (2010).
14. Williamson, C. E., Dodds, W., Kratz, T. K. & Palmer, M. A. Lakes and streams as sentinels of environmental change in terrestrial and atmospheric processes. *Front. Ecol. Environ.* **6**, 247–254 (2008).
15. Bambrick, H., Dear, K., Woodruff, R., Hanigan, I. & McMichael, A. *The Impacts of Climate Change on Three Health Outcomes: Temperature-Related Mortality and Hospitalisations, Salmonellosis and Other Bacterial Gastroenteritis, and Population at Risk from Dengue*. (Australian Government, Australian Government, Australia, 2008).
16. Reid, G. K. *et al.* Climate change and aquaculture: considering biological response and resources. *Aquac. Environ. Interact.* **11**, 569–602 (2019).
17. Tong, S. & Ebi, K. Preventing and mitigating health risks of climate change. *Environ. Res.* **174**, 9–13 (2019).
18. Paavola, J. Health impacts of climate change and health and social inequalities in the UK. *Environ. Health* **16**, 113 (2017).

19. Sorensen, C., Murray, V., Lemery, J. & Balbus, J. Climate change and women's health: Impacts and policy directions. *PLOS Med.* **15**, e1002603 (2018).
20. Hayes, K., Blashki, G., Wiseman, J., Burke, S. & Reifels, L. Climate change and mental health: risks, impacts and priority actions. *Int. J. Ment. Health Syst.* **12**, 28 (2018).
21. Kipp, A., Cunsolo, A., Gillis, D., Sawatzky, A. & Harper, S. L. The need for community-led, integrated and innovative monitoring programmes when responding to the health impacts of climate change. *Int. J. Circumpolar Health* **78**, 1517581 (2019).
22. Kint, P., Mahesh, G. & Panwar, Y. Mapping of Zebrafish Research: A Global Outlook. *Zebrafish* **10**, 510–517 (2013).
23. Cornet, C. *et al.* ZeGlobalTox: An Innovative Approach to Address Organ Drug Toxicity Using Zebrafish. *Int. J. Mol. Sci.* **18**, 864 (2017).
24. Cao, Y. *et al.* Regime shifts in shallow lake ecosystems along an urban-rural gradient in central China. *Sci. Total Environ.* **733**, 139309 (2020).
25. Yang, J., Luo, X., Jin, C., Xiao, X. & Xia, J. (Cecilia). Spatiotemporal patterns of vegetation phenology along the urban–rural gradient in Coastal Dalian, China. *Urban For. Urban Green.* **54**, 126784 (2020).
26. Rickards, N. *et al.* Understanding Future Water Challenges in a Highly Regulated Indian River Basin—Modelling the Impact of Climate Change on the Hydrology of the Upper Narmada. *Water* **12**, 1762 (2020).
27. Central Water Commission. Status of Trace & Toxic Metals in Indian Rivers. (2019).

28. Aitken, S. N., Yeaman, S., Holliday, J. A., Wang, T. & Curtis-McLane, S. Adaptation, migration or extirpation: climate change outcomes for tree populations. *Evol. Appl.* **1**, 95–111 (2008).
29. Harvey, B. P. *et al.* Evolution of Marine Organisms under Climate Change at Different Levels of Biological Organisation. *Water* **6**, 3545–3574 (2014).
30. Madeira, D., Narciso, L. & Cabral, H. Thermal tolerance and potential impacts of climate change on coastal and estuarine organisms. *J. Sea Res.* **70**, 32–41 (2012).
31. Both, C., Bouwhuis, S., Lessells, C. M. & Visser, M. E. Climate change and population declines in a long-distance migratory bird. *Nature* **441**, 81–83 (2006).
32. Agyeman, J., Schlosberg, D., Craven, L. & Matthews, C. Trends and Directions in Environmental Justice: From Inequity to Everyday Life, Community, and Just Sustainabilities. *Annu. Rev. Environ. Resour.* **41**, 321–340 (2016).
33. Pulido, L. Geographies of race and ethnicity II: Environmental racism, racial capitalism and state-sanctioned violence. *Prog. Hum. Geogr.* **41**, 524–533 (2017).
34. Schuelke-Leech, B.-A. A model for understanding the orders of magnitude of disruptive technologies. *Technol. Forecast. Soc. Change* **129**, 261–274 (2018).
35. Martin, A. *et al.* Justice and conservation: The need to incorporate recognition. *Biol. Conserv.* **197**, 254–261 (2016).
36. Guerin, G. R., Wen, H. & Lowe, A. J. Leaf morphology shift linked to climate change. *Biol. Lett.* **8**, 882–886 (2012).

37. McCauley, S. J., Hammond, J. I. & Mabry, K. E. Simulated climate change increases larval mortality, alters phenology, and affects flight morphology of a dragonfly. *Ecosphere* **9**, e02151 (2018).
38. Claireaux, G., Webber, D. M., Kerr, S. R. & Boutilier, R. G. Physiology and Behaviour of Free-Swimming Atlantic COD (*Gadus Morhua*) Facing Fluctuating Salinity and Oxygenation Conditions. *J. Exp. Biol.* **198**, 61–69 (1995).
39. Haas, T. C., Blum, M. J. & Heins, D. C. Morphological responses of a stream fish to water impoundment. *Biol. Lett.* **6**, 803–806 (2010).
40. Cureton, J. C. & Broughton, R. E. Rapid morphological divergence of a stream fish in response to changes in water flow. *Biol. Lett.* **10**, 20140352 (2014).
41. Kruitwagen, G., Hecht, T., Pratap, H. B. & Wendelaar Bonga, S. E. Changes in morphology and growth of the mudskipper (*Periophthalmus argentilineatus*) associated with coastal pollution. *Mar. Biol.* **149**, 201–211 (2006).
42. Sun, P. L., Hawkins, W. E., Overstreet, R. M. & Brown-Peterson, N. J. Morphological Deformities as Biomarkers in Fish from Contaminated Rivers in Taiwan. *Int. J. Environ. Res. Public Health* **6**, 2307–2331 (2009).
43. Jacquin, L., Petitjean, Q., Côte, J., Laffaille, P. & Jean, S. Effects of Pollution on Fish Behavior, Personality, and Cognition: Some Research Perspectives. *Front. Ecol. Evol.* **8**, (2020).
44. Beever, E. A. *et al.* Behavioral flexibility as a mechanism for coping with climate change. *Front. Ecol. Environ.* **15**, 299–308 (2017).

45. Seebacher, F. & Post, E. Climate change impacts on animal migration. *Clim. Change Responses* **2**, 5 (2015).
46. Zhang, H. *et al.* Hypoxia-driven changes in the behavior and spatial distribution of pelagic fish and mesozooplankton in the northern Gulf of Mexico. *J. Exp. Mar. Biol. Ecol.* **381**, S80–S91 (2009).
47. Sfakianakis, D. G., Leris, I., Laggis, A. & Kentouri, M. The effect of rearing temperature on body shape and meristic characters in zebrafish (*Danio rerio*) juveniles. *Environ. Biol. Fishes* **92**, 197–205 (2011).
48. Hallare, A. V., Schirling, M., Luckenbach, T., Köhler, H.-R. & Triebkorn, R. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. *J. Therm. Biol.* **30**, 7–17 (2005).
49. Suriyampola, P. S. *et al.* Water flow impacts group behavior in zebrafish (*Danio rerio*). *Behav. Ecol.* **28**, 94–100 (2017).
50. Limonta, G. *et al.* Microplastics induce transcriptional changes, immune response and behavioral alterations in adult zebrafish. *Sci. Rep.* **9**, 15775 (2019).
51. Abdallah, S. J., Thomas, B. S. & Jonz, M. G. Aquatic surface respiration and swimming behaviour in adult and developing zebrafish exposed to hypoxia. *J. Exp. Biol.* **218**, 1777–1786 (2015).
52. Shukla, R. & Bhat, A. Morphological divergences and ecological correlates among wild populations of zebrafish (*Danio rerio*). *Environ. Biol. Fishes* **100**, 251–264 (2017).
53. Dai, Y.-J. *et al.* Zebrafish as a model system to study toxicology. *Environ. Toxicol. Chem.* **33**, 11–17 (2014).

54. Barrionuevo, W. R. & Burggren, W. W. O₂ consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O₂. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **276**, R505–R513 (1999).
55. Malek, R. L., Sajadi, H., Abraham, J., Grundy, M. A. & Gerhard, G. S. The effects of temperature reduction on gene expression and oxidative stress in skeletal muscle from adult zebrafish. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **138**, 363–373 (2004).
56. Ton, C., Stamatiou, D. & Liew, C.-C. Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol. Genomics* **13**, 97–106 (2003).
57. International Organization for Standardization. *Water Quality—Determination of the Acute Lethal Toxicity of Substances to a Freshwater Fish [Brachydanio Rerio Hamilyon-Buchanan (Teleostei, Cyprinidae)]—Part 1: Static Method*. (International Organization for Standardization: Geneva, Switzerland, 1996).
58. Kelly, J. R. & Benson, S. A. Inconsistent ethical regulation of larval zebrafish in research. *J. Fish Biol.* **97**, 324–327 (2020).
59. Engeszer, R. E., Patterson, L. B., Rao, A. A. & Parichy, D. M. Zebrafish in The Wild: A Review of Natural History And New Notes from The Field. *Zebrafish* **4**, 21–40 (2007).
60. Laale, H. W. The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. *J. Fish Biol.* **10**, 121–173 (1977).
61. Welcomme, R. L. *International Introductions of Inland Aquatic Species*. (FAO Fish, Rome, Italy, 1988).

62. Pritchard, V. L. Behaviour and morphology of the zebrafish, *Danio rerio*. (University of Leeds, 2001).
63. Bhat, A. Patterns in the distribution of freshwater fishes in rivers of Central Western Ghats, India and their associations with environmental gradients. *Hydrobiologia* **529**, 83–97 (2004).
64. Spence, R., Gerlach, G., Lawrence, C. & Smith, C. The behaviour and ecology of the zebrafish, *Danio rerio*. *Biol. Rev.* **83**, 13–34 (2008).
65. United States Geological Survey. “*Danio Rerio*”. *Nonindigenous Aquatic Species*. vol. 294 (United States Geological Survey, Gainesville, FL, USA, 2013).
66. Trigueiro, N. S. de S., Canedo, A., Braga, D. L. de S., Luchiari, A. C. & Rocha, T. L. Zebrafish as an Emerging Model System in the Global South: Two Decades of Research in Brazil. *Zebrafish* **17**, 412–425 (2020).
67. Rahman, M. M. *et al.* Status of groundwater arsenic contamination in all 17 blocks of Nadia district in the state of West Bengal, India: A 23-year study report. *J. Hydrol.* **518**, 363–372 (2014).
68. Mazumder, D. N. G. *et al.* Chronic arsenic toxicity from drinking tubewell water in rural West Bengal. *Bull. World Health Organ.* **66**, 499–506 (1988).
69. Mazumder, D. N. G. *et al.* Dietary arsenic exposure with low level of arsenic in drinking water and biomarker: A study in West Bengal. *J. Environ. Sci. Health Part A* **49**, 555–564 (2014).
70. Shelton, D. S. *et al.* Collective Behavior in Wild Zebrafish. *Zebrafish* **17**, 243–252 (2020).

71. Suriyampola, P. S. *et al.* Zebrafish Social Behavior in the Wild. *Zebrafish* **13**, 1–8 (2016).
72. Whi^{TE}ley, A. R. *et al.* Population genomics of wild and laboratory zebrafish (*Danio rerio*). *Mol. Ecol.* **20**, 4259–4276 (2011).
73. IndiKosh.
74. BIS. Drinking Water—Specification (Second Revision of IS 10500: 2012). (2012).
75. World Health Organization. Exposure to Arsenic: A Major Public Health Concern; WHO/CED/PHE/EPE/19.4.1. (2019).
76. World Health Organization. Chromium in Drinking Water; WHO/SDE/WSH/03.04/04. (2003).
77. World Health Organization. Lead in Drinking-Water; WHO/SDE/WSH/03.04/09/Rev/1. (2011).
78. World Health Organization. Iron in Drinking-Water; WHO/SDE/WSH/03.04/08. (2003).
79. Egan, R. J. *et al.* Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* **205**, 38–44 (2009).
80. Miller, N. & Gerlai, R. From Schooling to Shoaling: Patterns of Collective Motion in Zebrafish (*Danio rerio*). *PLOS ONE* **7**, e48865 (2012).
81. Rodriguez, A. *et al.* ToxTrac: A fast and robust software for tracking organisms. *Methods Ecol. Evol.* **9**, 460–464 (2018).
82. Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T. & Andersson, M. ToxId: an efficient algorithm to solve occlusions when tracking multiple animals. *Sci. Rep.* **7**, 14774 (2017).
83. McHugh, M. L. Interrater reliability: the kappa statistic. *Biochem. Medica* **22**, 276–282 (2012).

84. R Core Team. R: A language and environment for statistical computing. (2022).
85. Zelditch, M. L., Swiderski, D. L., Sheets, H. D. & Fink, W. L. *Geometric Morphometrics for Biologists*. (Elsevier Academic Press, London, UK, 2004).
86. Rohlf, F. J. TpsUtil. Department of Ecology and Evolution, State University of New York: Stony Brook, NY, USA (2012).
87. Rohlf, F. J. TpsDig. Department of Ecology and Evolution, State University of New York: Stony Brook, NY, USA (2013).
88. Jantzen, C. E., Annunziato, K. M. & Cooper, K. R. Behavioral, morphometric, and gene expression effects in adult zebrafish (*Danio rerio*) embryonically exposed to PFOA, PFOS, and PFNA. *Aquat. Toxicol.* **180**, 123–130 (2016).
89. Suriyampola, P. S. & Eason, P. K. The Effects of Landmarks on Territorial Behavior in a Convict Cichlid, *Amatitlania siquia*. *Ethology* **121**, 785–792 (2015).
90. Rohlf, F. J. TpsRelw. Department of Ecology and Evolution, State University of New York: Stony Brook, NY, USA (2010).
91. Adams, D., Collyer, M., Kaliontzopoulou, A. & Baken, E. geomorph: Geometric Morphometric Analyses of 2D and 3D Landmark Data. (2024).
92. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using **lme4**. *J. Stat. Softw.* **67**, (2015).
93. McHenry, M. J. & Lauder, G. V. Ontogeny of form and function: Locomotor morphology and drag in zebrafish (*Danio rerio*). *J. Morphol.* **267**, 1099–1109 (2006).

94. Duan, Z. *et al.* Barrier function of zebrafish embryonic chorions against microplastics and nanoplastics and its impact on embryo development. *J. Hazard. Mater.* **395**, 122621 (2020).
95. Wu, N. C. & Seebacher, F. Bisphenols alter thermal responses and performance in zebrafish (*Danio rerio*). *Conserv. Physiol.* **9**, coaa138 (2021).
96. Hallauer, J. *et al.* The Effect of Chronic Arsenic Exposure in Zebrafish. *Zebrafish* **13**, 405–412 (2016).
97. Tu, H. *et al.* Role of neurexin2a in lead-induced locomotor defect in developing zebrafish. *Aquat. Toxicol.* **194**, 167–175 (2018).
98. Petersen, B. D. *et al.* Antibiotic drugs alter zebrafish behavior. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **242**, 108936 (2021).
99. Vignet, C. *et al.* Long-term disruption of growth, reproduction, and behavior after embryonic exposure of zebrafish to PAH-spiked sediment. *Environ. Sci. Pollut. Res.* **21**, 13877–13887 (2014).
100. Lyche, J. L. *et al.* Natural Mixtures of Persistent Organic Pollutants (POP) Increase Weight Gain, Advance Puberty, and Induce Changes in Gene Expression Associated with Steroid Hormones and Obesity in Female Zebrafish. *J. Toxicol. Environ. Health A* **73**, 1032–1057 (2010).
101. Ribas, L. *et al.* Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proc. Natl. Acad. Sci.* **114**, E941–E950 (2017).

102. Gavrilescu, M., Demnerová, K., Aamand, J., Agathos, S. & Fava, F. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. *New Biotechnol.* **32**, 147–156 (2015).
103. Widmer, S., Moore, F. B.-G. & Bagatto, B. The effects of chronic developmental hypoxia on swimming performance in zebrafish. *J. Fish Biol.* **69**, 1885–1891 (2006).
104. Yu, R. M. K. & Wu, R. S. S. Hypoxia Affects Sex Differentiation and Development, Leading to a Male-Dominated Population in Zebrafish (*Danio rerio*). *Environ. Sci. Technol.* **40**, 3118–3122 (2006).
105. Roy, T. & Bhat, A. Population, sex and body size: determinants of behavioural variations and behavioural correlations among wild zebrafish *Danio rerio*. *R. Soc. Open Sci.* **5**, 170978 (2018).
106. Baumann, L. *et al.* Prochloraz causes irreversible masculinization of zebrafish (*Danio rerio*). *Environ. Sci. Pollut. Res.* **22**, 16417–16422 (2015).
107. Gonçalves, Í. F. S. *et al.* Toxicity testing of pesticides in zebrafish—a systematic review on chemicals and associated toxicological endpoints. *Environ. Sci. Pollut. Res.* **27**, 10185–10204 (2020).
108. Roberge, J.-M. & Angelstam, P. Usefulness of the Umbrella Species Concept as a Conservation Tool. *Conserv. Biol.* **18**, 76–85 (2004).
109. Kalinkat, G. *et al.* Flagship umbrella species needed for the conservation of overlooked aquatic biodiversity. *Conserv. Biol.* **31**, 481–485 (2017).
110. Inderjit & Weston, L. A. Are Laboratory Bioassays for Allelopathy Suitable for Prediction of Field Responses? *J. Chem. Ecol.* **26**, 2111–2118 (2000).

111. Schoenfuss, H. L. *et al.* Complex mixtures, complex responses: Assessing pharmaceutical mixtures using field and laboratory approaches. *Environ. Toxicol. Chem.* **35**, 953–965 (2016).
112. Underwood, A. J. Toxicological testing in laboratories is not ecological testing of toxicology. *Hum. Ecol. Risk Assess. Int. J.* **1**, 178–182 (1995).
113. Hill, A. J., Teraoka, H., Heideman, W. & Peterson, R. E. Zebrafish as a Model Vertebrate for Investigating Chemical Toxicity. *Toxicol. Sci.* **86**, 6–19 (2005).
114. Kalueff, A. V. *et al.* Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. *Zebrafish* **10**, 70–86 (2013).
115. Tal, T., Yaghoobi, B. & Lein, P. J. Translational toxicology in zebrafish. *Curr. Opin. Toxicol.* **23–24**, 56–66 (2020).
116. Carvan III, M. J. *et al.* Oxidative stress in zebrafish cells: potential utility of transgenic zebrafish as a deployable sentinel for site hazard ranking. *Sci. Total Environ.* **274**, 183–196 (2001).
117. Bambino, K. & Chu, J. Chapter Nine - Zebrafish in Toxicology and Environmental Health. in *Current Topics in Developmental Biology* (ed. Sadler, K. C.) vol. 124 331–367 (Academic Press, 2017).
118. Wittwehr, C. *et al.* How Adverse Outcome Pathways Can Aid the Development and Use of Computational Prediction Models for Regulatory Toxicology. *Toxicol. Sci.* **155**, 326–336 (2017).
119. Suriyampola, P. S., Cacéres, J. & Martins, E. P. Effects of short-term turbidity on sensory preference and behaviour of adult fish. *Anim. Behav.* **146**, 105–111 (2018).

120. Sykes, D. J., Suriyampola, P. S. & Martins, E. P. Recent experience impacts social behavior in a novel context by adult zebrafish (*Danio rerio*). *PLOS ONE* **13**, e0204994 (2018).
121. de Villemereuil, P., Gaggiotti, O. E., Mouterde, M. & Till-Bottraud, I. Common garden experiments in the genomic era: new perspectives and opportunities. *Heredity* **116**, 249–254 (2016).
122. Nacci, D., Proestou, D., Champlin, D., Martinson, J. & Waits, E. R. Genetic basis for rapidly evolved tolerance in the wild: adaptation to toxic pollutants by an estuarine fish species. *Mol. Ecol.* **25**, 5467–5482 (2016).
123. Esbaugh, A. J., Brix, K. V. & Grosell, M. Na⁺ K⁺ ATPase isoform switching in zebrafish during transition to dilute freshwater habitats. *Proc. R. Soc. B Biol. Sci.* **286**, 20190630 (2019).
124. Piferrer, F. *et al.* Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture* **293**, 125–156 (2009).
125. Lorenzen, K., Beveridge, M. C. M. & Mangel, M. Cultured fish: integrative biology and management of domestication and interactions with wild fish. *Biol. Rev.* **87**, 639–660 (2012).
126. Schell, C. J. *et al.* The ecological and evolutionary consequences of systemic racism in urban environments. *Science* **369**, eaay4497 (2020).
127. Baker, K., Eichhorn, M. P. & Griffiths, M. Decolonizing field ecology. *Biotropica* **51**, 288–292 (2019).

128. Hazlett, M. A., Henderson, K. M., Zeitzer, I. F. & Drew, J. A. The geography of publishing in the Anthropocene. *Conserv. Sci. Pract.* **2**, e270 (2020).
129. Montgomery, B. L. Lessons from Microbes: What Can We Learn about Equity from Unculturable Bacteria? *mSphere* **5**, e01046-20 (2020).
130. Wordelman, D. M. L., Tabor. Plastic Demands Land. That's a Function of Colonialism. *Teen Vogue* <https://www.teenvogue.com/story/how-plastic-is-a-function-of-colonialism> (2018).
131. *Five Kinds of Capital: Useful Concepts for Sustainable Development.* (2003). doi:10.22004/ag.econ.15595.
132. Duff, N. M., Sommerfeld, R. E. & Litvak, M. K. Discriminating Sex in Zebrafish (*Danio rerio*) Using Geometric Morphometrics. *Zebrafish* **16**, 207–213 (2019).
133. Lutte, A. H. *et al.* Persistent increase in ecto-5'-nucleotidase activity from encephala of adult zebrafish exposed to ethanol during early development. *Neurotoxicol. Teratol.* **70**, 60–66 (2018).
134. Akhter, A., Rahaman, M., Suzuki, R., Murono, Y. & Tokumoto, T. Next-generation and further transgenerational effects of bisphenol A on zebrafish reproductive tissues. *Heliyon* **4**, e00788 (2018).
135. Blank, S. *The Four Steps to the Epiphany: Successful Strategies for Products That Win.* (John Wiley & Sons, 2020).
136. Freeman, J. & Engel, J. S. Models of Innovation: Startups and Mature Corporations. *Calif. Manage. Rev.* **50**, 94–119 (2007).

137. Citigroup. Closing the Racial Inequality Gaps, the Economic Cost of Black Inequality in the U.S. *Citi GPS, Global Perspectives & Solutions* (2020).
138. Berkes, F. & Ross, H. Community Resilience: Toward an Integrated Approach. *Soc. Nat. Resour.* **26**, 5–20 (2013).
139. Meerow, S., Newell, J. P. & Stults, M. Defining urban resilience: A review. *Landsc. Urban Plan.* **147**, 38–49 (2016).

CHAPTER FOUR
FROM PLAY DATE TO STRESS FATE: JUVENILE SOCIAL PLAY RESCUES
ADULT STRESS REACTIVITY INDEPENDENT OF PERINEURONAL NET
EXPRESSION IN SYRIAN HAMSTERS.

Abstract

Social play is a crucial experience for species-typical neural and behavioral development. Deprivation of juvenile social play increases stress vulnerability, agonistic behavior, and disrupts neural pruning in the prelimbic (PL) and infralimbic (IL) areas of the ventral medial prefrontal cortex in adult Syrian hamsters. We investigated whether juvenile social play deprivation alters perineuronal net (PNN) expression on parvalbumin (PV) neurons in the PL, IL, and basal lateral amygdala (BLA). We reared Syrian hamsters during postnatal day 21-43 in peer isolation, in peer isolation with daily social play encounters (dyadic play), or with litter mates in group-housed cages. In adulthood, we exposed subjects to acute social defeat stress and assessed their stress-related changes in social behavior. We found that 1-hour of dyadic play throughout the juvenile period was sufficient to rescue exaggerated submissive & defensive behavior in a conditioned defeat test, but not rescue social approach in a social avoidance test in both males and females. In females, we found lower PNN/PV co-expression in the PL among subjects who were peer isolated or received dyadic play compared to group housed subjects. Females exposed to dyadic play also had lower PNN/PV co-expression in the IL compared to all other play treatment groups. Interestingly, both males and females who consistently lost dyadic play encounters showed lower PNN/PV co-expression in the PL, IL, and BLA and more submissive and defensive behavior when re-introduced to a social group of peers. We show that opportunities for social play can promote resistance to social stressors in adulthood. Further, we show that deprivation of social play impairs the expression of competent agonistic social behaviors both in juvenile periods and in adulthood. These findings suggest

that opportunities for social play in juveniles alters neuronal development in the medial prefrontal cortex, which promotes competent social behavior in adulthood.

1. Introduction

Social play during the juvenile period is a crucial component of neural and behavioral development in gregarious species¹. Social play provides animals opportunities to learn social cues, switch social roles, and respond with reciprocal behavior in multiple contexts, which is thought to promote competent and adaptive social behavior in adulthood²⁻⁵. Because of ongoing neural development during the juvenile period, social play is thought to be particularly important for sculpting flexible and appropriate behavior in adulthood^{3,5-8}. Play in children can facilitate parent engagement; promote safe, stable, and nurturing relationships; encourage the development of numerous competencies, including executive functioning skills; and improve life course trajectories⁹⁻¹³. Therefore, there is a strong need to identify the neurobiological mechanisms by which social play leads to positive outcomes.

Deprivation of social play during the juvenile period impairs the expression of competent social behavior. Both play deprivation and juvenile social isolation disorganize adult behavior in rats, including male sexual behavior^{2,6,8,14,15}. Both male and female rats deprived of play opportunities also show altered agonistic behavior, which includes both elevated aggression and increased defensive behavior toward conspecifics^{3,6,8,16-20}. Interestingly, they also show a decreased ability to inhibit impulsive responses²¹. Further, hamsters deprived of social play during early juvenile development, postnatal day (PND)

21-25, exhibit increased aggression in adulthood towards a novel juvenile intruder compared to play-exposed animals ²²⁻²⁵. In young children, imaginative social play is directly associated with young girls' self-reported positive mood and coping ability in situations requiring self-regulation of impulsivity or aggression ²⁶⁻²⁸. Further, imaginative social play with others can facilitate cognitive growth including divergent thinking, problem-solving, and representational competence ^{26,28}. In dolphins, opportunities for social play between calves enhance the acquisition and maintenance of flexible problem-solving skills, the emergence and strengthening of social and communicative competencies, and the establishment of social relationships ²⁹. Collectively, these findings indicate that social play is critically important for the development of competent social behavior, and the lack of opportunities for social play contributes to inefficient social interactions, including elevated aggression.

In addition to promoting social competency, social play has been suggested to increase resistance to stressful situations ^{3,5}. Young marmosets that engage in more rough-and-tumble play show reduced cortisol responses in repeated social separation stress tests compared to those that play less ³⁰. In Belding ground squirrels, intermediate to high rates of social play predict more cautious behavior towards a novel threatening intruder, increased boldness, exploration, and decrease in time it required to escape and unfamiliar environment compared to those with less social play experience ^{31,32}. Indeed, rats and mice exposed to juvenile social isolation exhibit increased anxiety-like responses and depression-like behavior, including excessive social vigilance in agonistic encounters, compared to those raised in standard conditions ^{17,33}. Isolated male rats that were exposed

to an aggressive conspecific in adulthood also showed a longer latency to submit and elevated plasma corticosterone and epinephrine compared to non-isolates ⁸. Similarly, isolated rats had decreased spatial flexibility in escape behavior and displayed less defensive behavior toward a resident aggressor, which resulted in higher received aggression compared to those with play exposure ³. This suggests that social isolation impairs the ability to select the appropriate behavioral response to prevent the escalation of aggression. Previous research from our laboratory has shown that male and female Syrian hamsters deprived of social play during the juvenile period show greater durations of submissive and defensive behavior in a conditioned defeat test in adulthood compared to play-exposed controls ³⁴. Altogether, these findings suggest that juvenile social play promotes flexible responses to social challenges in adulthood and decreases stress vulnerability.

The ventral medial prefrontal cortex (vmPFC) plays a crucial role in mediating agonistic behavior. Specifically, vmPFC activation elicits aggressive behavior in rodents via projections to the mediobasal and lateral hypothalamus ^{16,35}. The vmPFC also plays a critical role in stress-related changes in agonistic behavior as pharmacological inactivation of the vmPFC enhances the conditioned defeat response in male hamsters ^{36,37}. In hamsters, rough-and-tumble play increases c-Fos expression in a range of brain regions that are associated with adult forms of offensive aggression, including in layers II/III of the anterior cingulate, prelimbic (PL), and infralimbic (IL) regions of the mPFC ^{38,39}. In addition, social play deprivation decreases neural pruning in apical dendrites of pyramidal cells in the PL and IL during the juvenile period in hamsters ⁴⁰ and in rats ⁴¹. Interestingly, while deficits

in pruning in the orbitofrontal cortex are temporary and return to normal after the juvenile period, deficits in pruning in the PL and IL last into adulthood ⁴¹. Deprivation of juvenile social play induces several other structural changes within the vmPFC including a reduction in synaptic plasticity proteins ³³ and decreased glial cell density ¹⁶. Together, these data suggest that play deprivation causes morphological changes in neural ensembles within the vmPFC that regulate agonistic behavior and responses to social stress.

vmPFC neurons with projections to the basolateral amygdala (BLA) are also critical for expression and suppression of fear responses ^{42,43}. Patients suffering from stress-related disorders such as post-traumatic stress disorder show functional abnormalities in the vmPFC ⁴⁴. In mice, the degree of the oscillatory activity between vmPFC and BLA, predicts susceptibility to social defeat stress ⁴⁵. The IL and PL also appear to play opposing roles in the expression and suppression of stress-related behavior. For example, lesioning the IL prevents the development of stress resilience in animals reared in an enriched environment ⁴⁶. Similarly, chemogenetic activation of BLA-projecting IL neurons promotes resistance to acute social defeat stress ⁴⁷. Conversely, neuronal tracing approaches have shown that stress-susceptible mice show greater activity in BLA-projecting PL neurons when exposed to social defeat stress ⁴⁸. Reciprocal interactions between the BLA and the vmPFC are primarily in layers II/III of the PL and IL with BLA efferents providing feedforward inhibition onto layer II somatostatin (SOM) cells that inhibit parvalbumin (PV) cells and disinhibit pyramidal neurons ^{49,50}. Therefore, PV cells can gate responses to long-range signal transmission between the BLA and vmPFC. Given the dysregulation of agonistic behavior and heightened stress vulnerability seen in play-

deprived animals, it is possible that the lack of juvenile social play disrupts experience-dependent plasticity in PV cells in the vmPFC.

One marker for developmental neural plasticity is expression of perineuronal nets (PNNs). PNNs are extracellular matrix structures consisting of chondroitin sulfate proteoglycans that surround neurons and stabilize synaptic connectivity ^{51,52}. In adult rodents PNNs often surround PV neurons and are abundant in the vmPFC and BLA ⁵³. PNNs have been shown to gate experience-dependent neural plasticity in sensory-processing brain regions during developmental sensitive periods ⁵⁴. In addition, PNNs play a crucial role in establishing and maintaining emotional memories. Victims of childhood abuse show upregulation of oligodendrocyte progenitor cells with greater density and morphological complexity of PNNs around these cells in the vmPFC ⁵⁵. Further, the extinction of fear memories in adult mice can be accelerated by degrading PNNs in the BLA ⁵⁶ or auditory cortex ⁵⁷. On the other hand, chronic restraint stress in adult mice has been shown to decrease PNN expression and decrease glutamatergic and GABAergic transmissions in the PFC which was associated with increased anxiety-like behavior in mice ⁵⁸. While PNNs modulate the development of emotional responses, it's unknown whether they serve as a marker for social play-dependent neural plasticity. Interestingly, elevated aggressive behavior exhibited by mice exposed to juvenile isolation is associated with greater PNN expression on PV+ neurons in the vmPFC, suggesting disrupted PNN maturation may contribute to the social deficits seen in play-deprived animals ¹⁷. PNNs may thus gate experience-dependent synaptic plasticity in the vmPFC and BLA during social play and thereby modulate neuronal responses to social stress in adulthood.

The functions of social play remain elusive and manipulating social play has been particularly difficult ⁷. Some studies have housed hamsters individually after weaning on PND 21–25 ^{23,25,59}. Others have housed juvenile animals with their mothers to avoid the effects of complete social isolation ^{40,60}. Furthermore, the amount of time animals are isolated prior to play encounters varies as some studies isolate animals for a short amount of time ^{61–63}, while others isolate animals for a longer period ^{22,24,34,59}. Interestingly, the amount of time hamsters spent playing was proportional to the amount of time they were socially isolated prior to a play bout ^{64,65}. We have previously shown that depriving hamsters of social play opportunities on PND 21-43 increases the effects of social defeat stress in adulthood ^{34,40,67}. Others have shown that 1-hour of social play per day in rats is sufficient to rescue exploration in an open field and decrease weight loss compared to those that were completely play deprived ²¹. Recently, it was shown that 1-hour of social play per day partially rescued inhibitory synaptic currents in the PFC that was related to cognitive deficits on a reversal learning task. Together, this suggests that brief social play interactions during critical developmental periods is sufficient to rescue the effects of peer isolation.

Here we asked whether giving juvenile hamsters 1-hour of daily social play was sufficient to rescue an exaggerated response to social defeat stress in adulthood. Further, we investigated whether juvenile social play deprivation alters PNN/PV co-expression in the PL, IL, and BLA, and whether PNN expression could be rescued by 1-hour of daily social play. We predicted that subjects given opportunities for social play would exhibit less defeat-induced changes in social behavior than play-deprived hamsters. We also

expected that animals given opportunities for social play would show reduced PNN expression in the PL, IL, and BLA compared to play-deprived subjects.

2. Method

2.1. Subjects

Male and female Syrian hamsters ($N = 96$) were obtained from our breeding colony, which was derived from animals purchased from Charles River Laboratories (Wilmington, MA). All animals were housed in polycarbonate cages measuring 20 x 40 x 20 cm with wire mesh tops and corn cob bedding. Food, water, and cotton nesting materials were available *ad libitum*. All animals were kept on a 14:10 reverse light cycle where the dark portion of the cycle began at 1300 hours and ended at 2300 hours. Animals were tested within the first 3 hours of the dark cycle to minimize circadian variation in agonistic behavior^{67,68}. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and are in accordance with ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experimental Design

After weaning on PND 21, subjects were divided into four treatment groups with 24 animals (12 males and 12 females) per group (**Figure 4.1**). A Peer-Isolated group was raised with their dam (mother) during PND 21-43 with no opportunities for social play. A Dyadic Play group was similarly raised with their dam but given 1-hour of social play per day with a sex-matched conspecific. A Group Play condition included hamsters that were housed with 3 littermates after weaning. A Handled Control condition included hamsters that were housed with littermates after weaning but and did not undergo social defeat stress.

We restricted access to social partners during PND 21-43 because this is the period of peak social play in juvenile hamsters ^{22,34}. Also, we housed Peer-Isolated and Dyadic Play subjects with their dam to avoid complete social isolation but restrict opportunities for social play because dams do not play with their offspring ⁶⁰. At PND 43, we combined Peer-Isolated and Dyadic Play subjects with 3 of their same-sex littermates from the Group Play condition. The re-housing at PND 43 functioned to restrict play manipulation to the juvenile stage.

At PND 60, subjects were individually housed for 7-8 days before exposure to social defeat stress. During these 7-8 days, subjects were handled for approximately 2-5 minutes daily to acclimate subjects to human contact. Female subjects were evaluated for signs of Estrus by placing a cotton swab against the vaginal area and checking for the presence of stringy vaginal discharge ⁶⁹. We then exposed subjects to acute social defeat stress which occurred on Diestrus I for females. Stress-related changes in social behavior were assessed 24 hours (Diestrus II) later using a conditioned defeat test and a social avoidance test. Subjects were humanely sacrificed 24 hours after behavioral testing (Proestrus) and brains collected for immunohistochemical analysis (**Figure 4.1**).

2.3. Behavioral Testing

Dyadic Play Dates: Hamsters were placed into a clean, neutral cage with corn cob bedding. Hamsters were sex-matched with a novel conspecific that was also peer isolated for 1 hour per day, five days per week during PND 21-43. Play encounters occurred in the same cage for 5 days and a new cage was used the following week. Hamsters were briefly anesthetized with isoflurane and shaved under their neck for identification before the first

and eleventh encounters. Subjects were paired with the same conspecific for each encounter, testing occurred in red light conditions, and all encounters were video recorded. Social play was scored using an established ethogram for hamster play behavior³⁴. Peer-Isolated hamsters were also shaved in parallel with Dyadic Play Date animals and placed in an empty cage for 1 hour daily.

Re-housing after play deprivation: Peer-Isolated and Dyadic Play Date hamsters were regrouped with littermates on PND 44. We filmed the first 15 minutes of regrouping and quantified the videos for agonistic, submissive, defensive, affiliative, and non-social behavior.

Social defeat: Social defeat stress consisted of 3, 5-minute aggressive interactions at 5-minute inter-trial intervals in which the subject lost to a novel, larger, same-sex aggressor. All encounters took place in the aggressor's home cage. Encounters were video recorded, and the latency for subjects to submit to the aggressor during the first social defeat encounter was quantified. To account for variation in how quickly the resident initiated aggression, we recorded the subjects' latency to submit relative to the initiation of aggression by the resident. A plastic barricade was used to separate the subjects from the resident aggressors after they submitted and received 5 attacks. We kept the subject separated and restricted to one side of the cage for the remainder of the 5-minute trial. The plexiglass barricade had holes in it so that the animals could still see, smell, and hear each other. The Handled Control group was exposed to cages of 3 unfamiliar hamsters after the residents had been removed. Because the effects of social defeat stress vary across the

estrous cycle, female subjects underwent social defeat on Diestrus 1 and were tested for conditioned defeat and social avoidance on Diestrus 2 ⁶⁹.

Conditioned Defeat: Conditioned defeat testing occurred 24 hours after social defeat stress exposure. Conditioned defeat testing involved a 5-minute social interaction in which a freely moving, non-aggressive, juvenile (PND 49-56) was placed into the subject's home cage. Encounters were video recorded, and we quantified the duration of submissive and defensive, aggressive, social, and non-social behavior displayed by the subject.

Social Avoidance Test: Approximately 30 minutes after conditioned defeat testing, subjects were tested for social avoidance in a neutral open arena (80cm x 80cm x 40cm). The arena was divided into four sections: the interaction zone, near side, far side, and far corners. We quantified the amount of time spent in each section and the duration of social vigilance ^{70,71}. Testing consisted of 3 phases that were 3 minutes each. During the first phase, subjects were placed in the empty arena and allowed to habituate. During the second phase an empty perforated box (7 x 14 x 7cm) was placed into the interaction zone and subjects were allowed to acclimate to the novel box. During the third phase (testing phase) an unfamiliar, adult, same-sex hamster was placed inside the perforated box to assess approach and avoidant behavior. The ratio of the subjects' time spent in the interaction zone between the acclimation and testing phases was calculated to determine whether subjects were relatively more avoidant of the unfamiliar hamster compared to the empty box.

Behavioral scoring: Behavioral tests were scored using either Noldus Observer (Noldus Information Technology) or BORIS software ⁷². All observers were trained to achieve at

least a 90% agreement on frequencies and durations of behavior prior to quantification. All observers were blind to treatment conditions while scoring.

2.4. Tissue Processing

Subjects were euthanized 24 hours after conditioned defeat and social avoidance testing using an overdose of isoflurane followed by cardiac perfusion with 100mL of 0.1M phosphate buffer (PB) and 100mL of 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde. Brains were then transferred into a 30% sucrose solution in 0.1M PB with 0.01% sodium azide. Brains were frozen using dry ice and stored at -80°C until sectioning into 40µm sections. PL and IL tissue was stored in 0.01% sodium azide solution in 0.05M PB at 4°C until processing for immunohistochemistry, while BLA tissue was sectioned directly onto slides and stored at -80°C until staining.

Immunohistochemistry:

Tissue was first washed 5 times for 5 minutes in 0.05M PB before being blocked in 10% normal goat serum in 0.02% PB-Triton X for 1 hour. Tissue was then incubated in primary antibodies for PV (1:1000, Sigma-Aldrich P3088, mouse monoclonal anti-parvalbumin antibody) in 0.02% PB-Triton X with 5% normal goat serum for 24 hours at room temperature. The next day, tissue was washed 5 times for 5 minutes in 0.05M PB before being incubated in a cocktail of secondary antibody for the PV primary antibody and a combined fluorescein antibody specific for *Wisteria floribunda* Agglutinin (WFA) in 0.02% PB-Triton X with 5% normal goat serum for 4 hours (1:500 Thermo Fisher Alexa fluor 594 goat anti-mouse A11032; 1:500, Vector Labs WFA, WFL, Fluorescein, FL-1351-2MG). Finally, tissue was again washed 5 times for 5 minutes with 0.05M PB. Stained

vmPFC tissue was mounted onto slides. All slides were covered with DAPI (SouthernBiotech, Lot: B0823-N643) mounting medium, coverslipped, and sealed using clear nail varnish. Slides were stored at 4°C until imaging.

PNN/PV Quantification:

Tissue was imaged bilaterally using an Olympus BX51 fluorescence microscope (**Figure 4.2**). We imaged layers II & III of the PL and IL with a clip region of 300 x 400 μm , and the BLA with a clip region of 500 x 500 μm . After images were collected, the files were then pipelined and analyzed using a software from RewireNeuro called Perineuronal net Intensity Program for the Standardization and Quantification of ECM Analysis (PIPSQUEAK) AI. This software was developed to detect and quantify the number of immunofluorescent positive cells (<https://rewireneuro.com/>). The software allowed us to use an artificial intelligence (AI) interface with the software to train the algorithm and count cells. The software counts PNN cells, PV cells, and then within 95% of the respective boundaries of the regions of interests (ROIs) of each PNN and PV cell, the software will count co-localized cells on the merged images. Researchers achieved 90% agreement with one another on cell counts, then established the AI software parameters that produced 90% agreement with researchers, and continuously monitored automated cell counts for false positives and false negatives. We analyzed approximately 6 sections of each brain region for each subject and analyzed the average number of PNNs or PV+ neurons per animal. All image quantification was performed by observers blind to treatment conditions.

2.5. Statistical Analysis

Testing the 50:50 ratio: Role reversal, which occurs when play partners take turns adopting complementary roles, is a common feature of rough-and-tumble social play^{73,74}. To test for symmetry of social play we classified play behavior as offensive or defensive. Offensive behavior that occurred during dyadic play encounters included pinning and flank marking, while defensive behavior included supines, evades, and flees. The proportion of offensive and defensive behavior can be used to calculate an index score that reflects which animals wins or loses the play bout⁷⁵⁻⁷⁷. Similarly, we calculated a winning index for each play bout (see below). To quantify the emergence of dominance relationships, we quantified the frequency of tail raises because this is a clear signal hamsters use for submission. However, tail raises were not considered a defensive play behavior. The winning index was averaged across the early, mid and late phases of play bouts. Finally, we compared scores between partners to determine whether play bouts had a clear winner and loser.

$$\text{Winning Index} = \frac{(\text{Pins} + \text{Flank Marks})/2}{(\text{Supines} + \text{Evades} + \text{Flees})/3}$$

All statistical analyses were performed using R statistical software⁷⁸. Where data were normally distributed, group effects and regression analyses were performed using linear mixed models using the lme4 package⁷⁹ with Tukey's post-hoc tests. When data were not normally distributed, overdispersion was tested to determine the appropriate distribution. Where data were found to fit a negative binomial distribution, we used generalized linear mixed models fit to a negative binomial distribution using the glmmTMB package⁸⁰. Where data were found to fit a Poisson distribution, we used

generalized linear mixed models fit to a Poisson distribution using the lme4 package ⁷⁹. We used repeated measures ANOVAs to investigate whether sex (male, female) and status (winner, loser) predicted differences in total play time, pins, and tail raises in dyadic play bouts ^{81,82}. If sphericity was violated, Greenhouse-Geisser was used as this has been shown to be a more conservative correction than Huynh-Feldt. We also used pairwise comparisons to investigate simple effects of between subject factors across each day of social play encounters. This approach allowed us to investigate if there was a sex or status difference across each day of dyadic play encounters for each dependent variable.

3. Results

3.1. Play Behavior

There were no sex differences in the total amount of time that hamsters in the Dyadic Play condition engaged in social play ($F_{1,70} = 0.44, p > 0.05$). However, in both males and females there was a significant change in the amount of time hamsters spent playing with one another ($F_{1,70} = 7.07, p < 0.01$) (**Figure 4.3**). Specifically, social play decreased over time such that both males and females spent more time playing during PND 24-34 compared to PND 38-42. This is consistent with previous research showing that social play decreases over time and slowly transitions into adult forms of aggression ^{7,25,66}.

We compared a winning index score across the early (PND 24-28), mid (PND 32-36), and late (PND 38-42) phases of the juvenile stage. In the early stage, play partners did not significantly differ in their winning index ($F_{1,20} = 0.96, p = 0.33$). However, in the mid phase, play bouts were no longer symmetrical and winners ($b = 12.44, SE = 6.635$) showed a significantly larger winning index than losers ($b = 1.6, SE = 4.69, F_{1,20} = 6.24, p = 0.021$).

However, this effect did not reach statistical significance for males and females separately (males: $F_{1,10}=1.8$, $p=0.21$; females: $F_{1,10}=2.16$, $p=0.17$). In the late phase, one partner was winning significantly more than the other ($F_{1,20}= 41.88$, $p< 0.001$), in both males ($F_{1,10}=36.94$, $p<0.001$) and females ($F_{1,10}=17.38$, $p=0.001$). Consistent with these findings the number of pins changed over time ($F_{1,20}= 16.47$, $p<0.001$). Specifically, pins were reciprocated between winners and losers during the early and mid-phases ($p>0.05$). However, during PND 38 ($F_{1,20}=18.33$, $p<0.001$), 40 ($F_{1,20}=4.98$, $p=0.04$), and 42 ($F_{1,20}= 4.47$, $p= 0.04$) one hamster pinned their partner more often than the other and winning became unilateral in both males and females. Interestingly, dominance relationships began to emerge as losers showed tail raise postures more often than winners during PND 38 ($F_{1,20}=8.38$, $p=0.01$), 40 ($F_{1,20}=7.99$, $p=0.01$), and 42 ($F_{1,20}= 19.21$, $p<0.001$). This gradual loss of reciprocity was also found for other types of play behavior.

3.2. Regrouping

We assessed agonistic and social behavior among Peer-Isolated and Dyadic Play subjects on PND 43 when they were re-grouped with same-age, same-sex conspecifics (**Figure 4.4**). We found that male hamsters displayed significantly more submissive and defensive behavior during regrouping than female hamsters ($\chi^2_{(1, N=42)}=7.31$, $p=0.007$). We further investigated group differences within males and found that Peer-Isolated males showed more submissive and defensive behavior than Dyadic Play males ($F_{(1,20)}=7.37$, $p=0.013$). Conversely, we found that female hamsters were more aggressive than males at regrouping ($\chi^2_{(1, N=42)}=5.88$, $p=0.015$). However, no significant differences were found in the frequency of attacks ($p=0.252$) or duration of affiliative behavior ($p=0.922$).

To assess how prior play experience affected agonistic behavior in Dyadic Play hamsters at regrouping, we compared agonistic behavior at regrouping between animals categorized as winners and losers in the late phase of play encounters. We found that winners showed significantly less submissive and defensive behavior than losers in both male and female hamsters ($\chi^2_{(1, N=23)}=12.21, p<0.001$). However, winners and losers did not statistically differ in the amount of aggressive behavior at regrouping ($p=0.661$).

After social groups were established, we found that some animals became hyper-aggressive and needed to be separated to prevent wounding. Specifically, 66.7% of Peer-Isolated females (8/12) and 33.3% (4/12) of the Peer-Isolated males had to be separated from their new social group and individually housed during PND 43-48. In contrast, 33.3% (4/12) of Dyadic Play females and 16.7% (2/12) Dyadic Play males had to be separated and individually housed during PND 43-48.

3.3. Defeats

We assessed the amount of aggression received during social defeat sessions to confirm that all subjects were exposed to sufficient social stress. While female hamsters received more aggression than male hamsters ($F_{1,67}=6.36, p=0.014$), the amount of aggression received did not depend on play treatment ($p=0.528$). We also found that individuals did not statistically differ in the number of attacks received ($p=0.257$).

We also quantified the latency for each subject to submit to the resident aggressor during the first social defeat session. A positive latency indicates subjects submitted after receiving aggression, while a negative latency indicates subjects submitted before receiving aggression. We found a trend where males showed greater adjusted latencies to

submit compared to females, though this trend did not reach statistical significance ($F_{1,65}=3.48, p=0.067$). However, play treatment did not alter latency to submit ($p=0.174$).

3.4. Conditioned Defeat

We found a significant main effect of group on submissive and defensive behavior in a conditioned defeat test ($\chi^2_{(3, N=99)}=80.25, p<0.001$) (**Figure 4.5**). Tukey post-hoc tests revealed that all three defeated groups showed more submissive behavior than Handled Control hamsters ($p<0.001$). We also found that Peer-Isolated subjects were significantly more submissive than Group Housed hamsters in both males and females ($p=0.031$). Also, daily play encounters partly rescued the conditioned defeat response and Dyadic Play subjects did not statistically differ from Group Housed subjects ($p=0.859$) or Peer-Isolates ($p=0.219$). Although males showed greater social vigilance than females ($\chi^2_{(1, N=99)}=9.35, p=0.002$), play treatment did not significantly alter the duration of vigilance behavior in animals exposed to social defeat ($p=0.325$).

We found a main effect of group on aggressive behavior during conditioned defeat in which Handled Control hamsters showed the most aggression ($\chi^2_{(3, N=99)}=11.33, p=0.010$) and the greatest number of attack ($\chi^2_{(3, N=99)}=11.03, p=0.012$). Specifically, they showed greater durations of aggressive behavior than Peer-Isolates ($p=0.022$), Dyadic Play ($p=0.038$), and Group Housed ($p=0.037$) hamsters. Female hamsters also showed significantly more aggressive behavior ($\chi^2_{(1, N=99)}=23.77, p<0.001$) and a greater number of attacks ($\chi^2_{(1, N=99)}=16.61, p<0.001$) than males. We also found a group by sex interaction for affiliative behavior ($F_{3,91}=3.54, p=0.018$) in which Handled Control males showed

more affiliation than all female groups (Peer-Isolated $p=0.002$; Dyadic Play Date $p=0.009$; Group Housed $p=0.004$) and Peer-Isolated males ($p<0.001$).

3.5. Social Avoidance

During the acclimation phase all groups showed similar approach and avoidance behavior toward the novel empty box. We did not find group differences in time spent in the interaction zone ($p=0.656$) or corners of the arena ($p=0.234$) in the acclimation phase. However, in the testing phase, we found a main effect of group ($\chi^2_{(3, N=100)}=41.99, p<0.001$) on time spent in the interaction zone where both Peer-Isolated and Dyadic Play hamsters spent less time in the interaction zone than Group Housed (Peer-Isolated $p=0.007$; Dyadic Play Date $p=0.016$) and Handled Control subjects (Peer-Isolated $p<0.001$; Dyadic Play Date $p<0.001$) (**Figure 4.6**). Also, groups exposed to social defeat spent more time in the corners of the arena than Handled Control hamsters during the testing phase ($\chi^2_{(1, N=100)}=34.06, p<0.001$). We also found that male hamsters spent significantly less time in the interaction zone ($\chi^2_{(1, N=100)}=22.23, p<0.001$), less time on the far side of the arena ($\chi^2_{(1, N=100)}=11.39, p<0.001$), and more time in the corners of the arena ($\chi^2_{(1, N=100)}=6.51, p=0.011$) than female hamsters regardless of their play exposure experience.

We found that defeated groups showed significantly more vigilance during social avoidance testing than Handled Control hamsters ($\chi^2_{(3, N=100)}=54.59, p<0.001$). We also found a trend where Dyadic Play hamsters were less vigilant than Peer-Isolated subjects, though this effect did not reach statistical significance ($p=0.072$). Vigilance among Dyadic Play hamsters did not statistically differ from Group Housed subjects ($p=0.299$). We also

found that male hamsters were more vigilant than females across all social play-exposed groups ($\chi^2_{(1, N=100)}=10.34, p=0.001$).

We also quantified the ratio of time spent in the interaction zone between acclimation and testing phases. We found that interaction ratios differed significantly between play treatment groups ($\chi^2_{(3, N=100)}=27.33, p<0.001$). Tukey post-hoc tests showed that both Peer-Isolated ($p=0.004$) and Dyadic Play ($p<0.001$) groups were significantly more socially avoidant than Handled Control hamsters. While Group Housed hamsters tended to be more socially avoidant than Handled Control subjects, this effect did not reach statistical significance ($p = 0.098$). We also found that male hamsters were significantly more socially avoidant than female hamsters across all groups ($\chi^2_{(1, N=100)}=25.61, p<0.001$).

3.6. Perineuronal Nets

We found a significant interaction between treatment group and sex on the proportion of PNNs surrounding PV+ neurons in the PL ($F_{3,89}=2.93, p=0.038$) (**Figure 4.7**). Specifically, Peer-Isolated and Dyadic Play females showed reduced PNN/PV co-expression compared to Group Play and Handled Control females, although Tukey post-hoc tests were not sensitive enough to detect a statistically significant difference (Peer Isolate vs Group Play $p=0.347$; Dyadic Play vs Group Play $p=0.242$). We did not find any significant differences in the number of PNNs ($p=0.386$) or PV+ neurons ($p=0.283$) in the PL.

We found a significant main effect of treatment group on the proportion of PNNs surrounding PV+ neurons in the IL ($F_{3,88}=3.263, p=0.025$) (**Figure 4.8**). Tukey post-hoc

tests revealed that Dyadic Play hamsters had a lower proportion of PNNs surrounding PV+ neurons in the IL than Peer-Isolated subjects ($p=0.027$). Similarly, Dyadic Play hamsters tended to have lower PNN/PV co-expression than Handled Control hamsters, though this effect did not reach statistical significance ($p=0.058$). There were no significant differences in the number of PNNs ($p=0.222$) or PV+ neurons ($p=0.364$) in the IL.

In the BLA we found no significant effect of play treatment on the expression of PNNs ($p=0.095$), PV+ neurons ($p=0.349$), or proportion of PNNs surrounding PV+ neurons ($p=0.596$) (**Figure 4.9**). While we did not find significant differences in PV+ neuron count between groups in the BLA ($p=0.483$), we did find that male hamsters had fewer PV+ neurons in the BLA than females ($F_{1,88}=4.06$, $p=0.047$).

3.7. PNN Predictions of Behavior

We asked whether individual differences in PNN and PNN/PV expression were associated with behavioral patterns at testing (**Figure 4.10A**). Here, we report associations that were significant or trending.

Dyadic Play Dates

We investigated differences in PNN expression in the PL, IL, and BLA between winners and losers of dyadic play encounters. We found that losers showed significantly lower PNN/PV expression in both the PL ($F_{1,18}=9.08$, $p=0.007$) and IL ($F_{1,18}=6.66$, $p=0.019$). This effect was driven by female subjects, however the two-way interaction between group and sex did not reach statistical significance (PL $p=0.295$; IL $p=0.119$). Similarly, both male and female losers also tended to have less PNN/PV expression in the BLA, though this effect did not reach statistical significance ($F_{1,18}=3.68$, $p=0.071$).

Regrouping Correlations

We tested whether PNN expression in the PL, IL, and BLA correlated with agonistic behavior during regrouping (**Figure 4.10B, C, D**). We found that, among males, the duration of submissive and defensive behavior was positively correlated with PNN number in the BLA ($F_{1,35}=7.59$, $p=0.009$, adjusted $R^2=0.36$). We also found a non-significant trend in which the proportion of PNNs surrounding PV+ PL neurons was negatively correlated with submissive behavior at regrouping in both males and females ($F_{1,38}=3.40$, $p=0.073$, adjusted $R^2=0.30$). The proportion of PNNs surrounding PV+ BLA neurons also tended to be positively correlated with aggressive behavior during regrouping in both males and females, though it did not reach statistical significance ($F_{1,35}=3.74$, $p=0.061$, adjusted $R^2=0.07$).

Conditioned Defeat

We tested whether agonistic behavior during conditioned defeat testing was associated with PNN expression (**Figure 4.10G**). We found a trend where PNN count in the PL was negatively correlated with aggression in both males and females, though this effect did not reach statistical significance ($F_{1,92}=3.37$, $p=0.069$, adjusted $R^2=0.27$). Interestingly, neither PNN nor PV expression predicted submissive and defensive behavior during conditioned defeat testing.

Social Avoidance Test

We correlated interaction zone ratio scores during social avoidance testing with PNN expression in the PL, IL, and BLA (**Figure 4.10E, F**). We found that PNN count in the IL was positively correlated with interaction zone ratio in females, where female

subjects with the greatest social approach had the most IL PNNs ($F_{1,92}=4.63$, $p=0.033$, adjusted $R^2=0.15$). Similarly, we found that the proportion of PNNs surrounding PV+ BLA neurons was also positively correlated with interaction zone ratios in both males and females, though this trend did not reach statistical significance ($F_{1,90}=3.69$, $p=0.068$, adjusted $R^2=0.14$).

4. Discussion

The present study sought to determine if 1-hour of daily play exposure was sufficient to rescue play deprivation-induced increases in stress vulnerability, and whether play exposure changed PNN expression. We found that deprivation of juvenile social play disrupts social interactions with unfamiliar animals in early adolescence and increases the conditioned defeat response in adulthood. These findings replicate our previous work that play deprivation increases submissive and defensive behavior in a conditioned defeat test^{34,40}. Here we extended this work by showing that 1 hour of daily social play was sufficient to restore a species-typical conditioned defeat response in both males and females. Interestingly, manipulating opportunities for social play also altered agonistic behavior when animals were placed into new social groups. Together, these findings suggest that social play is critical for the development of species-typical agonistic behavior during both the late juvenile period and in adulthood. Overall, our findings are consistent with literature that social play is essential for developing competent social behavior^{3,6,8,19,20}.

Our findings suggest that social play deprivation increases sensitivity to social defeat stress as measured by increased submissive and defensive behavior against a non-threatening juvenile during a conditioned defeat test. Also, reducing opportunities for

social play increased submissive and defensive behavior in males and increased aggression in females during the formation of new social groups. Interestingly, reducing opportunities for social play in female and male hamsters decreases their social interaction ratio in a social avoidance test, although 1 hour of daily play exposure did not rescue this form of stress-induced social avoidance. Altogether, this suggests that play deprivation disrupts agonistic behavior in new social groups, amplifies conditioned defeat responses, and enhances social avoidance. While dyadic play could rescue these deficits in conditioned defeat, 1 hour of dyadic play was insufficient to rescue social avoidance. It has been suggested that even 2-hours of social play may not be sufficient to compensate for the effects of social isolation in Syrian hamsters ⁶³. The amount of play needed to rescue the effects of isolation may be species-dependent, as 1-hour of social play was sufficient to rescue boldness behavior in an open field and decrease weight loss in rats ²¹. Further, if rats were exposed to a play partner that was injected with a drug to render them non-playful, but still mobile, they exhibited the same deficits as fully isolated rats ^{83,84}. Interestingly, probabilistic reversal learning (PRL) is commonly used to measure cognitive flexibility in rodents and humans. In PRL tasks, subjects must sample choice options and, from probabilistic feedback, find the current best choice which then changes without warning ⁸⁵. Recently, rats were either reared in isolation, in isolation with 1-hour of social play, or reared with a cage mate (PND 21-42). Rats reared in isolation had a decrease in perisomatic inhibitory synapses from PV cells with decreased inhibitory currents in the PFC and deficits in cognitive performance on reversal learning. Although, 1 hour of daily social play partially rescued cognitive performance in reversals in a probabilistic reversal learning

(PRL) task, which depended on the integrity of the PFC, by using a more simplified cognitive strategy than controls. Together, this indicates that while a short period of daily exposure to a peer can rescue some behavioral deficits, it is not a full rescue compared to continuous exposure to a playful partner⁸⁶. Another possibility is that the opportunity to play with multiple partners may be required for rescuing the effects of social stress in adulthood. Together, these findings suggest that peer isolation disrupts the development of contextually appropriate agonistic behavior and that providing opportunities for social play partly restores deficits in social behavior and cognitive performance.

Interestingly, the ability of daily dyadic play encounters to rescue effects of play deprivation depends on the outcome of play fights. The ‘50:50 rule’ suggests that individuals should take offensive and defensive roles in roughly equal proportion for play to remain appealing to both partners^{73,74}. However, reciprocity in playful behavior varies widely within and between species^{74,87}. In young male rhesus monkeys (*Macaca mulatta*), play-fighting roles tend to be fairly egalitarian at first, but as the partners grow older, one animal adopts the offensive role more often than the other⁸⁸. Rats have reciprocal play patterns with dynamic pins, attacks, supines, and role reversals. This complex pattern of rough and tumble play in young males will use rougher versions of rough-and-tumble play as a tool for negotiating dominance relationships^{7,89}. In this study, we found that the decrease in social play over time coincides with a reduction in reciprocity. By the late juvenile period, one animal clearly wins the encounter while the other uses a tail raise posture to indicate a subordinate social status. Winning or losing play encounters predicted behavior in a new social group, where female and male hamsters who lost more play fights

tended to show more submissive & defensive behavior during regrouping than hamsters who won. This suggests that the outcome of play encounters may be an important contributor to social behavior and stress vulnerability later in life. Others have shown that winning play fights enhances physical ability, increases chance for survival, and increases social status^{4,90-92}. We have previously shown that male and female hamsters that achieve a dominant social status show less stress vulnerability in a conditioned defeat test and a social avoidance test, respectively^{93,94}. Thus, social play experience may enhance the ability to win future aggressive encounters, which might improve stress resistance in adulthood.

Play-induced changes in PNN expression in the vmPFC may impact stress-related changes in social behavior. Among females, both Peer-Isolated and Dyadic Play hamsters showed diminished PNN/PV co-expression in the PL, with losers in the dyadic play showing the least amount of PNN/PV co-expression. Further, the proportion of PNNs that surround PV+ cells in the PL was negatively associated with aggressive behavior during the conditioned defeat test in both sexes. Females with fewer PV+ cells surrounded by PNNs also showed increased submissive behavior when re-housed into a new social group, which means that the losers in the dyadic play dates were more sensitive to re-socializing with littermates. Surprisingly, almost all the Peer-Isolated and a portion of the Dyadic Play females had to be individually housed prior to adulthood because they were hyper-aggressive and injured their cage mates. Together, these findings suggest the lack of social play and/or chronically losing play bouts may generate plasticity in the PL that sensitizes female aggression and social avoidance into adulthood. These findings are consistent with

other stressors that specifically disrupt inhibitory and excitatory neurotransmission in the PL. Rats subjected to peripubertal stress show decreased levels of synaptic cell adhesion molecules specific to GABAergic synapses, including PV cells, on PL neurons^{95,96}. Also, chronic unpredictable stress increased excitability of dopamine receptor-1 expressing pyramidal neurons of the PL, but not the IL⁹⁷. Similarly, dominant hamsters selectively activate BLA-projecting PL and IL neurons during social defeat and chemogenetic activation of these neurons reduces the conditioned defeat response^{47,98}. These studies suggest that various types of adverse experiences, including play deprivation, can produce synaptic plasticity in specific vmPFC neurons that alter stress-related changes in behavior.

vmPFC neurons also regulate aggressive behavior, although aggression depends on activity of specific neuronal ensembles. Rearing adolescent rats in isolation leads to increased aggressive behavior and decreased cellular markers of neural activity within the vmPFC, such as c-Fos and Arc⁹⁹. Activation of GABAergic interneurons diminish aggression in rodents, while inhibition of these interneurons increases aggression^{35,100}. This same pattern of control over aggressive behavior is seen in IL PV interneurons^{35,100,101}. Also, optogenetic stimulation of glutamatergic pyramidal cells in the vmPFC reduces aggression in mice, and optogenetic inhibition increases it¹⁰². In contrast, the elevated aggression in isolated adolescent rats has also been associated with increased c-Fos immunoreactivity in both CaMKII-expressing and GABA-expressing neurons in the PL and IL¹⁶. Further, optogenetic stimulation of vmPFC neurons projecting to either the lateral hypothalamus or medial basal hypothalamus increases bites in a resident-intruder test³⁵. These findings suggest that while global disruption of vmPFC activity decreases

inhibition of aggression, specific neural circuits within the vmPFC stimulate aggression. Together, these findings suggest the possibility that females reared in peer isolation, or those that consistently lost dyadic play dates, may have increased PL output because of disrupted PNN expression around PV+ cells, which alters agonistic behavior in the juvenile period and vulnerability to social defeat in adulthood.

Interestingly, we found that females given 1-hour of social play daily had lower PNN/PV co-expression in the IL compared to all other groups. The goal of the present study was to rescue deficits associated with play deprivation with 1-hour of social play daily, but this finding suggests that daily dyadic play generates patterns of neural plasticity that are different from playing in a social group. Because losing during dyadic play dates was associated with decreased PNN expression on PV+ neurons compared to winners, losers of play encounters may have decreased activity of PV+ neurons in the IL⁸⁶. Maturation of PNNs is implicated in the closing of the critical periods which may involve actual obstruction of neuritic extensions¹⁰³. Functionally, PNNs are implicated in the stabilization of synapses and consolidation of long-term memories^{56,103,104}. PNNs create a physical barrier to reduce synaptic plasticity around PV-expressing GABAergic cells^{105–107}. Impeding plasticity may assure efficient transfer of information within dedicated circuits, reflecting neural paths that are shielded from extraneous information of other neurons¹⁰⁷. We found that PNN number in the IL was positively correlated with interaction zone ratio during social avoidance testing in females, which is consistent with the role of the IL in suppressing fear responses^{42,108,109}. Interestingly, social avoidance was not associated with PNN expression on PV+ IL neurons, which suggests a role for PNN

expression on non-PV+ cells. One possibility is that increased PNN expression on IL pyramidal neurons enhances activity of IL-BLA neurons and suppresses defeat-induced changes in social behavior ^{47,110}.

The BLA is a critical site for synaptic plasticity underlying the acquisition of defeat-induced changes in social behavior ^{111,112}. While we did not find differences in PNN expression between play treatment groups, we did find that PNNs in the BLA were associated with individual differences in behavior during regrouping and social avoidance testing. Similar to our findings in the IL, PNN/PV co-expression in the BLA was associated with greater social approach during social avoidance testing. Because PNNs are thought to preferentially surround and stabilize mature PV+ interneurons ¹¹³, this could be reflective of PNNs stabilizing a pattern of local inhibition in the BLA. This may allow PV+ interneurons to exert greater inhibitory control over the BLA and reduce stress-related anxiety. We also found that hamsters with greater PNN/PV co-expression in the BLA were more aggressive during regrouping in both males and females. Interestingly, subjects with higher PNN count in the BLA independent of PV+ co-localization showed the most submissive and defensive behavior during regrouping. This highlights an important distinction between PNNs on PV+ and non-PV+ neurons. We suggest that while PNN expression on PV+ neurons in the BLA may attenuate anxiety responses, PNN expression on non-PV+ neurons may stabilize synaptic connections that increase anxiety responses. The expression of PV itself has also been shown to be activity dependent ¹¹⁴. As such, it is possible that PNNs are indeed surrounding PV+ neurons, but these interneurons are less active, and PV is undetectable using immunostaining. Future research will benefit from

investigating how PNNs modulate the activity in BLA inhibitory microcircuits to delineate the contribution of PV+ and PV- neurons to stress-related behavior.

5. Conclusion

Here we show that play deprivation increases stress vulnerability in a conditioned defeat test, and 1-hour of daily dyadic play was sufficient to rescue defeat-induced changes in agonistic aggression. Interestingly, consistently losing dyadic play bouts disrupts neural plasticity in the vmPFC and BLA, and female hamsters appear to be more sensitive to these changes than males. These findings suggest that early life experiences may alter PNN expression within stress sensitive brain regions which contributes to individual differences in social behavior. In this way, social play may help coordinate behavior in multiple domains where winning or losing broadly alters stress resistance and risk assessment in threatening situations. Beneficial directions for future research include delineating how PNN expression alters activity of select neural ensembles to produce individual differences in stress-related behavior. Further, future research should investigate what degree and context of social play is sufficient to fully rescue deficits in stress sensitivity and neural development. Our work delineates a critical role for social play in the development of competent social behavior and stress vulnerability, and sheds light on the role of PNNs on adult agonistic behavior.

References

1. Burghardt, G. M. *The Genesis of Animal Play: Testing the Limits*. (MIT Press, 2005).
2. Hol, T., Van den Berg, C. L., Van Ree, J. M. & Spruijt, B. M. Isolation during the play period in infancy decreases adult social interactions in rats. *Behav. Brain Res.* **100**, 91–97 (1999).
3. Von Frijtag, J. C., Schot, M., van den Bos, R. & Spruijt, B. M. Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. *Dev. Psychobiol.* **41**, 58–69 (2002).
4. Blumstein, D. T., Chung, L. K. & Smith, J. E. Early play may predict later dominance relationships in yellow-bellied marmots (*Marmota flaviventris*). *Proc. R. Soc. B Biol. Sci.* **280**, 20130485 (2013).
5. Nijhof, S. L. *et al.* Healthy play, better coping: The importance of play for the development of children in health and disease. *Neurosci. Biobehav. Rev.* **95**, 421–429 (2018).
6. Marquardt, A. E., VanRyzin, J. W., Fuquen, R. W. & McCarthy, M. M. Social play experience in juvenile rats is indispensable for appropriate socio-sexual behavior in adulthood in males but not females. *Front. Behav. Neurosci.* **16**, (2023).
7. Cooper, M. A., Grizzell, J. A., Whitten, C. J. & Burghardt, G. M. Comparing the ontogeny, neurobiology, and function of social play in hamsters and rats. *Neurosci. Biobehav. Rev.* **147**, 105102 (2023).
8. van den Berg, C. L. *et al.* Play is indispensable for an adequate development of coping with social challenges in the rat. *Dev. Psychobiol.* **34**, 129–138 (1999).

9. Ginsburg, K. R., and the Committee on Communications, & and the Committee on Psychosocial Aspects of Child and Family Health. The Importance of Play in Promoting Healthy Child Development and Maintaining Strong Parent-Child Bonds. *Pediatrics* **119**, 182–191 (2007).
10. Milteer, R. M. *et al.* The Importance of Play in Promoting Healthy Child Development and Maintaining Strong Parent-Child Bond: Focus on Children in Poverty. *Pediatrics* **129**, e204–e213 (2012).
11. Weisberg, D. S., Hirsh-Pasek, K., Golinkoff, R. M., Kittredge, A. K. & Klahr, D. Guided Play: Principles and Practices. *Curr. Dir. Psychol. Sci.* **25**, 177–182 (2016).
12. Yogman, M. *et al.* The Power of Play: A Pediatric Role in Enhancing Development in Young Children. *Pediatrics* **142**, e20182058 (2018).
13. Zelazo, P. D., Blair, C. B. & Willoughby, M. T. *Executive Function: Implications for Education. NCER 2017-2000. National Center for Education Research* <https://eric.ed.gov/?id=ED570880> (2016).
14. Da silva, N. L., Ferreira, V. M. M., De padua carobrez, A. & Morato, G. S. Individual Housing From Rearing Modifies the Performance of Young Rats on the Elevated Plus-Maze Apparatus. *Physiol. Behav.* **60**, 1391–1396 (1996).
15. Gerall, H. D., Ward, I. L. & Gerall, A. A. Disruption of the male rat's sexual behaviour induced by social isolation. *Anim. Behav.* **15**, 54–58 (1967).
16. Biro, L. *et al.* Structural and functional alterations in the prefrontal cortex after post-weaning social isolation: relationship with species-typical and deviant aggression. *Brain Struct. Funct.* **222**, 1861–1875 (2017).

17. Biro, L. *et al.* Post-weaning social isolation in male mice leads to abnormal aggression and disrupted network organization in the prefrontal cortex: Contribution of parvalbumin interneurons with or without perineuronal nets. *Neurobiol. Stress* **25**, 100546 (2023).
18. Oliveira, V. E. de M., Neumann, I. D. & de Jong, T. R. Post-weaning social isolation exacerbates aggression in both sexes and affects the vasopressin and oxytocin system in a sex-specific manner. *Neuropharmacology* **156**, 107504 (2019).
19. Potegal, M. & Einon, D. Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. *Dev. Psychobiol.* **22**, 159–172 (1989).
20. Einon, D. & Potegal, M. Enhanced defense in adult rats deprived of playfighting experience as juveniles. *Aggress. Behav.* **17**, 27–40 (1991).
21. Einon, D. F., Morgan, M. J. & Kibbler, C. C. Brief periods of socialization and later behavior in the rat. *Dev. Psychobiol.* **11**, 213–225 (1978).
22. Cervantes, M. C., Taravosh-Lahn, K., Wommack, J. C. & Delville, Y. Characterization of offensive responses during the maturation of play-fighting into aggression in male golden hamsters. *Dev. Psychobiol.* **49**, 87–97 (2007).
23. Delville, Y., Melloni, R. H. & Ferris, C. F. Behavioral and Neurobiological Consequences of Social Subjugation during Puberty in Golden Hamsters. *J. Neurosci.* **18**, 2667–2672 (1998).
24. Wommack, J. C., Salinas, A., Melloni, R. H. & Delville, Y. Behavioural and neuroendocrine adaptations to repeated stress during puberty in male golden hamsters. *J. Neuroendocrinol.* **16**, 767–775 (2004).

25. Wommack, J. C. & Delville, Y. Repeated social stress and the development of agonistic behavior: individual differences in coping responses in male golden hamsters. *Physiol. Behav.* **80**, 303–308 (2003).
26. Emfinger, K. Numerical Conceptions Reflected during Multiage Child-Initiated Pretend Play. *J. Instr. Psychol.* **36**, 326–334 (2009).
27. Fiorelli, J. A. & Russ, S. W. Pretend Play, Coping, and Subjective Well-Being in Children. (2012).
28. *The Cambridge Handbook of Play: Developmental and Disciplinary Perspectives*. vol. Pg. 208 (Cambridge University Press, 2018).
29. Kuczaj, S. A. & Eskelinen, H. C. Why do Dolphins Play? *Anim. Behav. Cogn.* **2**, 113 (2014).
30. Mustoe, A. C., Taylor, J. H., Birnie, A. K., Huffman, M. C. & French, J. A. Gestational cortisol and social play shape development of marmosets' HPA functioning and behavioral responses to stressors. *Dev. Psychobiol.* **56**, 1229–1243 (2014).
31. Marks, K. A., Vizconde, D. L., Gibson, E. S., Rodriguez, J. R. & Nunes, S. Play behavior and responses to novel situations in juvenile ground squirrels. *J. Mammal.* **98**, 1202–1210 (2017).
32. Shehan, M. I., Hernandez, M., Rodriguez, J. D. & Nunes, S. Social play predicts caution in juvenile Belding's ground squirrels (*Urocitellus beldingi*). *J. Mammal.* **104**, 1408–1420 (2023).

33. Leussis, M. P. & Andersen, S. L. Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse* **62**, 22–30 (2008).
34. Kyle, S. C., Burghardt, G. M. & Cooper, M. A. Development of social play in hamsters: Sex differences and their possible functions. *Brain Res.* **1712**, 217–223 (2019).
35. Biro, L. *et al.* Task Division within the Prefrontal Cortex: Distinct Neuron Populations Selectively Control Different Aspects of Aggressive Behavior via the Hypothalamus. *J. Neurosci. Off. J. Soc. Neurosci.* **38**, 4065–4075 (2018).
36. Markham, C. M., Luckett, C. A. & Huhman, K. L. The Medial Prefrontal Cortex is Both Necessary and Sufficient for the Acquisition of Conditioned Defeat. *Neuropharmacology* **62**, 933–939 (2012).
37. Morrison, K. E., Bader, L. R., McLaughlin, C. N. & Cooper, M. A. Defeat-induced activation of the ventral medial prefrontal cortex is necessary for resistance to conditioned defeat. *Behav. Brain Res.* **243**, 158–164 (2013).
38. Cheng, S.-Y., Taravosh-Lahn, K. & Delville, Y. Neural circuitry of play fighting in golden hamsters. *Neuroscience* **156**, 247–256 (2008).
39. Delville, Y., De Vries, G. J. & Ferris, C. F. Neural connections of the anterior hypothalamus and agonistic behavior in golden hamsters. *Brain. Behav. Evol.* **55**, 53–76 (2000).
40. Burleson, C. A. *et al.* Social play in juvenile hamsters alters dendritic morphology in the medial prefrontal cortex and attenuates effects of social stress in adulthood. *Behav. Neurosci.* **130**, 437–447 (2016).

41. Bell, H. C., Pellis, S. M. & Kolb, B. Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. *Behav. Brain Res.* **207**, 7–13 (2010).
42. Himmler, B. T. *et al.* Juvenile social experience and differential age-related changes in the dendritic morphologies of subareas of the prefrontal cortex in rats. *Synapse* **72**, e22022 (2018).
43. Giustino, T. F. & Maren, S. The Role of the Medial Prefrontal Cortex in the Conditioning and Extinction of Fear. *Front. Behav. Neurosci.* **9**, (2015).
44. Stevenson, C. W. Role of amygdala–prefrontal cortex circuitry in regulating the expression of contextual fear memory. *Neurobiol. Learn. Mem.* **96**, 315–323 (2011).
45. Quirk, G. J., Garcia, R. & González-Lima, F. Prefrontal Mechanisms in Extinction of Conditioned Fear. *Biol. Psychiatry* **60**, 337–343 (2006).
46. Kumar, S. *et al.* Prefrontal cortex reactivity underlies trait vulnerability to chronic social defeat stress. *Nat. Commun.* **5**, 4537 (2014).
47. Lehmann, M. L. & Herkenham, M. Environmental Enrichment Confers Stress Resiliency to Social Defeat through an Infralimbic Cortex-Dependent Neuroanatomical Pathway. *J. Neurosci.* **31**, 6159–6173 (2011).
48. Dulka, B. N. *et al.* Chemogenetic activation of an infralimbic cortex to basolateral amygdala projection promotes resistance to acute social defeat stress. *Sci. Rep.* **10**, 6884 (2020).
49. Grossman, Y. S. *et al.* Structure and function differences in the prelimbic cortex to basolateral amygdala circuit mediate trait vulnerability in a novel model of acute social defeat stress in male mice. *Neuropsychopharmacology* **47**, 788–799 (2022).

50. Anastasiades, P. G. & Carter, A. G. Circuit organization of the rodent medial prefrontal cortex. *Trends Neurosci.* **44**, 550–563 (2021).
51. Xu, H. *et al.* A Disinhibitory Microcircuit Mediates Conditioned Social Fear in the Prefrontal Cortex. *Neuron* **102**, 668-682.e5 (2019).
52. Fawcett, J. W., Oohashi, T. & Pizzorusso, T. The roles of perineuronal nets and the perinodal extracellular matrix in neuronal function. *Nat. Rev. Neurosci.* **20**, 451–465 (2019).
53. Xue, Y.-X. *et al.* Depletion of Perineuronal Nets in the Amygdala to Enhance the Erasure of Drug Memories. *J. Neurosci.* **34**, 6647–6658 (2014).
54. Lensjø, K. K., Christensen, A. C., Tennøe, S., Fyhn, M. & Hafting, T. Differential Expression and Cell-Type Specificity of Perineuronal Nets in Hippocampus, Medial Entorhinal Cortex, and Visual Cortex Examined in the Rat and Mouse. *eNeuro* **4**, (2017).
55. Pizzorusso, T. *et al.* Reactivation of Ocular Dominance Plasticity in the Adult Visual Cortex. *Science* **298**, 1248–1251 (2002).
56. Tanti, A. *et al.* Child abuse associates with increased recruitment of perineuronal nets in the ventromedial prefrontal cortex: a possible implication of oligodendrocyte progenitor cells. *Mol. Psychiatry* **27**, 1552–1561 (2022).
57. Gogolla, N., Caroni, P., Lüthi, A. & Herry, C. Perineuronal Nets Protect Fear Memories from Erasure. *Science* **325**, 1258–1261 (2009).
58. Banerjee, S. B. *et al.* Perineuronal Nets in the Adult Sensory Cortex Are Necessary for Fear Learning. *Neuron* **95**, 169-179.e3 (2017).

59. Li, X. *et al.* Perineuronal Nets Alterations Contribute to Stress-Induced Anxiety-Like Behavior. *Mol. Neurobiol.* (2023) doi:10.1007/s12035-023-03596-1.
60. Pellis, S. M. & Pellis, V. C. Play-fighting in the Syrian golden hamster *Mesocricetus auratus* waterhouse, and its relationship to serious fighting during postweaning development. *Dev. Psychobiol.* **21**, 323–337 (1988).
61. Guerra, R. F., Takase, E. & de O. Nunes, C. R. Play fighting of juvenile golden hamsters (*Mesocricetus auratus*): effects of two types of social deprivation and days of testing. *Behav. Processes* **47**, 139–151 (1999).
62. Pellis, S. M. & Pellis, V. C. Influence of dominance on the development of play fighting in pairs of male Syrian golden hamsters (*Mesocricetus auratus*). *Aggress. Behav.* **19**, 293–302 (1993).
63. Vieira, M. L., Garcia, M. P., Rau, D. D. W. & Prado, A. B. Effects of different opportunities for social interaction on the play fighting behavior in male and female golden hamsters (*Mesocricetus auratus*). *Dev. Psychobiol.* **47**, 345–353 (2005).
64. Vieira, M. L. & Otta, E. Play fighting in juvenile golden hamsters (*Mesocricetus auratus*): Effects of litter size and analysis of social interaction among males. *Behav. Processes* **43**, 265–273 (1998).
65. Vieira, M. L. Semelhanças e diferenças na estrutura da brincadeira social de hamsters dourados (*Mesocricetus auratus*) isolados e não isolados. *Biotemas* **14**, 19–36 (2001).
66. Vieira, M. L. & Otta, E. Efeitos de diferentes períodos de privação social sobre a brincadeira de hamsters dourados (*Mesocricetus auratus*). *Biotemas* **10**, 29–46 (1997).

67. Goldman, L. & Swanson, H. H. Developmental changes in pre-adult behavior in confined colonies of golden hamsters. *Dev. Psychobiol.* **8**, 137–150 (1975).
68. Landau, I. T. Light-dark rhythms in aggressive behavior of the male golden hamster. *Physiol. Behav.* **14**, 767–774 (1975).
69. Badura, L. L. & Nunez, A. A. Photoperiodic modulation of sexual and aggressive behavior in female golden hamsters (*Mesocricetus auratus*): Role of the pineal gland. *Horm. Behav.* **23**, 27–42 (1989).
70. Rosenhauer, A. M., McCann, K. E., Norvelle, A. & Huhman, K. L. An acute social defeat stressor in early puberty increases susceptibility to social defeat in adulthood. *Horm. Behav.* **93**, 31–38 (2017).
71. Golden, S. A., Covington, H. E., Berton, O. & Russo, S. J. A standardized protocol for repeated social defeat stress in mice. *Nat. Protoc.* **6**, 1183–1191 (2011).
72. Trainor, B. C. *et al.* Sex differences in stress-induced social withdrawal: Independence from adult gonadal hormones and inhibition of female phenotype by corncob bedding. *Horm. Behav.* **63**, 543–550 (2013).
73. Friard, O. & Gamba, M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol. Evol.* **7**, 1325–1330 (2016).
74. Altmann, S. A. A Field Study of the Sociobiology of Rhesus Monkeys, *Macaca Mulatta**. *Ann. N. Y. Acad. Sci.* **102**, 338–435 (1962).
75. Palagi, E. *et al.* Rough-and-tumble play as a window on animal communication. *Biol. Rev.* **91**, 311–327 (2016).

76. Bauer, E. B. & Smuts, B. B. Cooperation and competition during dyadic play in domestic dogs, *Canis familiaris*. *Anim. Behav.* **73**, 489–499 (2007).
77. Byosiére, S.-E., Espinosa, J. & Smuts, B. Investigating the function of play bows in adult pet dogs (*Canis lupus familiaris*). *Behav. Processes* **125**, 106–113 (2016).
78. Ward, C., Bauer, E. B. & Smuts, B. B. Partner preferences and asymmetries in social play among domestic dog, *Canis lupus familiaris*, littermates. *Anim. Behav.* **76**, 1187–1199 (2008).
79. R Core Team. R: A language and environment for statistical computing. (2022).
80. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using **lme4**. *J. Stat. Softw.* **67**, (2015).
81. Brooks, M. E. *et al.* glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R J.* **9**, 378–400 (2017).
82. Hand, D. J. & Taylor, C. C. *Multivariate Analysis of Variance and Repeated Measures*. (1987).
83. Krzanowski, W. J. *Principles of Multivariate Analysis: A User's Perspective*. (Oxford, 1988).
84. Einon, D. F., Humphreys, A. P., Chivers, S. M., Field, S. & Naylor, V. Isolation has permanent effects upon the behavior of the rat, but not the mouse, gerbil, or guinea pig. *Dev. Psychobiol.* **14**, 343–355 (1981).
85. Einon, D. F. & Morgan, M. J. A critical period for social isolation in the rat. *Dev. Psychobiol.* **10**, 123–132 (1977).

86. Latuske, P., von Heimendahl, M., Deiana, S., Wotjak, C. T. & du Hoffmann, J. Sustained MK-801 induced deficit in a novel probabilistic reversal learning task. *Front. Pharmacol.* **13**, 898548 (2022).
87. Bijlsma, A. *et al.* Social Play Behavior Is Critical for the Development of Prefrontal Inhibitory Synapses and Cognitive Flexibility in Rats. *J. Neurosci.* **42**, 8716–8728 (2022).
88. Cordoni, G. & Palagi, E. Ontogenetic Trajectories of Chimpanzee Social Play: Similarities with Humans. *PLOS ONE* **6**, e27344 (2011).
89. Symons, D. *Play and Aggression: A Study of Rhesus Monkeys*. (Columbia University Press, New York, 1978).
90. Pellis, S. M. *et al.* The effects of orbital frontal cortex damage on the modulation of defensive responses by rats in playful and nonplayful social contexts. *Behav. Neurosci.* **120**, 72–84 (2006).
91. Byers, J. A. & Walker, C. Refining the Motor Training Hypothesis for the Evolution of Play. *Am. Nat.* **146**, 25–40 (1995).
92. Fagen, R. & Fagen, J. Play behaviour and multi-year juvenile survival in free-ranging brown bears, *Ursus arctos*. *Evol. Ecol. Res.* **11**, 1053–1067 (2009).
93. Pellis, S. M., Burghardt, G. M., Palagi, E. & Mangel, M. Modeling play: distinguishing between origins and current functions. *Adapt. Behav.* **23**, 331–339 (2015).
94. Cooper, M. A. *et al.* Gonadal steroid hormone receptors in the medial amygdala contribute to experience-dependent changes in stress vulnerability. *Psychoneuroendocrinology* **129**, 105249 (2021).

95. Morrison, K. E. *et al.* Maintenance of dominance status is necessary for resistance to social defeat stress in Syrian hamsters. *Behav. Brain Res.* **270**, 277–286 (2014).
96. Tzanoulinou, S. *et al.* Neuroligin-2 Expression in the Prefrontal Cortex is Involved in Attention Deficits Induced by Peripubertal Stress. *Neuropsychopharmacology* **41**, 751–761 (2016).
97. Varoqueaux, F., Jamain, S. & Brose, N. Neuroligin 2 is exclusively localized to inhibitory synapses. *Eur. J. Cell Biol.* **83**, 449–456 (2004).
98. Anderson, E. M., Gomez, D., Caccamise, A., McPhail, D. & Hearing, M. Chronic unpredictable stress promotes cell-specific plasticity in prefrontal cortex D1 and D2 pyramidal neurons. *Neurobiol. Stress* **10**, 100152 (2019).
99. Dulka, B. N., Bress, K. S., Grizzell, J. A. & Cooper, M. A. Social Dominance Modulates Stress-induced Neural Activity in Medial Prefrontal Cortex Projections to the Basolateral Amygdala. *Neuroscience* **388**, 274–283 (2018).
100. Wall, V. L., Fischer, E. K. & Bland, S. T. Isolation rearing attenuates social interaction-induced expression of immediate early gene protein products in the medial prefrontal cortex of male and female rats. *Physiol. Behav.* **107**, 440–450 (2012).
101. Bai, F. *et al.* Prelimbic area to lateral hypothalamus circuit drives social aggression. *iScience* **26**, 107718 (2023).
102. Li, X. *et al.* Dysregulation of prefrontal parvalbumin interneurons leads to adult aggression induced by social isolation stress during adolescence. *Front. Mol. Neurosci.* **15**, (2022).

103. Takahashi, A., Nagayasu, K., Nishitani, N., Kaneko, S. & Koide, T. Control of Intermale Aggression by Medial Prefrontal Cortex Activation in the Mouse. *PLOS ONE* **9**, e94657 (2014).
104. Burket, J. A., Urbano, M. R. & Deutsch, S. I. Sugarcoated perineuronal nets regulate “GABAergic” transmission: Bittersweet hypothesis in autism spectrum disorder. *Clin. Neuropharmacol.* **40**, 120–130 (2017).
105. Thompson, E. H. *et al.* Removal of perineuronal nets disrupts recall of a remote fear memory. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 607–612 (2018).
106. Balmer, T. S. Perineuronal Nets Enhance the Excitability of Fast-Spiking Neurons. *eNeuro* **3**, (2016).
107. Berretta, S., Pantazopoulos, H., Markota, M., Brown, C. & Batzianouli, E. T. Losing the sugar coating: potential impact of perineuronal net abnormalities on interneurons in schizophrenia. *Schizophr. Res.* **167**, 18–27 (2015).
108. Burket, J. A., Webb, J. D. & Deutsch, S. I. Perineuronal Nets and Metal Cation Concentrations in the Microenvironments of Fast-Spiking, Parvalbumin-Expressing GABAergic Interneurons: Relevance to Neurodevelopment and Neurodevelopmental Disorders. *Biomolecules* **11**, 1235 (2021).
109. Sierra-Mercado, D., Padilla-Coreano, N. & Quirk, G. J. Dissociable Roles of Prelimbic and Infralimbic Cortices, Ventral Hippocampus, and Basolateral Amygdala in the Expression and Extinction of Conditioned Fear. *Neuropsychopharmacology* **36**, 529–538 (2011).

110. Suzuki, S. *et al.* The infralimbic and prelimbic medial prefrontal cortices have differential functions in the expression of anxiety-like behaviors in mice. *Behav. Brain Res.* **304**, 120–124 (2016).
111. Courtin, J. *et al.* Prefrontal parvalbumin interneurons shape neuronal activity to drive fear expression. *Nature* **505**, 92–96 (2014).
112. Day, D. E., Cooper, M. A., Markham, C. M. & Huhman, K. L. NR2B subunit of the NMDA receptor in the basolateral amygdala is necessary for the acquisition of conditioned defeat in Syrian hamsters. *Behav. Brain Res.* **217**, 55–59 (2011).
113. Jasnow, A. M. & Huhman, K. L. Activation of GABAA receptors in the amygdala blocks the acquisition and expression of conditioned defeat in Syrian hamsters. *Brain Res.* **920**, 142–150 (2001).
114. Guadagno, A., Belliveau, C., Mechawar, N. & Walker, C.-D. Effects of Early Life Stress on the Developing Basolateral Amygdala-Prefrontal Cortex Circuit: The Emerging Role of Local Inhibition and Perineuronal Nets. *Front. Hum. Neurosci.* **15**, (2021).
115. Patz, S., Grabert, J., Gorba, T., Wirth, M. J. & Wahle, P. Parvalbumin Expression in Visual Cortical Interneurons Depends on Neuronal Activity and TrkB Ligands during an Early Period of Postnatal Development. *Cereb. Cortex* **14**, 342–351 (2004).

CHAPTER FIVE
DOMINANCE STATUS MODULATES ACTIVITY IN MEDIAL AMYGDALA
CELLS WITH PROJECTIONS TO THE BED NUCLEUS OF THE STRIA
TERMINALIS

Abstract

The medial amygdala (MeA) controls several types of social behavior via its projections to other limbic regions. Cells in the posterior dorsal and posterior ventral medial amygdala (MePD and MePV, respectively) project to the bed nucleus of the stria terminalis (BNST) and these pathways respond to chemosensory cues and regulate aggressive and defensive behavior. Because the BNST is also essential for the display of stress-induced anxiety, a MePD/MePV-BNST pathway may modulate both aggression and responses to stress. In this study we tested the hypothesis that dominant animals would show greater neural activity than subordinates in BNST-projecting MePD and MePV cells after winning a dominance encounter as well as after losing a social defeat encounter. We created dominance relationships in male and female Syrian hamsters (*Mesocricetus auratus*), used cholera toxin b (CTB) as a retrograde tracer to label BNST-projecting cells, and collected brains for c-Fos staining in the MePD and MePV. We found that c-Fos immunoreactivity in the MePD and MePV was positively associated with aggression in males, but not in females. Also, dominant males showed a greater proportion of c-Fos⁺/CTB⁺ double-labeled cells compared to their same-sex subordinate counterparts. Another set of animals received social defeat stress after acquiring a dominant or subordinate social status and we stained for stress-induced c-Fos expression in the MePD and MePV. We found that dominant males showed a greater proportion of c-Fos⁺/CTB⁺ double-labeled cells in the MePD after social defeat stress compared to subordinates. Also, dominants showed a longer latency to submit during social defeat

than subordinates. Further, in males, latency to submit was positively associated with the proportion of c-Fos+ /CTB+ double-labeled cells in the MePD and MePV. These findings indicate that social dominance increases neural activity in BNST-projecting MePD and MePV cells and activity in this pathway is also associated with proactive responses during social defeat stress. In sum, activity in a MePD/MePV-BNST pathway contributes to status-dependent differences in stress coping responses and may underlie experience-dependent changes in stress resilience.

1. Introduction

Dominance relationships are characterized by experience-dependent changes in aggressive and submissive behavior and function to reduce social conflict for limited resources¹⁻⁴. They are created when one animal consistently wins a competitive interaction against a familiar opponent and the subordinate animal submits preemptively. The formation of dominance relationships leads to widespread changes in the neurochemicals and neural circuits that control aggressive and submissive behavior⁵⁻⁹. In addition, dominant and subordinate animals differ in how they respond to stress with dominants often showing stress resistance and subordinates showing stress vulnerability¹⁰⁻¹⁵. Together, these studies suggest that the specific neural circuits that underlie experience-dependent changes in aggression may also contribute to differences in stress responsivity between dominants and subordinates.

The medial amygdala (MeA) is a central node in a social behavior neural network and activity of MeA cells plays an essential role in aggressive, defensive, submissive, reproductive, and parental behavior¹⁶⁻²¹. Anterior regions of the MeA have robust

connections with the main and accessory olfactory system and play an essential role in processing social chemosensory cues^{22,23}. Neurons in the dorsal aspects of the posterior MeA (MePD) have been shown to regulate reproductive and aggressive behavior^{18,24–26}. On the other hand, ventral aspects of the posterior MeA (MePV) control defensive and aggressive behavior^{16,19,27}. The MeA contains a heterogeneous population of cell types with GABAergic projection cells common in the MePD, glutamatergic projection cells common in the MePV, and sparse GABAergic interneurons throughout^{26,28–31}. Efferent output of the MeA targets several limbic structures including the medial preoptic area, premammillary nucleus, basomedial amygdala, central amygdala, ventral medial hypothalamus (VMH), and bed nucleus of the stria terminalis (BNST)^{32,33}. These efferent pathways from the medial amygdala play a central role in regulating several types of social behavior including aggressive, mating, and parental behavior³⁴. Neurons in the MePD also express a high density of steroid hormone receptors, which makes this subdivision particularly sensitive to internal and motivational states.

In addition to providing a substrate for motivation to alter social behavior, the MeA also plays a key role in adversely motivated responses. Acute restraint stress increases neural activity in the MePD and MePV cells and lesions of the MeA decrease activation of the hypothalamic-pituitary-adrenal (HPA) axis^{35,36}. Acute social defeat stress activates MePD cells that express corticotropin-releasing factor type-2 receptors (CRF-R2)³⁷. Also, intermittent social defeat stress leads to a long-term increase in mRNA for brain-derived neurotrophic factor in the posterior MeA³⁸. Because similar MeA pathways control multiple types of social behavior³⁹, plasticity in the neural

ensembles regulating one aspect of behavior such as aggression may lead to changes in other types of behavior such as stress reactivity. However, whether experience-dependent changes in MeA activity lead to individual differences in stress reactivity remains largely unknown.

Cells in the MePD and MePV send robust projections to caudal portions of the BNST and modulate aggressive behavior. Winning aggressive encounters produces NMDA-dependent synaptic potentiation in a MePV-BNST pathway and potentiates aggression in future interactions, a phenomenon known as attack priming²⁷. Likewise, optogenetic stimulation of BNST-projecting MeA cells positive for neuropeptide Y1 receptors elicits territorial aggression⁴⁰. Also, in male Syrian hamsters, the vast majority (87%) of BNST-projecting cells in the posterior MeA are positive for androgen receptors, which suggests this pathway is sensitive to changes in testosterone activity⁴¹. While these findings indicate that a MePD/MePV-BNST pathway can regulate aggressive behavior, this pathway also modulates approach-avoidance behavior. Activation of BNST-projecting MePV cells positive for dopamine D1 receptors elicits approach to threatening stimuli¹⁹. Consistent with these findings, stress-induced social anxiety can be affected by neuropeptide activity in the BNST. For example, pharmacological blockade of oxytocin receptors in anteromedial BNST increases social approach and decreases social vigilance in female California mice exposed to social defeat stress⁴². Further, blockade of vasopressin 1A (V1a) receptors in the medioventral BNST reduces social approach in male California mice exposed to social defeat stress⁴³. Altogether, these findings indicate

that a MePD/MePV-BNST pathway is well-positioned to regulate stress-induced changes in social behavior.

Syrian hamsters are territorial rodents that form stable dominance relationships with familiar same-sex opponents⁴⁴⁻⁴⁷. Hamsters also exhibit a striking change in agonistic behavior following acute social defeat stress. When male hamsters are exposed to a larger, more aggressive animal for up to 15 min and lose the fight, they show a complete loss of territorial aggression the next day and a substantial increase in submissive and defensive behavior when tested with a novel, younger, non-aggressive animal⁴⁸. This defeat-induced change in social behavior is called the conditioned defeat response. We have shown that after male hamsters acquire a dominant social status, they exhibit a reduced conditioned defeat response compared to their subordinate counterparts⁴⁹. Interestingly, dominant males also show increased c-Fos expression in MePV cells following either social defeat or restraint stress^{35,50}. Because neurotransmission in the MeA during social defeat is necessary for the acquisition of the conditioned defeat response⁵¹, neural ensembles in the MePD and/or MePV may contribute to resistance to conditioned defeat in dominant males. Consistent with this possibility is that pharmacological blockade of androgen receptors in the MeA during social defeat stress increases a subsequent conditioned defeat response in dominant males but not subordinate males¹¹. While female hamsters do not show a robust conditioned defeat response, they do demonstrate defeat-induced changes in behavior by showing elevated avoidance when tested in a novel arena with an unfamiliar animal restrained behind a partition⁵². While we have found that dominant females show less defeat-induced social

avoidance compared to subordinate females, the role of MeA neurons in this status-dependent change in behavior is unknown ¹¹.

Because dominance status alters defeat-induced neural activity in MeA cells and neurotransmission in the BNST is necessary for the conditioned defeat response, a MePD/MePV-BNST pathway is a prime candidate for mediating status-dependent changes in conditioned defeat. The aim of this study was to determine whether the process of establishing a dominance relationship changes neural activity in MePD and MePV cells projecting to the BNST and whether status-dependent differences in neural activity are maintained when animals experience social defeat stress. We tested the prediction that dominant hamsters would show increased neural activity in BNST-projecting MePD and MePV cells during the development of social dominance compared to their subordinate counterparts. Then, we tested the prediction that dominant hamsters would show increased neural activity in BNST-projecting MePD and MePV cells during social defeat stress compared to their subordinate counterparts.

2. Methods

2.1. Subjects

Male and female Syrian hamsters (*Mesocricetus auratus*) were obtained from our breeding colony, which was derived from animals purchased from Charles River Laboratories (Wilmington, MA). Subjects were young adults and 9 weeks old (130–150 g) at the start of the study. One week prior to an experiment, animals were housed individually in polycarbonate cages (12 cm × 27 cm x 16 cm) with corncob bedding,

cotton nesting materials, and wire mesh tops. Because Syrian hamsters are highly territorial, low-ranking animals can be wounded when housed in a social group, and adults thrive in the laboratory when housed individually. In our breeding colony, juvenile animals are raised in social groups with environmental enrichment, such as plastic shelters and paper cups. Food and water were available ad libitum. Cages were not changed for one week prior to an experiment to allow individuals to scent mark their territory and animals were handled daily to habituate them to the stress of human contact. Hamster females have a remarkably consistent 4-day estrous cycle, and we monitored the estrous cycle for 8 days prior to start of the study. We evaluated animals for signs of Estrus by placing a cotton swab against the vaginal area and identified the day of Estrus by the presence of a stringy vaginal discharge^{53,54}. Subsequent days of the estrous cycle were Diestrus I, Diestrus II, and Proestrus. Animals were housed in a temperature controlled colony room (21 ± 2 °C) and kept on a 14:10 h light:dark cycle to facilitate gonad development and aggressive behavior. All behavioral protocols were performed during the first three hours of the dark phase of their light:dark cycle to minimize circadian variation in aggressive behavior^{55,56}. All procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee and are in accordance with ARRIVE guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Experimental design

In study 1 we predicted that male and female dominant hamsters would show elevated c-Fos immunoreactivity in BNST-projecting MePD and MePV cells following

the formation of a dominance relationship compared to their same-sex subordinate counterparts. We paired male and female hamsters in daily dyadic social encounters with a same-sex partner. We administered a unilateral microinjection of the retrograde transporter cholera toxin subunit B (CTB) in the BNST to label cells in a MePD/MePV-BNST pathway (Fig. 5.1). Because CTB shows peak retrograde transport after 7–10 days, we interrupted dyadic encounters for stereotaxic surgery at the midpoint of dominance encounters. We humanely euthanized animals and collected brains 90 min after the final dominance encounter, which corresponded to Proestrus for females.

In study 2 we predicted that male and female dominant hamsters would show elevated c-Fos immunoreactivity in BNST-projecting MePD and MePV cells following social defeat stress compared to their same-sex subordinate counterparts. We paired male and female hamsters in daily dyadic social encounters with a same-sex partner and administered a CTB microinjection into the BNST as described in study 1. Then, animals were exposed to acute social defeat stress 48 h after the final dominance encounter. We humanely euthanized animals and collected brains 90 min following social defeat, which corresponded to Diestrus I for females.

2.3. Dominance encounter

To create dominance relationships in same-sex pairs of hamsters, we weight-matched animals and assigned them to resident-intruder dyads. Using a resident-intruder format, animals received 12 dominance encounters with their partner over a 20-day period. Because aggression levels are inconsistent when hamsters are in the Estrus phase of their cycle^{53,54,57,58}, we cycle-matched females and skipped encounters when both

animals were in Estrus. This format resulted in 3 encounters followed by one ‘skip’ day until all dyads received 12 encounters. To use identical protocols for male and female hamsters, male dyads received 12 encounters in a similar format. Because of the 4 days skipped for estrus and the 4-day surgical recovery period, 12 encounters occurred over 20 days. Encounters were 10-min in duration prior to the identification of a clear winner and loser, and then encounters were shortened to 5-min in duration to allow the maintenance of a dominance relationship with minimal risk for physical wounding. For each dyadic encounter we recorded the latency to attack, the number of attacks, the latency to show submissive posture, and the number of flees. As a result, we determined dominance status at each encounter and used their status at the final encounter to categorize animals for analyses. Researchers achieved 90% agreement on these measures of agonistic behavior prior to scoring behavior.

2.4. Stereotaxic surgery and microinjection

After the 6th dominance encounter, animals were anesthetized with isoflurane, mounted in a stereotaxic instrument, and injected unilaterally with the fluorescently labeled retrograde tracer cholera toxin b (CTB; Alexa Flour CTB 488). Injections targeted caudal portions of the BNST using stereotaxic coordinates of 1.5 mm anterior and 1.35 mm lateral to bregma and 6.2 mm below dura. Injections deposited 100 nL of CTB solution over a 10-minute period, and the needle was left in place an additional 10 min to maximize diffusion following the injection. Animals were given 4 days of surgical recovery before resuming their daily dominance encounters. We have previously

found that hamster dominance relationships are very stable and not affected by an interruption for surgery and recovery^{59,60}.

2.5. Social defeat

Social defeat stress consisted of 3, 5-minute aggressive interactions at 5-minute inter-trial intervals in which the subject lost to a larger, same-sex aggressor. Novel aggressors were used for each encounter and interactions occurred in the aggressor's home cage. Although aggressors were pre-screened for territorial aggression, variation existed in the latency with which they initiated aggression. Also, there was variability in the extent to which subjects defended themselves and the latency with which they submitted to the aggressor. To account for variation in how animals responded to the first social defeat encounter, we quantified the latency for resident aggressors to initiate aggression (e.g. side aggressive posture, chase, or attack), and the latency for subjects to submit (e.g. tail-up posture or a full supine posture). Then, we calculated the relative latency to submit by subtracting the latency to initiate aggression from the latency to submit. This calculation resulted in a negative value when animals submitted prior to receiving aggression. Researchers achieved 90% agreement on these latency measures prior to quantifying social defeat encounters and behavior was scored blind to treatment conditions.

2.6. Histology and immunofluorescence microscopy

Animals were anesthetized with isoflurane and perfused transcardially with 100 mL of 0.1 M phosphate buffer (PB) followed by 100 mL of 4% paraformaldehyde.

Brains were removed and post-fixed in 4% paraformaldehyde for 24 h and then stored in 0.05 M PB with 30% sucrose and 0.01% sodium azide at 4 °C. Brains were sectioned on a freezing microtome at 40 µm and stored as free-floating, coronal sections in PB/sodium azide solution. To visualize CTB injection sites BNST sections were washed with distilled water, mounted on glass slides, and cover-slipped using a mounting medium with DAPI. Amygdala tissue sections were processed for c-Fos immunohistochemistry. Sections were rinsed in five PB washes, incubated in a blocking solution of 1% goat serum for 25 min, and then incubated in a c-Fos primary antibody solution for 24 h at room temperature (1:5000 concentration in 0.05PB/0.2% Triton, rabbit anti-c-Fos, Santa Cruz, sc-52). Then, sections were rinsed in PB/Triton solution and incubated in a secondary antibody solution for 2 h (1:500 concentration of Alexa Flour 594 in PB/Triton, with 1% goat serum). Sections were rinsed in PB, mounted onto slides, and cover-slipped using a mounting medium with DAPI.

Quantification of c-Fos⁺, CTB⁺, and c-Fos⁺/CTB⁺ double-labeled cells was performed using an Olympus BX51 microscope. CTB injection sites were plotted on templates from the Syrian hamster stereotaxic atlas ⁶¹. Animals were excluded from statistical analysis if the center of their CTB injection was outside the BNST. Images of CTB fluorescence and c-Fos immunoreactivity were collected throughout the rostral-caudal extent of the MePD and MePV, which corresponds to 1.5–2.0 mm posterior to bregma in the Syrian hamster stereotaxic atlas ⁶¹. We collected images at 10x magnification, using a 500 × 500 µm clip region, and with 3–6 images collected per animal. We centered the clip region on a population of CTB cells within the boundaries

of the MePD or MePV and observed that CTB⁺ cells were most abundant in the medial portion of the nucleus. This procedure resulted in green CTB⁺ cells and red c-Fos⁺ cells, and merged images were evaluated for c-Fos⁺ /CTB⁺ double-labeled cells (Fig. 5.2). We used MCID image analysis software to quantify single-labeled c-Fos⁺ cells and CTB⁺ cells above background in a semi-automated manner, and we quantified c-Fos⁺ /CTB⁺ double-labeled cells manually. Researchers achieved 90% agreement with one another on cell counts and then established MCID settings that produced 90% agreement with researchers. All images were quantified blind to treatment conditions.

2.7. Data analysis

Agonistic behavior during the development of dominance relationships is exceedingly unidirectional, which leads to data with unequal variances. To analyze changes in agonistic behavior during dominance encounters we used 2-way repeated measures ANOVAs with a Greenhouse-Geisser correction. For histological data on c-Fos⁺ cells, CTB⁺ cells, and c-Fos⁺ /CTB⁺ double-labeled cells we used generalized linear mixed models. When histological data failed tests for a normal distribution, we used a negative binomial distribution and a Wald chi-square test to check for overdispersion. Also, we performed linear regressions to explore whether agonistic behavior during the final dominance encounter, as well as dominance status and sex, predicted the number of c-Fos⁺ cells in the MePD and MePV. Similarly, we performed linear regressions to explore whether relative latency to submit during social defeat stress, as well as dominance status and sex, predicted the proportion of c-Fos⁺ /CTB⁺ double-labeled cells.

Analyzes were performed in SPSS version 29 or R data analysis software. Data are reported with means and standard errors. Effect sizes are reported as partial eta squared for ANOVAs and linear regressions and reported as Cohen's d for post-hoc T-tests. P values were set at .05.

3. Results

3.1. Formation of dominance relationships

Animals were matched with a partner and paired in daily dominance encounters in a resident-intruder format. Because hamsters are highly territorial, both males and females readily form stable dominance relationships using this procedure. Dominance relationships were assessed daily and animals were categorized as dominant or subordinate according to the direction of agonistic behavior on the 12th encounter. We found a main effect of aggressive behavior such that dominant males showed a higher rate of attacks compared to subordinate males ($F(1,27) = 41.09$, $p = .0001$, $\eta^2 = .603$; Fig. 5.3a). Also, subordinate males showed a higher rate of flees compared to dominant males ($F(1,27) = 19.68$, $p = .0001$, $\eta^2 = .422$). The rate of attacks did not significantly vary over time ($F(1,5.0) = 1.50$, $p = .195$, $\eta^2 = .053$), although the rate of flees showed a significant interaction with time and dominance status ($F(1,2.9) = 2.93$, $p = .041$, $\eta^2 = .098$; Fig. 5.3b). These findings reveal that dominant males consistently initiated more attacks than subordinate males, while the amount of flees increased over time in subordinate males.

Female hamsters also formed stable dominance relationships. Dominant females showed a higher rate of attacks than subordinate females ($F(1,27) = 90.42$, $p = .00001$,

$\eta^2 = .770$), and subordinates showed a higher rate of flees than dominants ($F(1,27) = 27.05$, $p = .0001$, $\eta^2 = .500$). The rate of attacks in females significantly varied over time and indicates that females were least aggressive during encounters 3, 6, 9 and 12, which corresponds to the proestrus phase of their estrous cycle ($F(1,3.8) = 4.15$, $p = .004$, $\eta^2 = .133$). The rate of flees showed a significant interaction with time and dominance status such as subordinate females showed the fewest number of flees during the proestrus phase of their estrus cycle, while dominants did not flee ($F(1,5.0) = 2.84$, $p = .018$, $\eta^2 = .095$). Altogether, these findings indicate that dominance relationships develop gradually in male hamsters, while they form quickly in female hamsters with agonistic behavior fluctuating over the estrous cycle.

3.2. Study 1: MeA-BNST activity during dominance encounters

To investigate differences in activity of BNST-projecting MePD/MePV neurons during the maintenance of dominance relationships, we collected brains from a group of animals for c-Fos immunoreactivity after the 12th dominance encounter. We found no significant effect of dominance status or sex on the number of MePD c-Fos+ cells (status: $\chi^2 = .205$, $df = 1$, $p = .651$; sex: $\chi^2 = .074$, $df = 1$, $p = .785$; Fig. 5.4a). In contrast, we found a main effect of sex such that males showed more c-Fos+ cells in the MePV compared to females ($\chi^2 = 4.66$, $df = 1$, $p = .031$), although there was no effect of dominance status ($\chi^2 = .194$, $df = 1$, $p = .660$; Fig. 5.4d). We also analyzed the number of CTB+ cells in the MePD/MePV-BNST pathway. We found that dominance status and sex did not significantly alter the number of CTB+ cells in the MePD (status: $\chi^2 = .014$, $df = 1$, $p = .906$; sex: $\chi^2 = 1.06$, $df = 1$, $p = .302$; Fig. 5.4b). However, we found that females

had more CTB+ cells in the MePV compared to males ($\chi^2 = 4.35$, $df = 1$, $p = .037$). This difference may have occurred because females had greater diffusion of CTB into the caudal BNST compared to males, although the number of MePV CTB+ cells did not differ between dominants and subordinates (status: $\chi^2 = .052$, $df = 1$, $p = .821$).

There was a main effect of dominance status and sex on the proportion of c-Fos+ /CTB+ double-labeled cells in the MePD (status: $\chi^2 = 9.24$, $df = 1$, $p = .002$; sex: $\chi^2 = 7.74$, $df = 1$, $p = .005$; Fig. 5.4c). Although the interaction did not reach statistical significance ($\chi^2 = 3.16$, $df = 1$, $p = .075$), these findings indicating that dominant males showed a 3-fold increase in the proportion of double-labeled cells compared to subordinate males (Cohen's $d = 2.06$), while the effect of dominance status in females was much weaker (Cohen's $d = 0.27$). We found a similar pattern of results in the MePV. There was a main effect of dominance status and sex on the proportion of c-Fos+ /CTB+ double-labeled cells in the MePV (status: $\chi^2 = 7.38$, $df = 1$, $p = .007$; sex: $\chi^2 = 28.21$, $df = 1$, $p = .00001$; Fig. 5.4f). These results indicate that dominant males had a 3-fold increase in double-labeled cells in the MePV compared to subordinates (Cohen's $d = 1.63$), while the effect of dominance status in females was much more modest (Cohen's $d = 0.56$).

We performed additional analyses to determine whether changes in c-Fos expression were associated with patterns of agonistic behavior during the final dominance encounter. We found that the c-Fos immunoreactivity in BNST-projecting MePD/MePV cells was not significantly associated with aggression in dominants or with submission in subordinates ($p > .05$ all tests). However, the total number of c-Fos+ cells

was associated with status-dependent differences in agonistic behavior. Specifically, linear regressions on the number of MePD c-Fos+ cells and the number of attacks initiated by dominants during the 12th encounter revealed a significant interaction with sex ($F(3,11) = 5.76$, $p = .035$, $\eta^2 = .344$). A similar interaction with sex was found for the association between MePV c-Fos+ cells and attacks ($F(3,11) = 20.50$, $p = .0001$, $\eta^2 = .651$). These findings indicate that the number of MePD and MePV c-Fos+ cells predicted the number of attacks initiated by dominant males but not dominant females (Fig. 5.5a,b). Interestingly, we found different associations between MePD and MePV c-Fos expression and submissive behavior in subordinates. We found a main effect such that the number of MePD c-Fos+ cells predicted the number of flees in subordinate animals during the 12th encounter (flee: $F(3,10) = 6.33$, $p = .031$, $\eta^2 = .388$; Fig. 5.5c). The number of c-Fos+ cells in the MePV did not significantly predict the number of flees in subordinate animals ($F(3,10) = 3.55$, $p = .089$, $\eta^2 = .262$; Fig. 5.5d). Overall, these analyses indicate that c-Fos immunoreactivity in the MePD and MePV during the dominance encounters was positively associated with aggression in dominant males, while MePD c-Fos immunoreactivity in the MePD was also positively associated with submission in subordinate males and females.

3.3. Study 2: MeA-BNST activity during social defeat stress

Next, to address stress-induced activation of the MePD/MePV-BNST pathway, a group of dominant and subordinate animals received acute social defeat stress. Interestingly, there was considerable variation in the extent to which subjects defended themselves against the resident aggressor during the first of three social defeat

encounters. In fact, 16 of 29 animals initially attacked a resident aggressor, which resulted in mutual aggression prior to the subject losing the fight. Further, all subjects submitted immediately in the 2nd and 3rd social defeat encounters. Overall, 13 of 15 dominants attacked a resident aggressor prior to losing, while 3 of 14 subordinates, all of which were females, attacked resident aggressors ($\chi^2 = 12.46$, $df = 1$, $p = .001$). In addition, we calculated a relative latency to submit and found a main effect of status such that dominant hamsters (49.3 ± 13.6 s) had a longer relative latency to submit compared to subordinates (9.3 ± 34.4 s) (Kruskal-Wallis $\chi^2 = 9.07$, $df = 1$, $p = .003$). Dominant males (26.1 ± 7.1 s) had a significantly longer relative latency to submit compared to subordinate males (-3.1 ± 6.4 s) (Mann-Whitney $U = 6.0$, $df = 1$, $p = .009$). The relative latency to submit in dominant (75.8 ± 25.2 s) and subordinate (21.7 ± 70.9 s) females approached statistical significance (Mann-Whitney $U = 9.0$, $df = 1$, $p = .053$).

First, we examined total c-Fos immunoreactivity in the MePD and MePV following social defeat stress. We found no significant effect of dominance status or sex on the number of c-Fos+ cells in the MePD (status: $F(1,25) = .160$, $p = .693$; sex: $F(1,25) = 0.996$, $p = .328$; Fig. 5.6a). Similarly, there was no significant effect of dominance status or sex on the number MePV c-Fos+ cells (status: $F(1,25) = 3.77$, $p = 0.064$; sex: $F(1,25) = 1.37$, $p = .252$; Fig. 5.6d). We also found no significant differences in the number of CTB+ cells, which indicates the number of labeled BNST-projecting MePD/MePV cells did not differ by treatment conditions. Specifically, there were no significant effects of dominance status or sex on the number of CTB+ cells in either the

MePD (status: $\chi^2 = .706$, $df = 1$, $p = .401$; sex: $\chi^2 = .131$, $df = 1$, $p = .718$; Fig. 5.6b) or MePV (status: $\chi^2 = .898$, $df = 1$, $p = .343$; sex: $\chi^2 = .595$, $df = 1$, $p = .440$; Fig. 5.6e).

Importantly, we found a significant effect of dominance status on the proportion of c-Fos+ /CTB+ double-labeled cells in the MePD ($F(1,25) = 10.16$, $p = .004$), although the interaction with sex did not reach statistical significance ($F(3,25) = 3.14$, $p = .089$; Fig. 5.6c). This result indicates that dominant males had a 2.5-fold increase in double-labeled cells in the MePD compared to subordinates (Cohen's $d = 1.48$), while the effect of dominance status in females was much more modest (Cohen's $d = 0.71$). In contrast, there was no effect of dominance status on the proportion of c-Fos+ /CTB+ double-labeled cells in the MePV ($F(1,25) = 2.29$, $p = .143$; Fig. 5.6f). Altogether, these findings indicate that dominant males showed greater c-Fos expression in BNST-projecting MePD cells compared to their subordinate counterparts and dominant females showed a similar trend.

We performed linear regressions to test whether relative latency to submit predicted the proportion of c-Fos+ /CTB+ double-labeled cells. We found that sex altered the relationship between relative latency to submit and the number of c-Fos+ /CTB+ cells in the MePD ($F(2,21) = 5.62$, $p = .027$, $\eta^2 = .221$). This finding indicates that relative latency to submit was positively associated with the proportion of c-Fos+ /CTB+ double-labeled cells in the MePD in male hamsters ($R^2 = .479$, $F(1,13) = 11.94$, $p = .004$), but not in females (Fig. 5.7a,b). Interestingly, both dominant and subordinate males showed a positive association. In contrast, there was no significant effect of sex on the relationship between relative latency to submit and the number of c-Fos+ /CTB+ cells in the MePV

($F(2,21) = 2.52$, $p = .127$, $\eta^2 = .147$). In addition, relative latency to submit did not significantly predict the proportion of c-Fos+ /CTB+ double-labeled cells in the MePV ($F(1,27) = 1.29$, $p = .266$, $\eta^2 = .104$) (Fig. 5.7c,d).

4. Discussion

This project was focused on whether the development of dominance relationships changes neural activity in BNST-projecting cells in the MePD and MePV. We found that dominant animals showed increased neural activity in MePD-BNST and MePV-BNST neural pathways during an aggressive interaction with their subordinate counterpart. This effect was most pronounced in males such that dominants showed a 3-fold increase in c-Fos immunoreactivity in BNST-projecting cells in both the MePD and MePV. Interestingly, dominant animals also showed increased neural activity in BNST-projecting MePD cells when they were exposed to social defeat stress. Dominant animals were also more likely to defend themselves against an aggressor during social defeat and had a longer latency to submit than subordinate animals. Specifically, neural activity in MePD-BNST and MePV-BNST pathways positively predicted latency to submit during social defeat stress in males. Altogether, these findings suggest activation of MePD-BNST neurons is associated with both the development of social dominance and how dominant animals respond to social stress.

Both males and females formed stable dominance relationships, although the formation and maintenance of dominance relationships is characterized by different patterns of aggressive and submissive behavior. Dominant males show consistent levels of aggression and subordinate males show gradual increases in submissive behavior. On

the other hand, females show mutual aggression prior to one animal losing and aggression in dominants fluctuates with their estrous cycle. Mutual aggression in hamsters is characterized by a rolling fight in which both animals assume an attack posture, tumble and bite until one animal gives up and flees ⁶². Mutual aggression is more common in females compared to males and this difference in aggression increases instability in female dominance relationships ¹¹. Interestingly, in primate dominance hierarchies, animals with unstable dominance relationships show elevated basal cortisol concentrations ⁶³ and increased levels of proinflammatory cytokines ⁶⁴ compared to monkeys with more certainty in their dominance rank. These findings indicate that rank-related differences in stress reactivity are dependent on the stability of the dominance hierarchy ⁶⁵.

Males and females also differ in the contribution of gonadal steroid hormones to experience-dependent changes in agonistic behavior. In males, a surge in post-victory testosterone and activation of androgen receptors increases the probability of winning future contests, a phenomenon called the winner effect ⁶⁶. The winner effect in California mice depends on a surge in plasma testosterone and an upregulation of androgen receptors in the nucleus accumbens and BNST, which is most likely to occur when animals successfully defend their home territory ^{67,68}. Pharmacological blockade of androgen receptors during daily dominance encounters also increases the conditioned defeat response in dominant male hamsters ¹¹. While females have a much lower density of androgen receptors than males ⁶⁹, there are other neuroendocrine factors that might contribute to experience-dependent changes in female aggression such as 5 α -reductase,

aromatase, and estrogen receptor alpha ⁷⁰. Because MeA has a high density of androgen and estrogen receptors, sex differences in gonadal steroid hormone receptors may contribute to sex differences in activity of MePD/MePV-BNST neurons. Overall, the sex differences in stability of dominance relationships and feedback of gonadal steroid hormones may contribute to why dominant females showed 1) reduced activation of MePD/MePV-BNST cells during dominance encounters compared to dominant males, 2) no association between MePD and MePV neural activity and aggressive behavior, 3) diminished defeat-induced activation of MePD/MePV-BNST cells compared to dominant males and, 4) no association between relative latency to submit during social defeat and activation of MePD/MePV-BNST cells. Another important consideration for sex differences in activation of MePD/MePV-BNST cells is that females were tested in the Proestrus phase of their cycle in study 1 and in Diestrus I in study 2. While aggression and social avoidance vary across the estrous cycle in hamsters ^{52,53}, it is unknown whether neural activity in the MeA also varies. However, it seems unlikely that the difference in estrous cycle phase between study 1 and 2 prevented our ability to detect whether dominant females reactivate a MePD/MePV-BNST pathway during social defeat because we found no difference between dominant and subordinate females in either study. Nevertheless, it remains possible that females may activate BNST-projecting cells in the MePD or MePV during other phases of their cycle.

The posterior MeA has a complex set of cell types that regulate aggressive, submissive, and defensive behavior. Optogenetic stimulation of GABAergic cells in the MePD leads to increased aggression in male mice ¹⁸. On the other hand, optogenetic

stimulation of MePV glutamatergic cells projecting to the BNST and lateral regions of the ventral medial hypothalamus (VMHL) increases the frequency and duration of aggression in an attack priming test, respectively ²⁷. Likewise, select MePV neural ensembles also modulate defensive behavior to threat cues via their connections to the BNST, dorsomedial regions of the VMH (VMHdm), and anterior hypothalamus ^{16,19}. Because aggressive and submissive behavior are likely regulated by separate MePD and MePV neural ensembles, it is not surprising that dominant and subordinate animals did not differ in total c-Fos expression. Our results are consistent with findings in long-tailed hamsters (*Tscheskia triton*) showing that dominant and subordinate males did not differ in c-Fos immunoreactivity in the MeA following a single 10-minute aggressive interaction ⁷¹. Similarly, outbred male mice show greater c-Fos immunoreactivity in the MePV after exposure to alpha male urine compared to subordinate male urine, although c-Fos expression was not dependent on the subject's own social status ⁷². While dominant and subordinate animals in this study did not differ in MePD or MePV c-Fos immunoreactivity during dominance interactions, we found interesting correlations between c-Fos expression and agonistic behavior. In males, MePD and MePV c-Fos expression was correlated with aggressive behavior in dominants. In contrast, c-Fos expression in MePD cells predicted the amount of submissive behavior in both males and females. The contribution of MePV activity to both aggression and submission in males, but not in females, may have led to the sex difference found in total MePV c-Fos expression. Overall, these findings are consistent with separate MePD and MePV neural

ensembles modulating different aspects of agonistic behavior during dynamic social interactions.

We found that formation of dominance relationships did not alter neural activity in BNST-projecting cells in the MePD or MePV of female hamsters, although activity of other MeA cell types might drive differences in behavior between dominant and subordinate females. MePD and MePV neurons send dense projections to the VMHL^{16,26}, which can modulate VMHL neural ensembles critical for aggression^{73,74}. Microinjection of vasopressin into the VMHL of male hamsters facilitates territorial aggression⁷⁵. Also, dominant male hamsters show increased V1a receptor density in the VMHL⁷⁶. However, an opposite relationship may exist for female hamsters because subordinate females show greater V1a receptor density than handled controls⁴⁴. Interestingly, a MePV-VMHL pathway contributes to experience-dependent changes in aggression^{27,77}. For example, Nordman et al.²⁷ showed that aggression priming strengthens MePV-VMHL synapses, and they used low frequency stimulation to reduce strength of MePV-VMHL synapses and blocked aggression priming. In addition, VMHL activity may contribute to effects of dominance status on stress-related behavior because dominant male hamsters show increased c-Fos immunoreactivity in VMHL neurons after social defeat compared to subordinates⁴⁹. In sum, although activity of a MePD/MePV-BNST pathway failed to differentiate dominant and subordinate females in this study, a MePD/MePV-VMHL pathway may contribute to the effects of social dominance in female hamsters and should be the focus of future studies.

The MeA is a key structure in the neural circuitry controlling the conditioned defeat response, although the different cell types in the MeA have made its precise contribution difficult to delineate. Muscimol treatment into the MeA blocks the conditioned defeat response when administered prior to social defeat or prior to conditioned defeat testing⁵¹. However, dominant hamsters show increased neural activity in the MePV during social defeat and a reduced conditioned defeat response^{49,50}. These findings suggest that neurotransmission in the MeA is necessary for defeat-induced changes in social anxiety, but that dominant hamsters activate select neurons during social defeat that reduce changes in anxiety-like behavior. Unexpectedly, we did not replicate our previous finding that dominant males have greater defeat-induced c-Fos immunoreactivity in the MePV compared to subordinate males. This discrepancy might be due to methodological differences such as using separate antibodies for immunofluorescence and brightfield microscopy or quantifying a smaller region of the MePD and MePV focused on the location of CTB+ cells. It seems unlikely that interrupting dominance encounters for stereotaxic surgery and CTB infusion would have alters c-Fos expression because we have previously used a similar procedure without disrupting the status-dependent changes in c-Fos immunoreactivity in the prelimbic and infralimbic cortex^{59,60}. Overall, we found that dominants show greater defeat-induced neural activity in a MePD-BNST pathway than subordinates, which suggests that defeat-induced activity of MePD-BNST cells may underlie the reduced conditioned defeat response in dominant males. However, MePD/MePV projections target posterior BNST which is known to modulate social investigation, aggression, and sexual behavior^{78,79}. In

contrast, fear and anxiety-related responses are critically dependent on neurotransmission in anterior BNST^{80,81}. In fact, pharmacological blockade of CRF-R2 in anterior BNST prior to testing reduces the expression of the conditioned defeat response⁸². Similarly, the central amygdala sends projections to anterior BNST, and disruption of this pathway reduces the conditioned defeat response⁸³. Future research will be needed to delineate the integration of central amygdala and MeA input within the BNST and the potential microcircuits in the BNST that integrate social experience and stress-related changes in behavior.

Defending oneself during social defeat encounters represents a type of proactive coping response that is associated with stress resistance. Sprague Dawley rats can be segregated into two distinct populations in which one phenotype exhibits passive coping behaviors during social defeat stress and assumes a supine, submissive posture with a short latency to submit, while another gradually develops a proactive response characterized by upright, defensive postures and a long latency to show a supine, submissive posture⁸⁴. Rats with a short latency to submit display more immobility in a forced swim test, CRF hypersecretion, and dysregulation of the HPA axis compared to long latency to submit animals, which indicates increased stress resistance in rats with proactive coping responses⁸⁵. In addition, animals with a long latency to submit show lower prepro-orexin mRNA in the lateral hypothalamus compared to short latency animals and chemogenetic inhibition of orexin cells promotes stress resilience to short latency rats⁸⁶. Similarly, Walker and colleagues⁸⁷ found rats that actively resist social defeat by countering the resident's attacks exhibit reduced corticosterone concentrations

and reduced c-Fos immunoreactivity in stress-sensitive brain regions including the MeA and BNST. We found that dominant animals were more likely than subordinates to initiate aggression during the first of three social defeat encounters. Also, dominants showed a longer latency to submit compared to their same-sex subordinate counterparts. In males, specifically, latency to submit was positively associated with neural activity in MePD and MePV cells projecting to the BNST. These findings suggest that dominant males actively resist social defeat stress, which may drive activation of a MePD/MePV-BNST pathway and reduce subsequent stress-related changes in behavior. Interestingly, dominant females showed modest activation of BNST-projecting MePD cells during social defeat stress, although neural activity in this pathway was not associated with latency to submit in females. While we have previously shown dominant females exhibit less defeat-induced social avoidance compared to subordinates ¹¹, activity in a MePD-BNST pathway may play a limited role in status-dependent changes in stress susceptibility in females.

5. Conclusions

Male and female hamsters show similar patterns of territorial aggression ^{54,88-90}, although the neurochemical regulation of aggression reveals substantial sex differences ^{9,44,91}. In addition, there is a dramatic sex differences in how hamsters respond to social defeat stress ^{48,53}. Further, sex-dependent neural plasticity during the development of dominance relationships likely leads to sex differences in effects of dominance status on neuronal responses to social defeat stress. In male hamsters, neural activity in a MePD/MePV-BNST pathway is a potential factor driving experience-dependent changes

in stress-related behavior. Interestingly, the activity of BNST-projecting MePD and MePV is specifically linked to active resistance during a social defeat encounter. This association is not characteristic of other pathways that modulate status-dependent changes in stress-related behavior as chemogenetic activation of infralimbic neurons with projections to the basolateral amygdala reduces the conditioned defeat response of subordinate hamsters but does not alter their proactive coping responses during social defeat⁹². Overall, activating a MePD/MePV-BNST pathway during social stress may promote active coping behavior, which is very often essential for stress resistance.

Author statement

This study was reviewed and approved by The Institutional Animal Care and Use Committee at The University of Tennessee, Knoxville. All procedures were performed and reported with adherence to the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals. The work described here has not been published previously and has not been submitted elsewhere for publication. All authors have approved the submitted version of the manuscript. The authors have no conflicts of interest in the conduct and reporting of the research.

Declaration of interest

None.

Acknowledgments

We thank our team of dedicated research assistants for their assistance with testing animals, quantifying behavior, and performing histology. This project was supported by

the US National Institutes of Health grant R15MH122946 to MAC. CW was supported by a Ford Foundation Predoctoral Fellowship.

Data availability

Data will be made available on request.

References

1. Drews, C. The Concept and Definition of Dominance in Animal Behaviour. *Behaviour* **125**, 283–313 (1993).
2. Holekamp, K. E. & Strauss, E. D. Aggression and dominance: an interdisciplinary overview. *Curr. Opin. Behav. Sci.* **12**, 44–51 (2016).
3. Blanchard, D. C. & Blanchard, R. J. Behavioral correlates of chronic dominance-subordination relationships of male rats in a seminatural situation. *Neurosci. Biobehav. Rev.* **14**, 455–462 (1990).
4. Bernstein, I. S. Dominance: The baby and the bathwater. *Behav. Brain Sci.* **4**, 419–429 (1981).
5. Williamson, C. M., Klein, I. S., Lee, W. & Curley, J. P. Immediate early gene activation throughout the brain is associated with dynamic changes in social context. *Soc. Neurosci.* **14**, 253–265 (2019).
6. Wang, F. *et al.* Bidirectional Control of Social Hierarchy by Synaptic Efficacy in Medial Prefrontal Cortex. *Science* **334**, 693–697 (2011).
7. Zhou, T. *et al.* History of winning remodels thalamo-PFC circuit to reinforce social dominance. *Science* **357**, 162–168 (2017).
8. Xing, B., Mack, N. R., Zhang, Y.-X., McEachern, E. P. & Gao, W.-J. Distinct Roles for Prefrontal Dopamine D1 and D2 Neurons in Social Hierarchy. *J. Neurosci.* **42**, 313–324 (2022).

9. Terranova, J. I. *et al.* Serotonin and arginine–vasopressin mediate sex differences in the regulation of dominance and aggression by the social brain. *Proc. Natl. Acad. Sci.* **113**, 13233–13238 (2016).
10. LeClair, K. B. *et al.* Individual history of winning and hierarchy landscape influence stress susceptibility in mice. *eLife* **10**, e71401 (2021).
11. Cooper, M. A. *et al.* Gonadal steroid hormone receptors in the medial amygdala contribute to experience-dependent changes in stress vulnerability. *Psychoneuroendocrinology* **129**, 105249 (2021).
12. Karamihalev, S. *et al.* Social dominance mediates behavioral adaptation to chronic stress in a sex-specific manner. *eLife* **9**, e58723 (2020).
13. Blanchard, D. C. *et al.* Visible burrow system as a model of chronic social stress: Behavioral and neuroendocrine correlates. *Psychoneuroendocrinology* **20**, 117–134 (1995).
14. Sapolsky, R. M. Hypercortisolism Among Socially Subordinate Wild Baboons Originates at the CNS Level. *Arch. Gen. Psychiatry* **46**, 1047–1051 (1989).
15. Snyder-Mackler, N. *et al.* Social status alters immune regulation and response to infection in macaques. *Science* **354**, 1041–1045 (2016).
16. Choi, G. B. *et al.* Lhx6 Delineates a Pathway Mediating Innate Reproductive Behaviors from the Amygdala to the Hypothalamus. *Neuron* **46**, 647–660 (2005).
17. Chen, P. B. *et al.* Sexually Dimorphic Control of Parenting Behavior by the Medial Amygdala. *Cell* **176**, 1206–1221.e18 (2019).

18. Hong, W., Kim, D.-W. & Anderson, D. J. Antagonistic Control of Social versus Repetitive Self-Grooming Behaviors by Separable Amygdala Neuronal Subsets. *Cell* **158**, 1348–1361 (2014).
19. Miller, S. M., Marcotulli, D., Shen, A. & Zweifel, L. S. Divergent medial amygdala projections regulate approach-avoidance conflict behavior. *Nat. Neurosci.* **22**, 565–575 (2019).
20. Yamaguchi, T. *et al.* Posterior amygdala regulates sexual and aggressive behaviors in male mice. *Nat. Neurosci.* **23**, 1111–1124 (2020).
21. Unger, E. K. *et al.* Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes. *Cell Rep.* **10**, 453–462 (2015).
22. Maras, P. M. & Petrulis, A. The anterior medial amygdala transmits sexual odor information to the posterior medial amygdala and related forebrain nuclei. *Eur. J. Neurosci.* **32**, 469–482 (2010).
23. Maras, P. M. & Petrulis, A. Anatomical connections between the anterior and posterodorsal sub-regions of the medial amygdala: integration of odor and hormone signals. *Neuroscience* **170**, 610–622 (2010).
24. Lehman, M. N., Winans, S. S. & Powers, J. B. Medial Nucleus of the Amygdala Mediates Chemosensory Control of Male Hamster Sexual Behavior. *Science* **210**, 557–560 (1980).
25. Kollack-Walker, S. & Newman, S. W. Mating and agonistic behavior produce different patterns of Fos immunolabeling in the male Syrian hamster brain. *Neuroscience* **66**, 721–736 (1995).

26. Canteras, N. S., Simerly, R. B. & Swanson, L. W. Organization of projections from the medial nucleus of the amygdala: A PHAL study in the rat. *J. Comp. Neurol.* **360**, 213–245 (1995).
27. Nordman, J. C. *et al.* Potentiation of Divergent Medial Amygdala Pathways Drives Experience-Dependent Aggression Escalation. *J. Neurosci.* **40**, 4858–4880 (2020).
28. Keshavarzi, S., Sullivan, R. K. P., Ianno, D. J. & Sah, P. Functional Properties and Projections of Neurons in the Medial Amygdala. *J. Neurosci.* **34**, 8699–8715 (2014).
29. Bian, X. Physiological and morphological characterization of GABAergic neurons in the medial amygdala. *Brain Res.* **1509**, 8–19 (2013).
30. Bian, X., Yanagawa, Y., Chen, W. R. & Luo, M. Cortical-Like Functional Organization of the Pheromone-Processing Circuits in the Medial Amygdala. *J. Neurophysiol.* **99**, 77–86 (2008).
31. Dong, H.-W., Petrovich, G. D. & Swanson, L. W. Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res. Rev.* **38**, 192–246 (2001).
32. Petrulis, A. Chapter 2 - Structure and function of the medial amygdala. in *Handbook of Behavioral Neuroscience* (eds. Urban, J. H. & Rosenkranz, J. A.) vol. 26 39–61 (Elsevier, 2020).
33. Pardo-Bellver, C., Cadiz-Moretti, B., Novejarque, A., Martinez-Garcia, F. & Lanuza, E. Differential efferent projections of the anterior, posteroventral, and posterodorsal subdivisions of the medial amygdala in mice. *Front. Neuroanat.* **6**, (2012).
34. Newman, S. W. The Medial Extended Amygdala in Male Reproductive Behavior A Node in the Mammalian Social Behavior Network. *Ann. N. Y. Acad. Sci.* **877**, 242–257 (1999).

35. Cooper, M. A. *et al.* Dominance status alters restraint-induced neural activity in brain regions controlling stress vulnerability. *Physiol. Behav.* **179**, 153–161 (2017).
36. Dayas, C. V., Buller, K. M. & Day, T. A. Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. *Eur. J. Neurosci.* **11**, 2312–2322 (1999).
37. Fekete, É. M. *et al.* Social defeat stress activates medial amygdala cells that express type 2 corticotropin-releasing factor receptor mRNA. *Neuroscience* **162**, 5–13 (2009).
38. Fanous, S., Hammer, R. P. & Nikulina, E. M. Short- and long-term effects of intermittent social defeat stress on brain-derived neurotrophic factor expression in mesocorticolimbic brain regions. *Neuroscience* **167**, 598–607 (2010).
39. Raam, T. & Hong, W. Organization of neural circuits underlying social behavior: A consideration of the medial amygdala. *Curr. Opin. Neurobiol.* **68**, 124–136 (2021).
40. Padilla, S. L. *et al.* Agouti-related peptide neural circuits mediate adaptive behaviors in the starved state. *Nat. Neurosci.* **19**, 734–741 (2016).
41. Been, L. E. & Petrulis, A. Chemosensory and hormone information are relayed directly between the medial amygdala, posterior bed nucleus of the stria terminalis, and medial preoptic area in male Syrian hamsters. *Horm. Behav.* **59**, 536–548 (2011).
42. Duque-Wilckens, N. *et al.* Oxytocin Receptors in the Anteromedial Bed Nucleus of the Stria Terminalis Promote Stress-Induced Social Avoidance in Female California Mice. *Biol. Psychiatry* **83**, 203–213 (2018).

43. Duque-Wilckens, N. *et al.* Inhibition of vasopressin V1a receptors in the medioventral bed nucleus of the stria terminalis has sex- and context-specific anxiogenic effects. *Neuropharmacology* **110**, 59–68 (2016).
44. Grieb, Z. A. *et al.* Sex-dependent effects of social status on the regulation of arginine-vasopressin (AVP) V1a, oxytocin (OT), and serotonin (5-HT) 1A receptor binding and aggression in Syrian hamsters (*Mesocricetus auratus*). *Horm. Behav.* **127**, 104878 (2021).
45. Albers, H. E. The regulation of social recognition, social communication and aggression: Vasopressin in the social behavior neural network. *Horm. Behav.* **61**, 283–292 (2012).
46. Drickamer, L. C. & Vandenbergh, J. G. Predictors of social dominance in the adult female golden hamster (*Mesocricetus auratus*). *Anim. Behav.* **21**, 564–570 (1973).
47. Drickamer, L. C., Vandenbergh, J. G. & Colby, D. R. Predictors of dominance in the male golden hamster (*Mesocricetus auratus*). *Anim. Behav.* **21**, 557–563 (1973).
48. Huhman, K. L. *et al.* Conditioned defeat in male and female syrian hamsters. *Horm. Behav.* **44**, 293–299 (2003).
49. Morrison, K. E., Curry, D. W. & Cooper, M. A. Social status alters defeat-induced neural activation in Syrian hamsters. *Neuroscience* **210**, 168–178 (2012).
50. Morrison, K. E. *et al.* Maintenance of dominance status is necessary for resistance to social defeat stress in Syrian hamsters. *Behav. Brain Res.* **270**, 277–286 (2014).
51. Markham, C. M. & Huhman, K. L. Is the medial amygdala part of the neural circuit modulating conditioned defeat in Syrian hamsters? *Learn. Mem.* **15**, 6–12 (2008).

52. Rosenhauer, A. M., McCann, K. E., Norvelle, A. & Huhman, K. L. An acute social defeat stressor in early puberty increases susceptibility to social defeat in adulthood. *Horm. Behav.* **93**, 31–38 (2017).
53. Solomon, M. B., Karom, M. C. & Huhman, K. L. Sex and estrous cycle differences in the display of conditioned defeat in Syrian hamsters. *Horm. Behav.* **52**, 211–219 (2007).
54. Wise, D. A. Aggression in the female golden hamster: Effects of reproductive state and social isolation. *Horm. Behav.* **5**, 235–250 (1974).
55. Huhman, K. L., Moore, T. O., Ferris, C. F., Mougey, E. H. & Meyerhoff, J. L. Acute and repeated exposure to social conflict in male golden hamsters: Increases in plasma POMC-peptides and cortisol and decreases in plasma testosterone. *Horm. Behav.* **25**, 206–216 (1991).
56. Landau, I. T. Light-dark rhythms in aggressive behavior of the male golden hamster. *Physiol. Behav.* **14**, 767–774 (1975).
57. Ciaccio, L. A., Lisk, R. D. & Reuter, L. A. Prelordotic behavior in the hamster: A hormonally modulated transition from aggression to sexual receptivity. *J. Comp. Physiol. Psychol.* **93**, 771–780 (1979).
58. Takahashi, L. K. & Lisk, R. D. Organization and expression of agonistic and socio-sexual behavior in golden hamsters over the estrous cycle and after ovariectomy. *Physiol. Behav.* **31**, 477–482 (1983).
59. Grizzell, J. A., Clarity, T. T., Graham, N. B., Dulka, B. N. & Cooper, M. A. Activity of a vmPFC-DRN Pathway Corresponds With Resistance to Acute Social Defeat Stress. *Front. Neural Circuits* **14**, (2020).

60. Dulka, B. N., Bress, K. S., Grizzell, J. A. & Cooper, M. A. Social Dominance Modulates Stress-induced Neural Activity in Medial Prefrontal Cortex Projections to the Basolateral Amygdala. *Neuroscience* **388**, 274–283 (2018).
61. Morin, L. P. & Wood, R. I. *A Stereotaxic Atlas of the Golden Hamster Brain*. (Academic Press, New York, 2001).
62. Albers, H. E., Huhman, K. L. & Meisel, R. L. 6 - Hormonal Basis of Social Conflict and Communication. in *Hormones, Brain and Behavior* (eds. Pfaff, D. W., Arnold, A. P., Fahrbach, S. E., Etgen, A. M. & Rubin, R. T.) 393–433 (Academic Press, San Diego, 2002). doi:10.1016/B978-012532104-4/50008-1.
63. Sapolsky, R. M. Cortisol concentrations and the social significance of rank instability among wild baboons. *Psychoneuroendocrinology* **17**, 701–709 (1992).
64. Vandeleest, J. J. *et al.* Decoupling social status and status certainty effects on health in macaques: a network approach. *PeerJ* **4**, e2394 (2016).
65. Milewski, T. M., Lee, W., Champagne, F. A. & Curley, J. P. Behavioural and physiological plasticity in social hierarchies. *Philos. Trans. R. Soc. B Biol. Sci.* **377**, 20200443 (2022).
66. Marler, C. A. & Trainor, B. C. The challenge hypothesis revisited: Focus on reproductive experience and neural mechanisms. *Horm. Behav.* **123**, 104645 (2020).
67. Fuxjager, M. J., Mast, G., Becker, E. A. & Marler, C. A. The ‘home advantage’ is necessary for a full winner effect and changes in post-encounter testosterone. *Horm. Behav.* **56**, 214–219 (2009).

68. Fuxjager, M. J. *et al.* Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. *Proc. Natl. Acad. Sci.* **107**, 12393–12398 (2010).
69. Rybka, K. A. *et al.* Androgen Regulation of Corticotropin Releasing Factor Receptor 1 in the Mouse Brain. *Neuroscience* **491**, 185–199 (2022).
70. Rosvall, K. A., Bentz, A. B. & George, E. M. How research on female vertebrates contributes to an expanded challenge hypothesis. *Horm. Behav.* **123**, 104565 (2020).
71. Pan, Y., Xu, L., Young, K. A., Wang, Z. & Zhang, Z. Agonistic encounters and brain activation in dominant and subordinate male greater long-tailed hamsters. *Horm. Behav.* **58**, 478–484 (2010).
72. Lee, W. *et al.* Effect of relative social rank within a social hierarchy on neural activation in response to familiar or unfamiliar social signals. *Sci. Rep.* **11**, 2864 (2021).
73. Falkner, A. L., Grosenick, L., Davidson, T. J., Deisseroth, K. & Lin, D. Hypothalamic control of male aggression-seeking behavior. *Nat. Neurosci.* **19**, 596–604 (2016).
74. Lin, D. *et al.* Functional identification of an aggression locus in the mouse hypothalamus. *Nature* **470**, 221–226 (2011).
75. Delville, Y., Mansour, K. M. & Ferris, C. F. Serotonin blocks vasopressin-facilitated offensive aggression: Interactions within the ventrolateral hypothalamus of golden hamsters. *Physiol. Behav.* **59**, 813–816 (1996).
76. Cooper, M. A., Karom, M., Huhman, K. L. & Elliott Albers, H. Repeated agonistic encounters in hamsters modulate AVP V1a receptor binding. *Horm. Behav.* **48**, 545–551 (2005).

77. Nordman, J., Ma, X. & Li, Z. Traumatic Stress Induces Prolonged Aggression Increase through Synaptic Potentiation in the Medial Amygdala Circuits. *eNeuro* **7**, ENEURO.0147-20.2020 (2020).
78. Perkins, A. E. *et al.* Analysis of *c-Fos* induction in response to social interaction in male and female Fisher 344 rats. *Brain Res.* **1672**, 113–121 (2017).
79. Rigney, N., Zbib, A., de Vries, G. J. & Petrulis, A. Knockdown of sexually differentiated vasopressin expression in the bed nucleus of the stria terminalis reduces social and sexual behaviour in male, but not female, mice. *J. Neuroendocrinol.* **34**, e13083 (2022).
80. Walker, D. L. & Davis, M. Double Dissociation between the Involvement of the Bed Nucleus of the Stria Terminalis and the Central Nucleus of the Amygdala in Startle Increases Produced by Conditioned versus Unconditioned Fear. *J. Neurosci.* **17**, 9375–9383 (1997).
81. Goode, T. D., Ressler, R. L., Acqa, G. M., Miles, O. W. & Maren, S. Bed nucleus of the stria terminalis regulates fear to unpredictable threat signals. *eLife* **8**, e46525 (2019).
82. Cooper, M. A. & Huhman, K. L. Corticotropin-releasing factor type II (CRF₂) receptors in the bed nucleus of the stria terminalis modulate conditioned defeat in Syrian hamsters (*Mesocricetus auratus*). *Behav. Neurosci.* **119**, 1042–1051 (2005).
83. Jasnow, A. M., Davis, M. & Huhman, K. L. Involvement of Central Amygdalar and Bed Nucleus of the Stria Terminalis Corticotropin-Releasing Factor in Behavioral Responses to Social Defeat. *Behav. Neurosci.* **118**, 1052–1061 (2004).

84. Wood, S. K. & Bhatnagar, S. Resilience to the effects of social stress: Evidence from clinical and preclinical studies on the role of coping strategies. *Neurobiol. Stress* **1**, 164–173 (2015).
85. Wood, S. K., Walker, H. E., Valentino, R. J. & Bhatnagar, S. Individual differences in reactivity to social stress predict susceptibility and resilience to a depressive phenotype: role of corticotropin-releasing factor. *Endocrinology* **151**, 1795–1805 (2010).
86. Grafe, L. A., Eacret, D., Dobkin, J. & Bhatnagar, S. Reduced Orexin System Function Contributes to Resilience to Repeated Social Stress. *eNeuro* **5**, (2018).
87. Walker, F. R., Masters, L. M., Dielenberg, R. A. & Day, T. A. Coping with defeat: acute glucocorticoid and forebrain responses to social defeat vary with defeat episode behaviour. *Neuroscience* **162**, 244–253 (2009).
88. Lerwill, C. J. & Makings, P. The agonistic behaviour of the golden hamster *Mesocricetus auratus* (waterhouse). *Anim. Behav.* **19**, 714–721 (1971).
89. Payne, A. P. & Swanson, H. H. Agonistic Behaviour Between Pairs of Hamsters of the Same and Opposite Sex in a Neutral Observation Area. *Behaviour* **36**, 259–269 (1970).
90. Floody, O. R. & Pfaff, D. W. Aggressive behavior in female hamsters: The hormonal basis for fluctuations in female aggressiveness correlated with estrous state. *J. Comp. Physiol. Psychol.* **91**, 443–464 (1977).
91. Terranova, J. I., Ferris, C. F. & Albers, H. E. Sex Differences in the Regulation of Offensive Aggression and Dominance by Arginine-Vasopressin. *Front. Endocrinol.* **8**, (2017).

92. Dulka, B. N. *et al.* Chemogenetic activation of an infralimbic cortex to basolateral amygdala projection promotes resistance to acute social defeat stress. *Sci. Rep.* **10**, 6884 (2020).

CHAPTER SIX
DISCUSSION AND SYNTHESIS

My dissertation investigated how stressful experiences induce neuronal and phenotypic plasticity in zebrafish and Syrian hamsters. The present studies characterized a wide range of morphological, neurochemical, and behavioral responses to physical and social stressors such as chemical toxicity, anthropogenic influence in natural environments, social play deprivation, and social defeat. Responses to these stressors included dysregulated social and anxiety-like behaviors, altered morphology, changes in markers of plasticity, and altered neural activity in limbic circuits. While these stressors induce profound responses in exposed individuals, these studies also identify environmental stimuli that mitigate negative outcomes. Specifically, social experiences such as juvenile social play and acquisition of a dominant social status can attenuate stress-related changes in behavior. The cellular mechanisms by which social experience reduces stress vulnerability involve neural plasticity and changes neural activity in key limbic regions including the medial frontal cortex (vmPFC), basolateral amygdala (BLA), medial amygdala (MeA), and bed nucleus of the stria terminalis (BNST). My key findings are summarized below.

3. Ecotoxicology & Anthropogenic Change: Synthesis of Specific Aims 1 & 2

1.1. Specific Aim 1: Identify behavioral plasticity within social networks induced by acute cadmium toxicity.

Pollution has been identified as the single greatest risk to human and environmental health and contributes to 16% of annual deaths worldwide ¹. Exposure to chemical pollutants such as cadmium toxicity has been linked to disease formation ^{2,3}, cognitive impairments ², sensory deficits ^{4,5}, increased aggression ⁶, and changes in species distribution ³. Group dynamics and decision-making within social groups can thus

be disrupted by individual exposure to chemical contaminants such as cadmium. We investigated how the presence of two cadmium-contaminated zebrafish can influence the behavior of unexposed group members. Further, we assessed whether these behavioral changes were caused by vision deficits.

We found that cadmium exposure induced indirect effects on group boldness where the presence of two cadmium-exposed zebrafish increased approach toward a novel object among unexposed group members. The presence of cadmium-exposed individuals also increased aggressive chase behaviors exhibited by unexposed female group members. Interestingly, despite altered agonistic activity within social groups we did not find significant differences in social network cohesion or connectivity between cadmium-exposed groups and controls. These findings suggest that the presence of cadmium-contaminated individuals in social groups can alter group behavior and decision-making without disrupting the group's social structure. In addition, cadmium-exposed fish showed decreased visual perception in an optomotor response assay compared to controls, which suggests that alterations in group behavior may be associated with sensory impairments among contaminated individuals.

Our findings indicate that the direct action of chemical pollutants such as cadmium on sensory systems can indirectly disrupt social dynamics within groups. The indirect effect on boldness and social behaviors among unexposed group members may arise from the disruptive effects of cadmium on social recognition ⁷, learning ⁸, aggression ⁹, and/or communication ¹⁰. The cadmium-induced impairments of exposed zebrafish may also alter the behavior of unexposed group members by emboldening

uncontaminated individuals to engage in risky behaviors such as increased approach to novel stimuli ¹¹, which can pose increased risk for social groups via greater predation risk ^{12–14}. Social interactions can impact how individuals interact with the physical environment, but if these social experiences are disrupted by sensory impairments it may alter appropriate group responses to novel stimuli ^{15–17}. It is possible that cadmium-induced visual impairments may also require animals to approach novel stimuli more closely to achieve adequate visual perception, which may encourage group members to increase proximity. Future research should investigate the mechanisms by which boldness or social investigation are altered in individuals both directly and indirectly exposed to cadmium.

The presence of cadmium-treated fish in groups altered social behavior of the unexposed majority whereby unexposed group members became more aggressive than control groups. Multilayer social network analysis revealed that these cadmium-induced changes in social networks were mediated by female fish where unexposed females housed with cadmium-exposed fish initiated more chasing of group members. Previous studies have shown that cadmium toxicity is more severe in female organisms than males, and our results suggest the possibility that female organisms may also be more susceptible to the indirect effects of cadmium exposure ^{18,19}. Future research will benefit from investigating both the direct and indirect effects of cadmium exposure on social interactions and how sex influences cadmium toxicity to provide a better understanding for how female organisms are disproportionately affected.

This study provides novel insights into the widespread effects of anthropogenic change by highlighting the effects that even indirect exposure to contaminants can have on organisms. While the present study investigated the sensory mechanisms and social factors by which cadmium toxicity may generate indirect effects on group behavior, future studies will benefit from investigating how habitat variation impacts how indirect cadmium exposure alters group dynamics. Future research should also assess the mechanisms by which female organisms are disproportionately affected by cadmium exposure. Such research is critical for understanding the consequences of anthropogenic change and protecting populations against the consequences of chemical pollutants.

1.2. Specific Aim 2: Determine how environmental factors induce behavioral and morphological plasticity in wild zebrafish populations.

Anthropogenic influence in natural environments is causing rapid and profound disruptions in organism health, behavior, and physiology^{1,20–22}. Aquatic environments experience greater disruption from anthropogenic influence than terrestrial environments, which highlights a critical need in conservation biology to determine how aquatic ecosystems are affected by anthropogenic change²³. The present study aimed to assess how environmental gradients in aquatic environments affect behavior and morphology in populations of wild zebrafish in India.

We found that zebrafish from a rural environment with less anthropogenic influence showed faster swimming speeds, greater distance traveled, and larger inter-individual distances than fish from an urban environment with more anthropogenic influence. We also found that fish sampled from the rural environment were longer and

had larger posterior-to-anterior body size ratios than fish from the urban environment. Our data are consistent with previous findings that behavioral and morphological plasticity in zebrafish can be induced by biotic and abiotic factors present at sampled field sites including plastic ^{24,25}, arsenic ²⁶, and lead ²⁷ pollution, as well as elevated temperatures ²⁸, hypoxia ^{29,30}, and water flow ^{29,31–33}. Interestingly, we found a male-biased sex ratio in sampled populations. Previous studies have shown that hypoxia ³⁴, sex-specific predation pressure ³⁵, endocrine disruptors ³⁶, and other pollutants ³⁷ may have contributed to the skewed sex ratio. While subjects were tested in holding containers, site differences in predation pressure may also interact with observed behaviors, as site 2 had a greater predation risk than site 1. While the present study highlights phenotypic variation between these populations of fish, delineating the mechanisms by which these factors alter morphology and behavior will be an important step toward untangling the complex interactions between environmental factors.

A notable limitation of the present study is the use of only two field sites. While the present study does highlight the degree of phenotypic variation within two field sites in relatively close geographic proximity, sampling from a wide range of field locations with contrasting environmental gradients will be invaluable for determining the contribution of different factors to phenotypic variability. Field studies such as this are also limited in their ability to derive conclusions about causal relationships between environmental factors and behavioral and morphological responses. As such, there is a critical need for further laboratory research investigating the effects of individual and multiple ecological variables on phenotypic variability.

The present study provides insight into the effect anthropogenic change and environmental disruptions can have in natural environments. Such impacts pose deleterious risks for organism health and fitness, highlighting a critical need to understand the mechanisms driving these outcomes. This study also highlights the utility of sentinel species such as zebrafish as biological indicators for adverse environmental events. The use of sentinel species presents a unique opportunity for implementation in underserved communities in need of alternative and cost-effective systems for monitoring water quality. Such methodologies may be valuable for detecting and mitigating risks of environmental perturbations on public and environmental health outcomes.

1.3. *Synthesis*

These two studies highlight the impact of environmental perturbations such as anthropogenic influence and cadmium toxicity on behavior and morphology in zebrafish. These environmental perturbations can produce both direct and indirect effects on behavioral outcomes, and thus even individuals who have not directly experienced contamination events may be subject to negative consequences. Phenotypic alterations may be induced by sensory alterations or potential changes in physical development such as altered growth of posterior body regions. Female fish may also be disproportionately affected by contamination events such as cadmium toxicity, highlighting a critical need to understand sex differences in responses to changing environments.

This work presents several directions for future research such as investigating the neuroendocrine and neurochemical mechanisms by which anthropogenic change alters morphological and behavioral phenotypes. Such studies should focus on changes in gene

expression in brain regions regulating social behaviors, disruption of neuroendocrine systems, and quantification of real-time binding of contaminants to target receptors. As many of the biotic and abiotic factors altered by anthropogenic change function as endocrine disrupting chemicals and can induce neurotoxic changes in acetylcholinesterase inhibition ³⁸, it is essential to first characterize the effects of these environmental factors on neural and endocrine systems.

Other future directions include replicating and extending current findings to determine causal links, and assessing whether findings have ecological validity in field settings. To determine whether cadmium indirectly increases aggression through sensory disruptions in exposed fish a series of sensory deprivation experiments should be conducted. Aggressive behaviors could be used to construct directed social networks among shoals of zebrafish where two members underwent visual or lateral line sensory ablation to confirm whether sensory impairments among group-members alone can induce the observed alterations in group behavior in the present study. Similarly, such social networks could be constructed among wild zebrafish populations from field sites with varying levels of cadmium contamination. Indirect effects could be observed by relocation or combined testing of fish from one population with another. Further, leveraging seasonal changes and periods of high water exchange, such as monsoon seasons, may provide a natural opportunity to investigate interactions between fish with different prior exposure to contaminants such as cadmium.

Future field studies should also sample from a wide variety of field sites with varying gradients of environmental factors to increase representative sampling and

develop a deeper understanding of the complex interactions between biotic and abiotic factors on phenotypic alterations. Further, populations could be sampled longitudinally to include periods when field sites experience alterations in water parameters such as fluctuations in temperature, flow, and seasonal agricultural runoff. Such repeated observations would allow identification of interactions that may otherwise go undetected. Finally, genetic techniques focused on epigenetic markers may identify the mechanisms by which phenotypic alterations persist in future generations.

Zebrafish are widely used in laboratory toxicological research due to their vast biomedical toolkit, rapid generation times, and genetic homology, as well as the availability of transgenic lines, and resources in databases such as the Zebrafish Information Network ³⁹⁻⁴². The studies in this dissertation highlight the utility of zebrafish as model organisms for ecotoxicology in the field. Indeed, their widespread geographic distribution, small size, adaptability, and presence near the surface in clear water makes zebrafish a relatively accessible organism to work with in field settings ^{33,43}. Assessing adverse environmental events in both field and laboratory settings will increase external validity by sampling organisms under naturalistic conditions with diverse biotic and abiotic factors ⁴⁴⁻⁴⁶, while also increasing throughput and cost-efficiency. As such, future research will benefit from the tight integration of laboratory and field studies investigating the effects of anthropogenic change and other adverse environmental events.

4. Social Experience Modifies Stress Vulnerability: Synthesis of Specific Aims 3 & 4

2.1. Specific Aim 3: Evaluate changes in perineuronal net (PNN) expression in the vmPFC and BLA of play-deprived hamsters.

Social play during the juvenile period is essential for species-typical neural and behavioral development⁴⁷. Previous studies have shown that both play deprivation and juvenile social isolation decrease social interaction and sexual activity in adulthood in rats^{48–52}. Similarly, social deprivation alters agonistic behavior including elevating aggression and increasing defensive behavior displayed towards conspecifics in both male and female rats^{51–57}. Social play deprivation and juvenile social isolation produce myriad structural changes in the vmPFC such as a reduction in synaptic plasticity proteins⁵⁸, decreased glial cell density⁵⁴, and reduced pruning of apical dendrites on pyramidal neurons in both rats and Syrian hamsters^{59–61}. It is possible that experience-dependent plasticity in vmPFC neurons modifies behavioral responses to stressful experiences in adulthood. We tested whether depriving hamsters of juvenile social play altered stress-related changes in social behavior in adulthood. In addition, we tested whether these behavioral alterations were associated with changes in expression of PNNs surrounding PV+ neurons in the IL, PL, and BLA. Further, we asked whether 1-hour of social play per day was sufficient to rescue play deprivation-induced disruption in social behavior and neural plasticity.

We found that peer isolated hamsters showed an increase in the conditioned defeat response, and this increase was rescued by 1-hour of social play per day during the juvenile period. Peer isolated hamsters also showed disrupted agonistic behavior in subsequent social encounters, including elevated defensive and submissive behavior in

males when they were introduced to a novel group. We also found that both peer isolated hamsters and subjects that received 1 hour of social play daily had decreased PNN expression on PV+ neurons in the PL compared to hamsters who were group housed with unlimited access to play partners. Interestingly, we found that only subjects given 1 hour of play daily had diminished PNN expression on PV+ neurons in the IL, which suggests that 1 hour of social play with the same partner each day may generate a different pattern of neuronal plasticity compared to playing with multiple partners in a social group. We also found that the expression of PNNs on PV+ neurons was associated with behaviors shown both in juveniles and adults. Elevated PNN/PV co-expression in PL neurons was associated with decreased submissive behavior at regrouping among males, but higher PNN count in the PL decreased aggression during conditioned defeat testing among females. We also found that increased PNN expression in the IL and increased PNN/PV co-expression in the BLA were associated with greater social approach during social avoidance testing. Together, our results show that juvenile social play has an important role in promoting stress resistance, and the expression of competent behavior during social challenges. Further, social play experience generates different patterns of neural plasticity in the vmPFC and BLA, and these changes may contribute to individual differences in behavior in adulthood.

Interestingly, opportunities for juvenile social play itself do not necessarily rescue all the effects of play deprivation. Although play fights in dyadic pairs of hamsters are highly reciprocal at postnatal day 21, by postnatal day 42 one hamster tends to show more offensive play behavior, while its partner shows more defensive play behavior. We

quantified the amount of wins and losses each hamster experienced during their 1-hour play dates and found that one individual in each dyad tended to win more play fights than the other during the late juvenile period (postnatal days 35-42). When introduced to a novel social group, hamsters that emerged from play dates as winners showed less submissive and defensive behaviors than losers. Thus, social roles during the later stages of play fighting account for individual differences in responses to social challenges. The finding that winners showed less submissive and defensive behavior during social integration is consistent with previous findings that socially dominant hamsters show less submissive & defensive behavior in a conditioned defeat test compared to subordinates⁶²⁻⁶⁵. Thus, the protective effects of juvenile social play on stress-related behavior may be modulated by the emergence of social dominance.

When analyzing neural differences between hamsters that win or lose play fights, we found that losing play fights was associated with decreased PNN expression on PV+ neurons in the PL, IL, and BLA. Interestingly, female hamsters were particularly sensitive to this decrease. It is possible that winning play fights establishes a pattern of PNN expression and synaptic connections within the PL, IL, and BLA that promotes resistance to social stress. This is consistent with the findings that greater activity in a vmPFC-BLA pathway promotes stress resilience⁶⁶⁻⁶⁸. While a winning index predicts PNN expression on PV+ neurons, winners and losers do not significantly differ in PNN expression or responses to social defeat stress. In addition, individual differences in PNN expression correlates with variation in social behavior. This suggests that while not all

effects of social play last into adulthood, neural phenotypes do persist and may contribute to responses to social stress in adulthood.

While we found that social play deprivation increases vulnerability to social defeat stress when measured in both a conditioned defeat test and a social avoidance test, 1 hour of play daily rescued only the conditioned defeat response. This suggests that separate measures of defeat-induced anxiety are differentially sensitive to play deprivation. These findings are consistent with other findings in our lab using a dominant/subordinate paradigm in which subordinate male hamsters show an elevated conditioned defeat response but no change in approach/avoidance behavior compared to their dominant counterparts⁶⁵. Expression of PNNs on PL and BLA neurons was also correlated with defensive and submissive behavior when animals were introduced to a novel group during the late juvenile period, which suggests that neuroplasticity in these limbic regions may influence the development of competent behavior during a social challenge.

This study demonstrates that juvenile social play promotes both resistance to stress-induced changes in agonistic behavior and the development of competent social behavior in adulthood. Additionally, being deprived of opportunities for social play leads to decreased PNN/PV co-expression in the PL and IL. Further, PNN expression surrounding PV+ neurons may alter synaptic connections in the PL, IL, and BLA, which produces individual variation in agonistic behaviors and differences in stress vulnerability. Beneficial directions for future research include investigating how the outcome of social play bouts contributes to differences in PL, IL, and BLA circuits.

Further, future research should investigate the amount and type of social play required to fully rescue deficits in neural plasticity and defeat-induced social avoidance. Future studies could also investigate the contribution of PNNs to agonistic behavior and stress vulnerability by knocking down PNNs in the PL, IL, or BLA. Overall, our work delineates a critical role for social play in the development of competent social behavior and stress vulnerability, and sheds light on the role of PNNs on the expression of adult agonistic behavior.

2.2. Specific Aim 4: Determine whether endogenous activity in a MeA-BNST circuit is associated with stress resilience in dominant hamsters.

While exposure to traumatic stressors can produce long-term increases in stress vulnerability, not all individuals who experience a traumatic stressor show long-term negative consequences^{69–72}. Stress vulnerability can be altered by environmental factors such as exercise, stressor controllability, and stress coping strategies^{70,73–75}. In addition, social experience such as status within a dominance hierarchy modifies stress vulnerability whereby socially dominant individuals exhibit stress resistance, while subordinate individuals show stress vulnerability^{69,70,76}. Socially dominant Syrian hamsters show greater stress-induced c-Fos expression in the MePV than subordinates, which suggests a potential role for activity of MePV neurons in status-dependent stress resistance⁷⁷. The BNST receives robust input from MePD and MePV neurons and is a key brain region regulating stress responses, fear, and social behavior^{78,79}. The MePD and MePV are thought to respond to social stimuli and thereby provide social information for the BNST to guide and sustain a stress response^{80,81}. In the present study we asked

whether the acquisition of social dominance changes neural activity in a MePD/MePV-BNST circuit and whether animals also show status-dependent activity in this pathway during social defeat stress.

We found that dominant male hamsters expressed greater c-Fos immunoreactivity in MePD and MePV neurons that project to the BNST after they win an aggressive encounter compared to subordinates and female hamsters. Likewise, dominant males also showed increased c-Fos expression in BNST-projecting neurons in the MePD when experiencing social defeat stress compared to all other subjects. We also found that the latency to submit to a resident aggressor was positively correlated with activation of BNST-projecting neurons in both the MePD and MePV among male, but not female hamsters. This suggests that dominant males activate this neural pathway when they resist social defeat. Together, repeated activation of the MePD-BNST pathway in dominant males as they acquire their social status may facilitate proactive coping responses to subsequent stressors.

Previous research in our laboratory has shown that the acquisition of a dominant social status increases the expression of androgen receptors in both the MePD and MePV of male hamsters^{82,83}. Ongoing research in our laboratory⁸⁴ has found that dominant male hamsters show greater c-Fos expression in androgen receptor-expressing neurons in the MePV than subordinates. We found that approximately 70% of BNST-projecting MeA neurons express androgen receptors in dominant males, whereas only 40% of these neurons express androgen receptors in subordinates. It is thus possible that development of a dominant social status increases androgen receptor expression in a MePD-BNST

circuit, which increases the stress-induced activity of this pathway and promotes stress resilience.

While we did not find differences in c-Fos expression in a MePD/MePV-BNST pathway among female hamsters, females do show status-dependent differences in stress vulnerability similar to males^{83,85}. However, unlike male hamsters, females tend not to show a robust conditioned defeat response, but dominant females show less avoidance during a social avoidance test than subordinates⁸³. In males, resilience in a conditioned defeat test may be linked to changes in androgen receptors within an MePD-BNST pathway. Conversely, dominant female hamsters do not show changes in androgen receptors or increased activity in an MePD-BNST pathway, and this is consistent with social dominance not modifying their conditioned defeat response. However, because dominant females show less avoidance in a social avoidance test compared to subordinates, future studies will need to elucidate the neural pathways driving stress resistance in dominant female hamsters. Future research may also benefit from investigating whether defeat-induced activation of MePD/MePV neurons expressing estrogen receptor alpha are associated with stress resistance in female hamsters. Finally, chemogenetic manipulation of MePD-BNST projecting neurons will be needed to delineate whether activity in this pathway is necessary or sufficient for the expression of status-dependent differences in stress vulnerability in Syrian hamsters.

2.3. Synthesis

These studies highlight ways by which social experience can alter stress vulnerability in adult Syrian hamsters whereby social interactions such as juvenile social

play or achieving a high rank in a dominance hierarchy can promote stress resistance. Interestingly, these two types of social experience likely interact because social play in Syrian hamsters is thought to be critical for the development of adult aggression⁸⁶. In fact, juvenile social play and territorial aggression in hamsters are regulated by similar neural circuits^{87,88}. In addition, play fighting has been suggested to help facilitate the establishment and maintenance of dominance relationships^{89,90}. It is thus possible that similar neural ensembles regulate social play and adult dominance relationships, and that experience-dependent plasticity in these neural ensembles modulates stress susceptibility. Future research could investigate whether juvenile social play leads to synaptic plasticity within a MePD/MePV-BNST pathway that enables greater neural activity during social challenges in adulthood. Such findings would provide novel insights into the intersection of social play and dominance relationships and the neural mechanisms by which they generate resistance to social stress.

While social play was reciprocal during postnatal days 21-34, during postnatal days 35-42 one hamster in each pair tended to win more play bouts and the other lost. We also found that animals who lost more fights showed an increase in tail-ups and flees during postnatal days 35-42, which suggests that during the final week of play encounters adult patterns of agonistic behavior emerged. When investigating behavior at regrouping between winners and losers of play fights, we found that losers showed more submissive and defensive behaviors than winners. This is similar to effects seen in adult hamsters where animals with a subordinate social status show increased submissive and defensive behavior in a conditioned defeat test⁷⁶. Due to the overlap in neural circuits regulating

both social play and adult territorial aggression^{87,88}, it is possible that winning play fights may generate similar patterns of neural plasticity as achieving a dominant social status in adulthood, and may contribute to individual differences in stress sensitivity. Dominant hamsters show greater activation in PL and IL neurons that project to the BLA during social defeat stress, which is necessary for stress resilience^{63,67}. Hamsters that lost play fights showed decreased PNN expression on PV+ neurons in the PL, IL, and BLA, which suggests that losing play fights may similarly influence neural plasticity in these circuits. Future studies could investigate whether this pattern of neural plasticity in hamsters that win or lose play fights is similar to effects seen in dominant or subordinate adults. This could be achieved through using tracing approaches to label BLA-projecting neurons in the PL and IL, and determining if these neurons are activated more when winning play fights compared to losing. Similarly, activation in these pathways could be assessed during regrouping to determine if activity of vmPFC-BLA neurons mediates expression of submissive and defensive behavior. Indeed, if winning and losing in social play encounters generates neural plasticity similar to dominance relationships, further research could also investigate whether social play generates synaptic plasticity in a MeA-BNST pathway.

To delineate causal relationships between activity within a MeA-BNST pathway and social status-dependent stress resistance in adult hamsters, future research could use a chemogenetic approach to stimulate or inhibit BNST-projecting MeA neurons during social encounters. We could inhibit or activate this pathway both during the formation of social dominance hierarchies and during social defeat to determine whether inhibition

induces subordinate-like stress vulnerability and whether activation induces dominant-like stress resistance. Additionally, these circuit manipulations could be carried out in juvenile animals, and we could determine whether they alter patterns of social play and stress-related behavior in juveniles and adults.

Future research directions that would extend the present studies should include additional markers of synaptic plasticity in limbic regions that govern stress responses and social behavior. Potential targets could include quantifying expression of brain-derived neurotrophic factor, reelin, synaptophysin, or measuring long-term potentiation of local and projection circuits using electrophysiology techniques. These markers would provide novel information about the mechanisms by which play deprivation and achieving a social dominance induces synaptic plasticity in specific neuronal phenotypes. The link between PNN expression and social behavior could also be further explored by assessing behavioral alterations following the degradation of PNNs using chondroitinase ABC enzyme or repeated ketamine administration⁹¹. Given the results of the present study, candidate regions for PNN degradation would be the PL, IL, and BLA. Because submissive behavior at regrouping was associated with decreased PNN/PV co-expression in the PL it is possible that PNN degradation in the PL would increase submissive behavior during social integration. Similarly, PNN count in the IL and PNN/PV co-expression in the BLA were positively correlated with social approach in a social avoidance test. It is possible that degrading PNNs in the IL and BLA would increase defeat-induced social avoidance. Such findings would suggest that PNNs in the PL, IL, and BLA play an important role in attenuating stress-related social anxiety responses in

Syrian hamsters and highlight a potential mechanism by which social experiences such as play and social dominance generate individual differences in stress vulnerability.

Both studies highlighted sex differences in experience-dependent neural plasticity where only dominant males exhibited increased activity in a MePD-BNST circuit during agonistic social encounters. Also, female hamsters who lost play fights were particularly sensitive to showing decreased PNN/PV co-expression in the PL and IL. Such findings highlight the need to further investigate the neural mechanisms regulating stress vulnerability and agonistic behavior in female subjects. The data in our lab suggest the cellular mechanisms controlling stress vulnerability in males do not generalize to females, yet the mechanisms governing these behavioral outcomes in females are unknown. To elucidate these neural pathways future studies could use retrograde and anterograde tracers to investigate additional putative pathways involved in regulating aggressive behavior such as outputs from the MeA to the ventromedial hypothalamus ⁶⁵. In addition, projections from the vmPFC to the MeA could be examined to determine how cortical input to the MeA may alter status-dependent c-Fos expression during social defeat stress ⁹³.

These studies collectively highlight the mechanisms by which social experience generates neural plasticity in specific neural ensembles to regulate stress vulnerability. While experience-dependent neuroplasticity in a MePD-BNST circuit plays a role in attenuating responses to social defeat stress in male hamsters, the neural pathways governing status-dependent stress resistance in female hamsters remain unclear. Similarly, social play experience in the juvenile period promotes stress resistance in both

males and females, and is associated with PNN expression in the PL, IL, and BLA. This research identifies cellular markers and specific neural pathways that modulate stress vulnerability and delineates a critical role for experience-dependent neural plasticity in generating individual differences in stress-related behaviors. Such findings are a critical step toward delineating the cellular mechanisms and neural circuits underlying stress-related psychopathology and creating novel treatment strategies for mitigating the harmful effects of traumatic stress.

5. Summary

Stressors such as environmental perturbations and social experiences elicit a wide range of stress responses across taxa, and individuals can differ considerably in their responses to adverse stimuli⁹⁴⁻⁹⁸. Stress-induced plasticity in behavioral, physiological, and neuroendocrine systems can have a long-term impact on an organism's fitness^{95,99-106}. The experiences individuals have in social play and dominance hierarchies can modify how they respond to stressors and generate individual differences in stress resilience^{69,76}. This work delineates individual and population-level differences in stress-related behavior across myriad stressors with a cross-species comparative lens. The work presented in this dissertation highlights the importance of integrating lab and field-based approaches to understanding ecologically relevant impacts of environmental challenges, while also promoting ecological and external validity in laboratory analyses. The findings presented in this dissertation also highlight the critical need to understand the negative consequences associated with different types of stressors, and how environmental factors may attenuate these risks. Future research delineating the mechanisms by which the

environmental factors modulate stress-related behavior should be beneficial for identifying treatments to mitigate stress-induced impairments.

This dissertation focused on experience-dependent neural plasticity to elucidate the mechanisms of individual variation in stress vulnerability. The findings presented here identify key biomarkers that distinguish individuals who are susceptible or resistant to social stressors. Developing a greater understanding of the mechanisms that modulate stress vulnerability will be beneficial for delineating risk factors for stress-related psychopathology and the development of novel treatment options. This dissertation contributes to a growing body of work to elucidate the myriad ways in which stressors impact organism health, neuronal systems, and behavior, and how the deleterious effects of stress can be mitigated.

References

1. Landrigan, P. J. *et al.* The Lancet Commission on pollution and health. *The Lancet* **391**, 462–512 (2018).
2. Satarug, S. *et al.* A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* **137**, 65–83 (2003).
3. Larison, J. R., Likens, G. E., Fitzpatrick, J. W. & Crock, J. G. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. *Nature* **406**, 181–183 (2000).
4. Yang, F. *et al.* Cadmium exposure disrupts the olfactory sensitivity of fire ants to semiochemicals. *Environ. Pollut.* **287**, 117359 (2021).
5. LeFauve, M. K. & Connaughton, V. P. Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish. *Curr. Zool.* **63**, 221–227 (2017).
6. Terçariol, S. G., Almeida, A. A. & Godinho, A. F. Cadmium and exposure to stress increase aggressive behavior. *Environ. Toxicol. Pharmacol.* **32**, 40–45 (2011).
7. Ward, A. J. W., Duff, A. J., Horsfall, J. S. & Currie, S. Scents and scents-ability: pollution disrupts chemical social recognition and shoaling in fish. *Proc. R. Soc. B Biol. Sci.* **275**, 101–105 (2008).
8. Galef, B. G. & Wigmore, S. W. Transfer of information concerning distant foods: A laboratory investigation of the ‘information-centre’ hypothesis. *Anim. Behav.* **31**, 748–758 (1983).
9. Scott, G. R. & Sloman, K. A. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* **68**, 369–392 (2004).

10. van der Sluijs, I. *et al.* Communication in troubled waters: responses of fish communication systems to changing environments. *Evol. Ecol.* **25**, 623–640 (2011).
11. Pilastro, A., Benetton, S. & Bisazza, A. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Anim. Behav.* **65**, 1161–1167 (2003).
12. Fleeger, J. W., Carman, K. R. & Nisbet, R. M. Indirect effects of contaminants in aquatic ecosystems. *Sci. Total Environ.* **317**, 207–233 (2003).
13. Köhler, H.-R. & Triebskorn, R. Wildlife Ecotoxicology of Pesticides: Can We Track Effects to the Population Level and Beyond? *Science* **341**, 759–765 (2013).
14. Candolin, U. & Wong, B. B. M. Mate choice in a polluted world: consequences for individuals, populations and communities. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20180055 (2019).
15. Saaristo, M. *et al.* Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. *Proc. R. Soc. B Biol. Sci.* **285**, 20181297 (2018).
16. Zala, S. M. & Penn, D. J. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Anim. Behav.* **68**, 649–664 (2004).
17. Green, A. J. & Planchart, A. The neurological toxicity of heavy metals: A fish perspective. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **208**, 12–19 (2018).
18. Satarug, S. *et al.* A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicol. Lett.* **137**, 65–83 (2003).
19. Satarug, S., Garrett, S. H., Sens, M. A. & Sens, D. A. Cadmium, Environmental Exposure, and Health Outcomes. *Environ. Health Perspect.* **118**, 182–190 (2010).

20. Urban, M. C. Accelerating extinction risk from climate change. *Science* **348**, 571–573 (2015).
21. Lema, S. C., Bock, S. L., Malley, M. M. & Elkins, E. A. Warming waters beget smaller fish: evidence for reduced size and altered morphology in a desert fish following anthropogenic temperature change. *Biol. Lett.* **15**, 20190518 (2019).
22. Aktar, S. *et al.* Individual and Combined Effects of Arsenic and Lead on Behavioral and Biochemical Changes in Mice. *Biol. Trace Elem. Res.* **177**, 288–296 (2017).
23. Burrows, M. T. *et al.* The Pace of Shifting Climate in Marine and Terrestrial Ecosystems. *Science* **334**, 652–655 (2011).
24. Duan, Z. *et al.* Barrier function of zebrafish embryonic chorions against microplastics and nanoplastics and its impact on embryo development. *J. Hazard. Mater.* **395**, 122621 (2020).
25. Wu, N. C. & Seebacher, F. Bisphenols alter thermal responses and performance in zebrafish (*Danio rerio*). *Conserv. Physiol.* **9**, coaa138 (2021).
26. Hallauer, J. *et al.* The Effect of Chronic Arsenic Exposure in Zebrafish. *Zebrafish* **13**, 405–412 (2016).
27. Tu, H. *et al.* Role of neurexin2a in lead-induced locomotor defect in developing zebrafish. *Aquat. Toxicol.* **194**, 167–175 (2018).
28. Ribas, L. *et al.* Heat-induced masculinization in domesticated zebrafish is family-specific and yields a set of different gonadal transcriptomes. *Proc. Natl. Acad. Sci.* **114**, E941–E950 (2017).

29. Shukla, R. & Bhat, A. Morphological divergences and ecological correlates among wild populations of zebrafish (*Danio rerio*). *Environ. Biol. Fishes* **100**, 251–264 (2017).
30. Widmer, S., Moore, F. B.-G. & Bagatto, B. The effects of chronic developmental hypoxia on swimming performance in zebrafish. *J. Fish Biol.* **69**, 1885–1891 (2006).
31. Suriyampola, P. S. *et al.* Zebrafish Social Behavior in the Wild. *Zebrafish* **13**, 1–8 (2016).
32. Suriyampola, P. S. *et al.* Water flow impacts group behavior in zebrafish (*Danio rerio*). *Behav. Ecol.* **28**, 94–100 (2017).
33. Shelton, D. S. *et al.* Collective Behavior in Wild Zebrafish. *Zebrafish* **17**, 243–252 (2020).
34. Hypoxia Affects Sex Differentiation and Development, Leading to a Male-Dominated Population in Zebrafish (*Danio rerio*) | Environmental Science & Technology.
<https://pubs.acs.org/doi/full/10.1021/es0522579>.
35. Roy, T. & Bhat, A. Population, sex and body size: determinants of behavioural variations and behavioural correlations among wild zebrafish *Danio rerio*. *R. Soc. Open Sci.* **5**, 170978 (2018).
36. Baumann, L. *et al.* Prochloraz causes irreversible masculinization of zebrafish (*Danio rerio*). *Environ. Sci. Pollut. Res.* **22**, 16417–16422 (2015).
37. Toxicity testing of pesticides in zebrafish—a systematic review on chemicals and associated toxicological endpoints | Environmental Science and Pollution Research.
<https://link.springer.com/article/10.1007/s11356-020-07902-5>.
38. Fitzgerald, J. A., Könnemann, S., Krümpelmann, L., Županič, A. & Vom Berg, C. Approaches to Test the Neurotoxicity of Environmental Contaminants in the Zebrafish

Model: From Behavior to Molecular Mechanisms. *Environ. Toxicol. Chem.* **40**, 989–1006 (2021).

39. Kalueff, A. V. *et al.* Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. *Zebrafish* **10**, 70–86 (2013).
40. Tal, T., Yaghoobi, B. & Lein, P. J. Translational toxicology in zebrafish. *Curr. Opin. Toxicol.* **23–24**, 56–66 (2020).
41. Carvan III, M. J. *et al.* Oxidative stress in zebrafish cells: potential utility of transgenic zebrafish as a deployable sentinel for site hazard ranking. *Sci. Total Environ.* **274**, 183–196 (2001).
42. Bambino, K. & Chu, J. Chapter Nine - Zebrafish in Toxicology and Environmental Health. in *Current Topics in Developmental Biology* (ed. Sadler, K. C.) vol. 124 331–367 (Academic Press, 2017).
43. Dai, Y.-J. *et al.* Zebrafish as a model system to study toxicology. *Environ. Toxicol. Chem.* **33**, 11–17 (2014).
44. Inderjit & Weston, L. A. Are Laboratory Bioassays for Allelopathy Suitable for Prediction of Field Responses? *J. Chem. Ecol.* **26**, 2111–2118 (2000).
45. Schoenfuss, H. L. *et al.* Complex mixtures, complex responses: Assessing pharmaceutical mixtures using field and laboratory approaches. *Environ. Toxicol. Chem.* **35**, 953–965 (2016).
46. Underwood, A. J. Toxicological testing in laboratories is not ecological testing of toxicology. *Hum. Ecol. Risk Assess. Int. J.* **1**, 178–182 (1995).

47. Kellman, J. & Radwan, K. Towards an expanded neuroscientific understanding of social play. *Neurosci. Biobehav. Rev.* **132**, 884–891 (2022).
48. Da silva, N. L., Ferreira, V. M. M., De padua carobrez, A. & Morato, G. S. Individual Housing From Rearing Modifies the Performance of Young Rats on the Elevated Plus-Maze Apparatus. *Physiol. Behav.* **60**, 1391–1396 (1996).
49. Gerall, H. D., Ward, I. L. & Gerall, A. A. Disruption of the male rat's sexual behaviour induced by social isolation. *Anim. Behav.* **15**, 54–58 (1967).
50. Hol, T., Van den Berg, C. L., Van Ree, J. M. & Spruijt, B. M. Isolation during the play period in infancy decreases adult social interactions in rats. *Behav. Brain Res.* **100**, 91–97 (1999).
51. Marquardt, A. E., VanRyzin, J. W., Fuquen, R. W. & McCarthy, M. M. Social play experience in juvenile rats is indispensable for appropriate socio-sexual behavior in adulthood in males but not females. *Front. Behav. Neurosci.* **16**, (2023).
52. Van den Berg, C. L. *et al.* Isolation changes the incentive value of sucrose and social behaviour in juvenile and adult rats. *Behav. Brain Res.* **106**, 133–142 (1999).
53. Biro, L. *et al.* Post-weaning social isolation in male mice leads to abnormal aggression and disrupted network organization in the prefrontal cortex: Contribution of parvalbumin interneurons with or without perineuronal nets. *Neurobiol. Stress* **25**, 100546 (2023).
54. Biro, L. *et al.* Structural and functional alterations in the prefrontal cortex after post-weaning social isolation: relationship with species-typical and deviant aggression. *Brain Struct. Funct.* **222**, 1861–1875 (2017).

55. Einon, D. & Potegal, M. Enhanced defense in adult rats deprived of playfighting experience as juveniles. *Aggress. Behav.* **17**, 27–40 (1991).
56. Potegal, M. & Einon, D. Aggressive behaviors in adult rats deprived of playfighting experience as juveniles. *Dev. Psychobiol.* **22**, 159–172 (1989).
57. Von Frijtag, J. C., Schot, M., van den Bos, R. & Spruijt, B. M. Individual housing during the play period results in changed responses to and consequences of a psychosocial stress situation in rats. *Dev. Psychobiol.* **41**, 58–69 (2002).
58. Leussis, M. P. & Andersen, S. L. Is adolescence a sensitive period for depression? Behavioral and neuroanatomical findings from a social stress model. *Synapse* **62**, 22–30 (2008).
59. Bell, H. C., Pellis, S. M. & Kolb, B. Juvenile peer play experience and the development of the orbitofrontal and medial prefrontal cortices. *Behav. Brain Res.* **207**, 7–13 (2010).
60. Burleson, C. A. *et al.* Social Play in Juvenile Hamsters Alters Dendritic Morphology in the Medial Prefrontal Cortex and Attenuates Effects of Social Stress in Adulthood. *Behav. Neurosci.* **130**, 437–447 (2016).
61. Pellis, S. M., Pellis, V. C., Ham, J. R. & Stark, R. A. Play fighting and the development of the social brain: The rat's tale. *Neurosci. Biobehav. Rev.* **145**, 105037 (2023).
62. Cooper, M. A. *et al.* Dominance status modulates activity in medial amygdala cells with projections to the bed nucleus of the stria terminalis. *Behav. Brain Res.* **453**, 114628 (2023).

63. Dulka, B. N., Bress, K. S., Grizzell, J. A. & Cooper, M. A. Social Dominance Modulates Stress-induced Neural Activity in Medial Prefrontal Cortex Projections to the Basolateral Amygdala. *Neuroscience* **388**, 274–283 (2018).
64. Morrison, K. E., Curry, D. W. & Cooper, M. A. Social status alters defeat-induced neural activation in Syrian hamsters. *Neuroscience* **210**, 168–178 (2012).
65. Morrison, K. E. *et al.* Maintenance of dominance status is necessary for resistance to social defeat stress in Syrian hamsters. *Behav. Brain Res.* **270**, 277–286 (2014).
66. Kumar, S. *et al.* Prefrontal cortex reactivity underlies trait vulnerability to chronic social defeat stress. *Nat. Commun.* **5**, 4537 (2014).
67. Dulka, B. N. *et al.* Chemogenetic activation of an infralimbic cortex to basolateral amygdala projection promotes resistance to acute social defeat stress. *Sci. Rep.* **10**, 6884 (2020).
68. Bremner, J. D., Elzinga, B., Schmahl, C. & Vermetten, E. Structural and functional plasticity of the human brain in posttraumatic stress disorder. in *Progress in Brain Research* (eds. De Kloet, E. R., Oitzl, M. S. & Vermetten, E.) vol. 167 171–186 (Elsevier, 2007).
69. Cooper, M. A., Clinard, C. T. & Morrison, K. E. Neurobiological mechanisms supporting experience-dependent resistance to social stress. *Neuroscience* **291**, 1–14 (2015).
70. Wood, S. K., Walker, H. E., Valentino, R. J. & Bhatnagar, S. Individual differences in reactivity to social stress predict susceptibility and resilience to a depressive phenotype: role of corticotropin-releasing factor. *Endocrinology* **151**, 1795–1805 (2010).

71. Wyrofsky, R. R. *et al.* Endocannabinoids, stress signaling, and the locus coeruleus-norepinephrine system. *Neurobiol. Stress* **11**, 100176 (2019).
72. Grafe, L. A. *et al.* Passive Coping Strategies During Repeated Social Defeat Are Associated With Long-Lasting Changes in Sleep in Rats. *Front. Syst. Neurosci.* **14**, (2020).
73. Greenwood, B. N. & Fleshner, M. Voluntary wheel running: a useful rodent model for investigating mechanisms of stress robustness and exercise motivation. *Curr. Opin. Behav. Sci.* **28**, 78–84 (2019).
74. Maier, S. F. Behavioral control blunts reactions to contemporaneous and future adverse events: Medial prefrontal cortex plasticity and a corticostriatal network. *Neurobiol. Stress* **1**, 12–22 (2015).
75. Wood, S. K. & Bhatnagar, S. Resilience to the effects of social stress: Evidence from clinical and preclinical studies on the role of coping strategies. *Neurobiol. Stress* **1**, 164–173 (2015).
76. Morrison, K. E. *et al.* Maintenance of dominance status is necessary for resistance to social defeat stress in Syrian hamsters. *Behav. Brain Res.* **270**, 277–286 (2014).
77. Morrison, K. E., Curry, D. W. & Cooper, M. A. Social status alters defeat-induced neural activation in Syrian hamsters. *Neuroscience* **210**, 168–178 (2012).
78. Raam, T. & Hong, W. Organization of neural circuits underlying social behavior: A consideration of the medial amygdala. *Curr. Opin. Neurobiol.* **68**, 124–136 (2021).
79. Lebow, M. A. & Chen, A. Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatry* **21**, 450–463 (2016).

80. Davis, M., Walker, D. L., Miles, L. & Grillon, C. Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. *Neuropsychopharmacology* **35**, 105–135 (2010).
81. Blanchard, R. J., Shepherd, J. K., John Rodgers, R., Magee, L. & Caroline Blanchard, D. Attenuation of antipredator defensive behavior in rats following chronic treatment with imipramine. *Psychopharmacology (Berl.)* **110**, 245–253 (1993).
82. Clinard, C. T., Barnes, A. K., Adler, S. G. & Cooper, M. A. Winning agonistic encounters increases testosterone and androgen receptor expression in Syrian hamsters. *Horm. Behav.* **86**, 27–35 (2016).
83. Cooper, M. A. *et al.* Gonadal steroid hormone receptors in the medial amygdala contribute to experience-dependent changes in stress vulnerability. *Psychoneuroendocrinology* **129**, 105249 (2021).
84. Whitten, C. J. *et al.* Activation of androgen receptor-expressing neurons in the posterior medial amygdala is associated with stress resistance in dominant male hamsters. *Horm. Behav.* ((Under Review)).
85. Whitten, C. J. *et al.* Sex differences in dominance relationships in Syrian hamsters. *Physiol. Behav.* **270**, 114294 (2023).
86. Cooper, M. A., Grizzell, J. A., Whitten, C. J. & Burghardt, G. M. Comparing the ontogeny, neurobiology, and function of social play in hamsters and rats. *Neurosci. Biobehav. Rev.* **147**, 105102 (2023).

87. Delville, Y., David, J. T., Taravosh-Lahn, K. & Wommack, J. C. Stress and the development of agonistic behavior in golden hamsters. *Horm. Behav.* **44**, 263–270 (2003).
88. Cheng, S.-Y., Taravosh-Lahn, K. & Delville, Y. Neural circuitry of play fighting in golden hamsters. *Neuroscience* **156**, 247–256 (2008).
89. Pellis, S. M., Field, E. F., Smith, L. K. & Pellis, V. C. Multiple differences in the play fighting of male and female rats. Implications for the causes and functions of play. *Neurosci. Biobehav. Rev.* **21**, 105–120 (1997).
90. Pellis, S. M. & Pellis, V. C. Influence of dominance on the development of play fighting in pairs of male Syrian golden hamsters (*Mesocricetus auratus*). *Aggress. Behav.* **19**, 293–302 (1993).
91. Venturino, A. *et al.* Microglia enable mature perineuronal nets disassembly upon anesthetic ketamine exposure or 60-Hz light entrainment in the healthy brain. *Cell Rep.* **36**, 109313 (2021).
92. Nordman, J. C. *et al.* Potentiation of Divergent Medial Amygdala Pathways Drives Experience-Dependent Aggression Escalation. *J. Neurosci.* **40**, 4858–4880 (2020).
93. Sotoudeh, N., Namavar, M. R., Bagheri, F. & Zarifkar, A. The medial prefrontal cortex to the medial amygdala connections may affect the anxiety level in aged rats. *Brain Behav.* **12**, e2616 (2022).
94. Mazza, V. *et al.* Coping with style: individual differences in responses to environmental variation. *Behav. Ecol. Sociobiol.* **73**, 142 (2019).

95. Koolhaas, J. M. *et al.* Coping styles in animals: current status in behavior and stress-physiology. *Neurosci. Biobehav. Rev.* **23**, 925–935 (1999).
96. Koolhaas, J. M. *et al.* Stress revisited: A critical evaluation of the stress concept. *Neurosci. Biobehav. Rev.* **35**, 1291–1301 (2011).
97. Korte, S. M., Koolhaas, J. M., Wingfield, J. C. & McEwen, B. S. The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci. Biobehav. Rev.* **29**, 3–38 (2005).
98. Matthews, G. & Campbell, S. E. Sustained performance under overload: personality and individual differences in stress and coping. *Theor. Issues Ergon. Sci.* **10**, 417–442 (2009).
99. Mumtaz, F., Khan, M. I., Zubair, M. & Dehpour, A. R. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. *Biomed. Pharmacother.* **105**, 1205–1222 (2018).
100. Patel, D., Kas, M. J., Chattarji, S. & Buwalda, B. Rodent models of social stress and neuronal plasticity: Relevance to depressive-like disorders. *Behav. Brain Res.* **369**, 111900 (2019).
101. Claireaux, G., Webber, D. M., Kerr, S. R. & Boutilier, R. G. Physiology and Behaviour of Free-Swimming Atlantic COD (*Gadus Morhua*) Facing Fluctuating Salinity and Oxygenation Conditions. *J. Exp. Biol.* **198**, 61–69 (1995).
102. Egan, R. J. *et al.* Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behav. Brain Res.* **205**, 38–44 (2009).
103. Chrousos, G. P. Stress and disorders of the stress system. *Nat. Rev. Endocrinol.* **5**, 374–381 (2009).

104. Haas, T. C., Blum, M. J. & Heins, D. C. Morphological responses of a stream fish to water impoundment. *Biol. Lett.* **6**, 803–806 (2010).
105. Sun, P. L., Hawkins, W. E., Overstreet, R. M. & Brown-Peterson, N. J. Morphological Deformities as Biomarkers in Fish from Contaminated Rivers in Taiwan. *Int. J. Environ. Res. Public Health* **6**, 2307–2331 (2009).
106. McCauley, S. J., Hammond, J. I. & Mabry, K. E. Simulated climate change increases larval mortality, alters phenology, and affects flight morphology of a dragonfly. *Ecosphere* **9**, e02151 (2018).

APPENDIX

Chapter 2 Figures & Tables

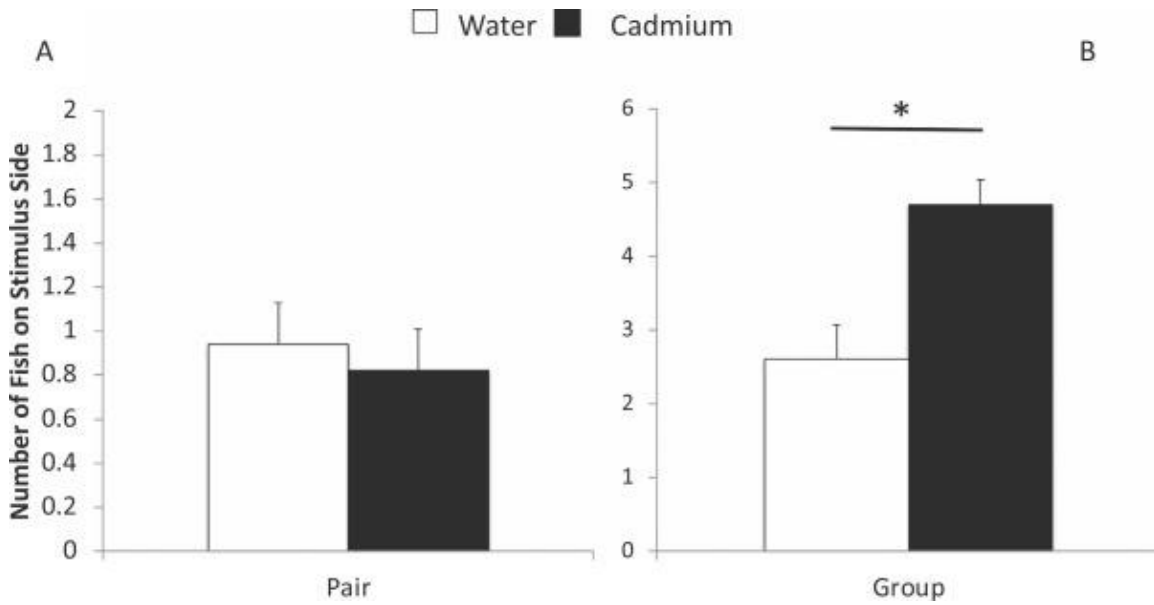


Fig 2. 1 Cadmium had no direct effect on pair boldness, but an indirect effect on group boldness. **(A)** Average number of fish in the pair condition on the tube or stimulus side. **(B)** Average number of fish in the group condition on the stimulus side. Error bars represent + 1SE (n = 17 or 18 in all conditions); statistically significant differences are noted (* $p < 0.05$).

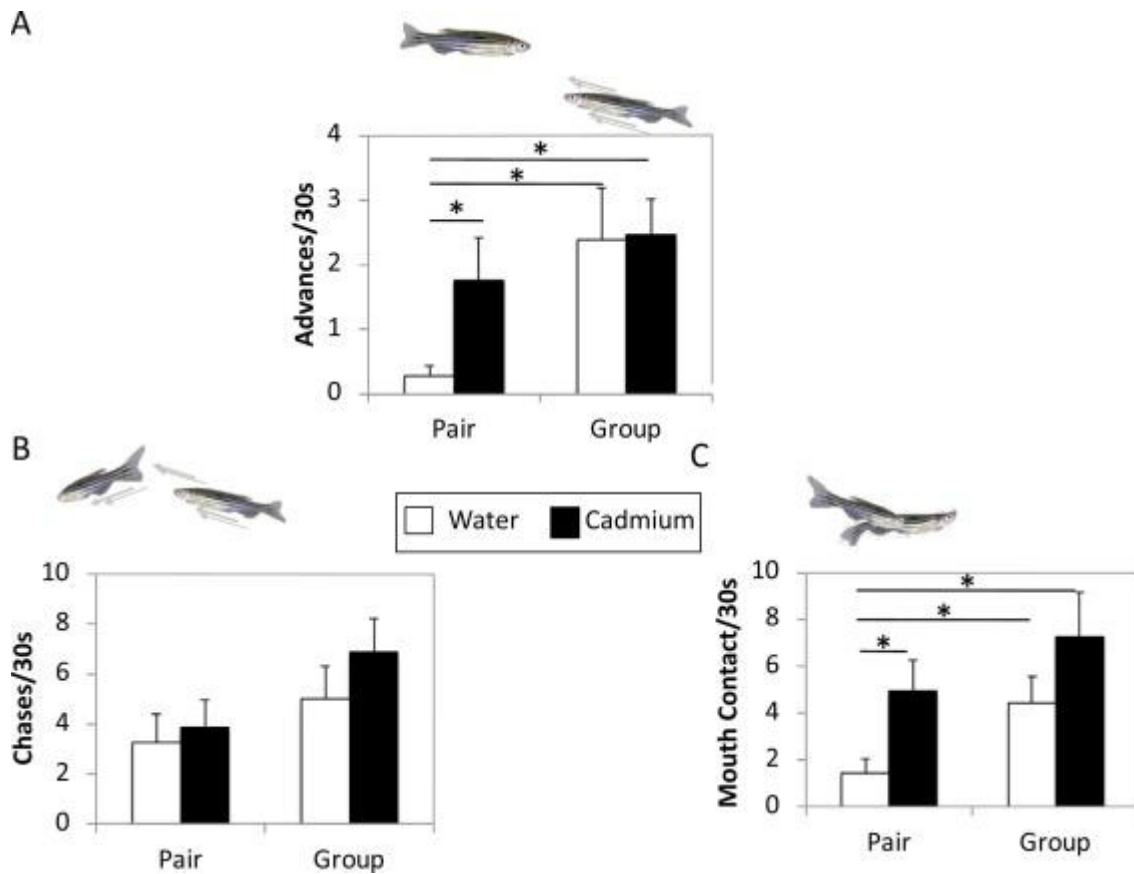


Fig 2. 2 Pair and group behavioral responses to the presence of cadmium-treated and control fish. **(A)** Average number of advances, **(B)** average number of chases and **(C)** average number of mouth contacts in 30 s for pair and groups of zebrafish with water-treated (white bars) and Cd-treated pairs (black bars). Error bars represent + 1 SE (n = 17 or 18 in all conditions); statistically significant differences are noted (* $p < 0.05$). The drawings provide a visual representation of the behavior measured for each figure.

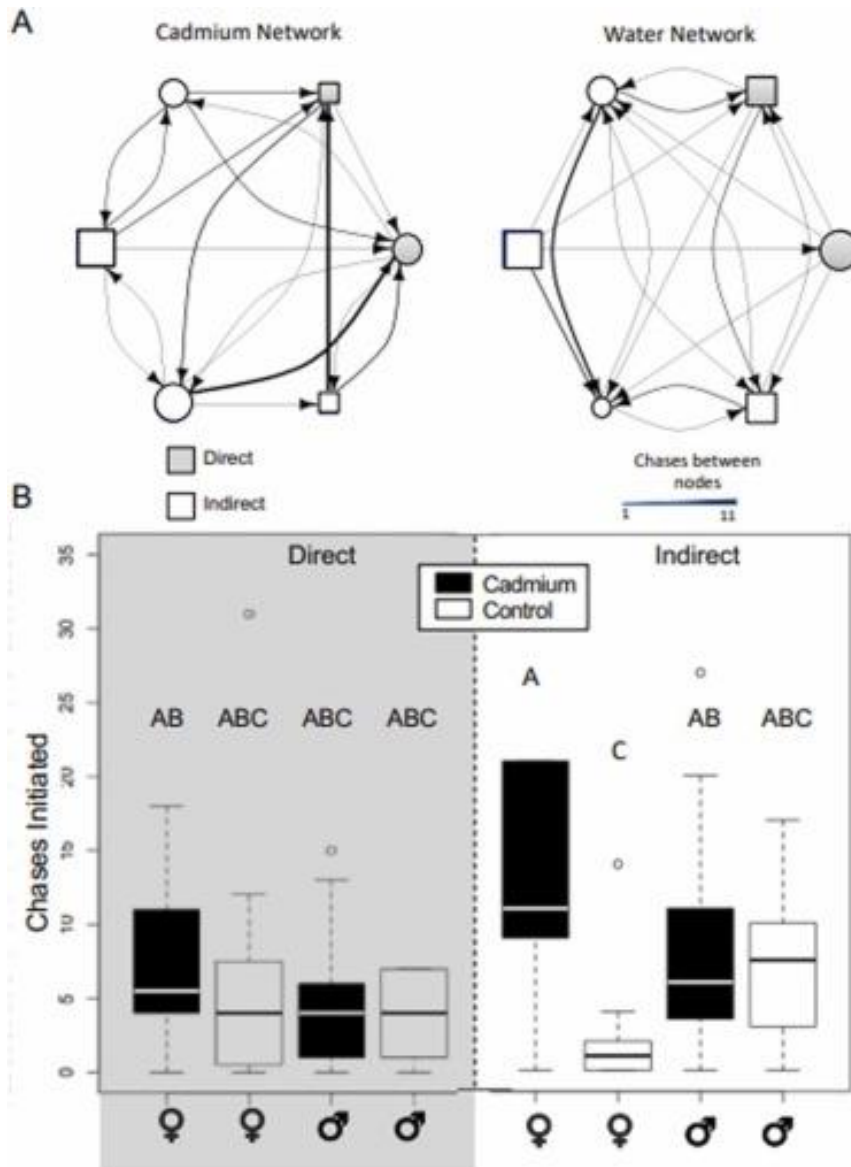


Fig 2. 3 Networks of cadmium and water shoals. **(A)** Multilayer social networks show the number of chases initiated between individuals with sex and exposure overlayed for a representative cadmium and control shoal. Node color denotes exposure type (gray = directly exposed, white = indirectly exposed), node shape denotes sex (circle $\circ$ = ♀, triangle Δ = ♂), and node size scales with average total chases initiated by each class of individuals. The width of the edges denotes the frequency of chases between dyads. Indirectly exposed female fish have larger nodes than other fish in the cadmium shoals, whereas the nodes are similar in size in the water shoals. Cadmium and water shoals had similar levels of connectivity ($t(16.61) = 0.54$, $p = 0.59$; $N = 10$ for cadmium and water shoals). **(B)** Cadmium-induced network changes are mediated by female fish ($\chi^2(df=1, N = 80) = 5.37$, $p = 0.02$). Indirectly exposed female cadmium fish initiated more chases than control indirectly exposed female fish (Tukey, $p < 0.01$). Medians with interquartile ranges ($N = 10$ per treatment). Letters denote significant Tukey pairwise comparisons at $p < 0.05$. $^{\circ}$ represents outliers.

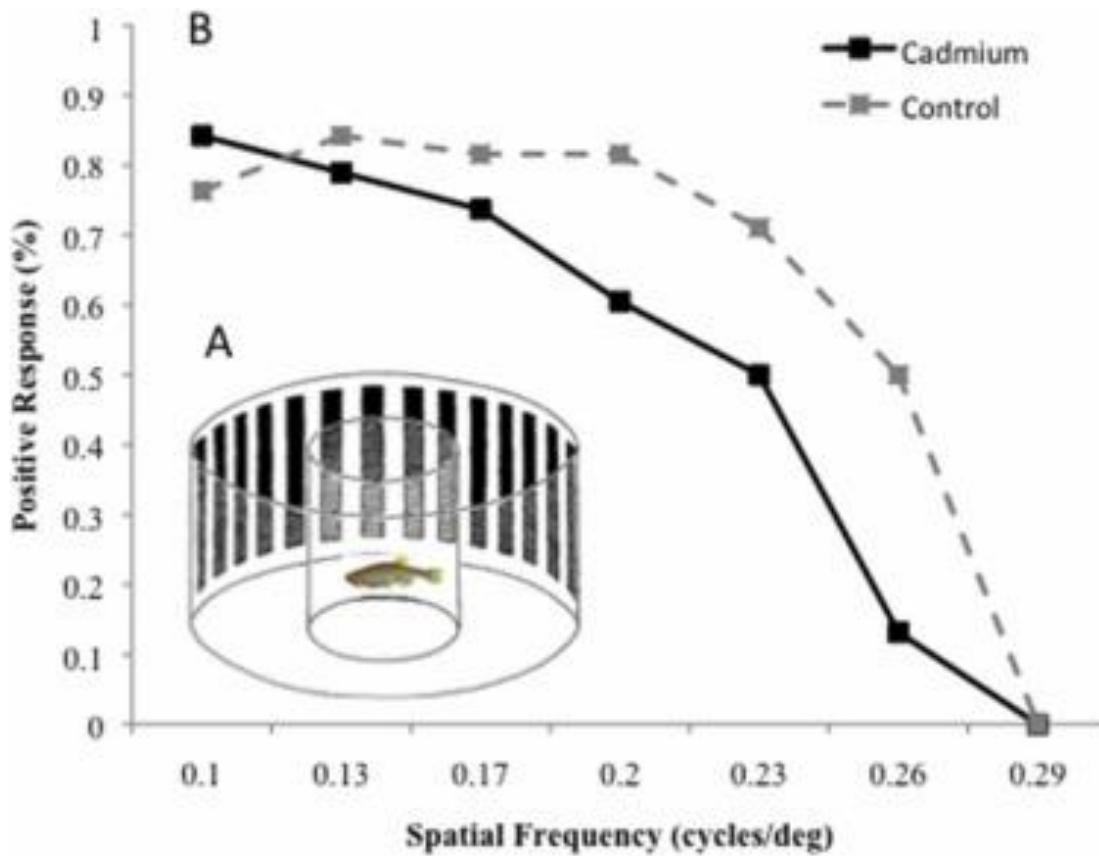


Fig 2. 4 Cadmium directly affects optomotor responses of fish. **(A)** Optomotor arena, a brightly lit (720 lux) rotating drum with a stimulus of repeating pattern of black and white bars. **(B)** Cadmium fish showed a decreased optomotor response in comparison to control fish. Both treatments showed decreased response with increased spatial frequencies.

Chapter 3 Figures & Tables

Table 3. 1 Description of conditions at both field sites.

Site	Site 1	Site 2
District	Cooch Behar	Nadia
Pollutants	As (<0.01 mg/L), Iron (2.3-6.5mg/L), Lead (0-16.6 µg/L) [27], minimal human-made debris	As (0.1-0.5) mg/L [67,69], Iron (0-9.1 mg/L), Chromium (0-147.8 µg/L) [27], abundant human-made debris: plastic bottles, Styrofoam plates, bags, shoes [52]
Water use	People were observed bathing, washing clothes, and irrigating crops with water from the field site	People tried to minimize contact with the water. When contact was made with water, people described burning and itching sensations
Land use	Homes, rice cultivation, sand mining, livestock grazing, unpaved roads	Homes, buildings, shops, paved roads
Water flow	Flowing [71]	Stagnant [71]
pH	7.4 [71]	6.9-7.8 [70,71]
Total dissolved solids (ppt)	0.2 [71]	0.3 [70,71]
Dissolved oxygen (ppm)	3.2-5.4 [79]	1.5 [52]
Nitrate (ppm)	0.0 [70]	0.0 [70]
Nitrite (ppm)	0.5 [70]	0.0 [70]
Temperature (°C)	19-25.6 [71]	21.4-25.6 [70,71]
Elevation (m)	12.2 [71]	41.4 [70,71]
Substrate	Mud [71]	Silt [71]
Predation pressure	Less [71]	Greater [71]
Population density	~700 persons/km ² [52]	~1250 persons/km ² [52]

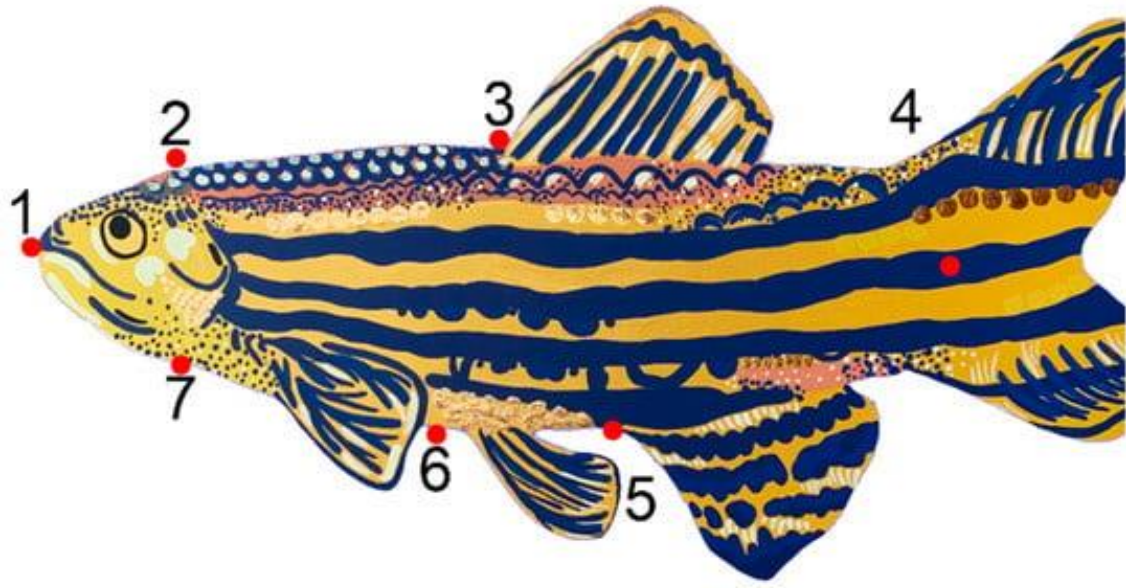


Fig 3. 1 A total of seven digitized landmarks were placed on distinct points of zebrafish for use in morphometric analysis. Landmarks denote: (1) mouth, (2) posterior dorsal skull, (3) anterior dorsal fin, (4) caudal fin, (5) anterior anal fin, (6) ventral surface, and (7) ventral anterior skull. Art created by Gillian McHaney.

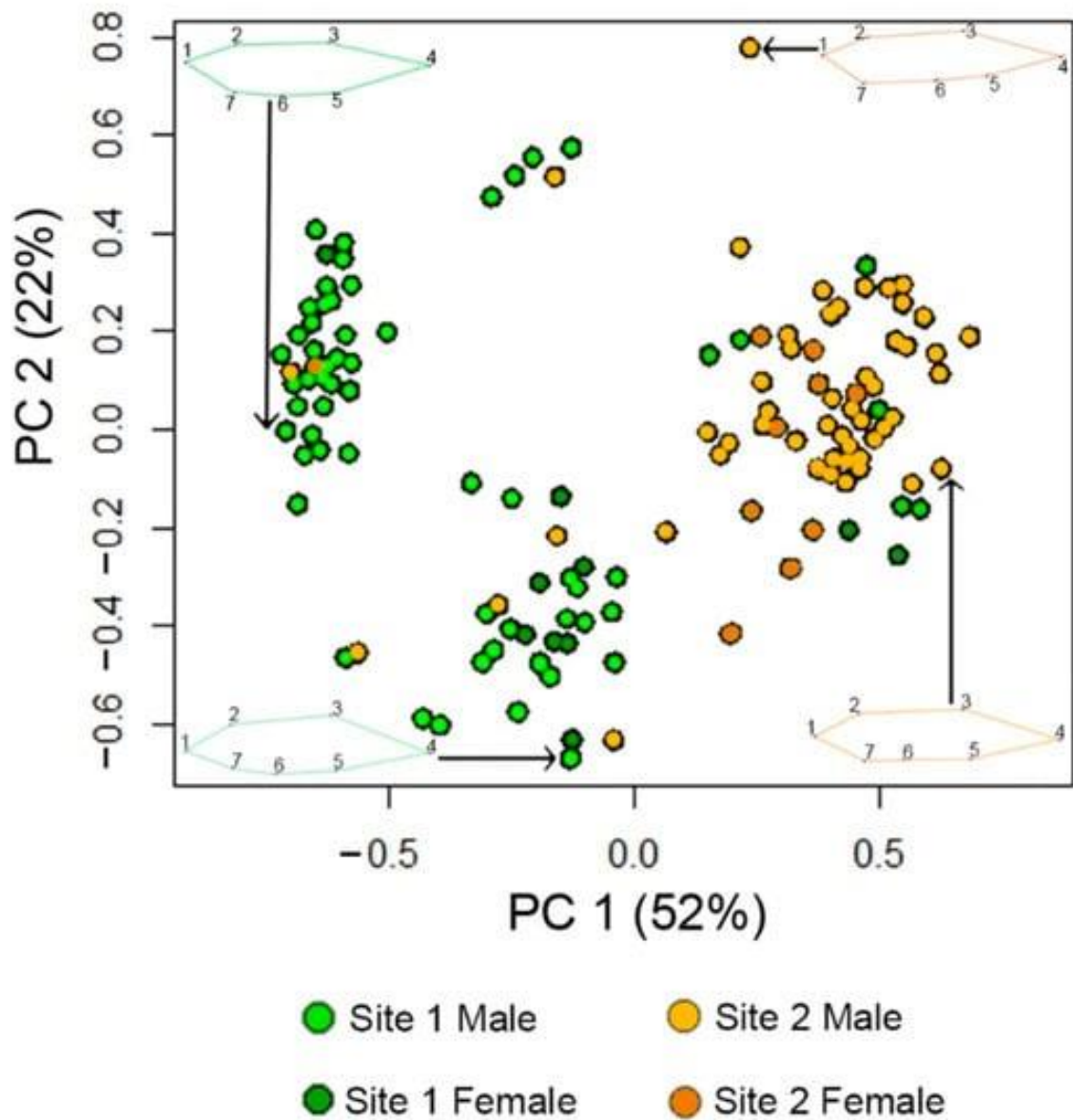


Fig 3. 2 Procrustes PCA plot of variance in partial warps between levels of Population and Sex. The first two Principal Components account for 74% of the variance in consensus shape between populations. The landmarks of specimens at the negative and positive ends of the first and second axes are shown not scaled to size. Site 1 fish are labeled in green, and Site 2 fish are labeled in orange. Lighter shades denote male fish and darker shades denote female fish.

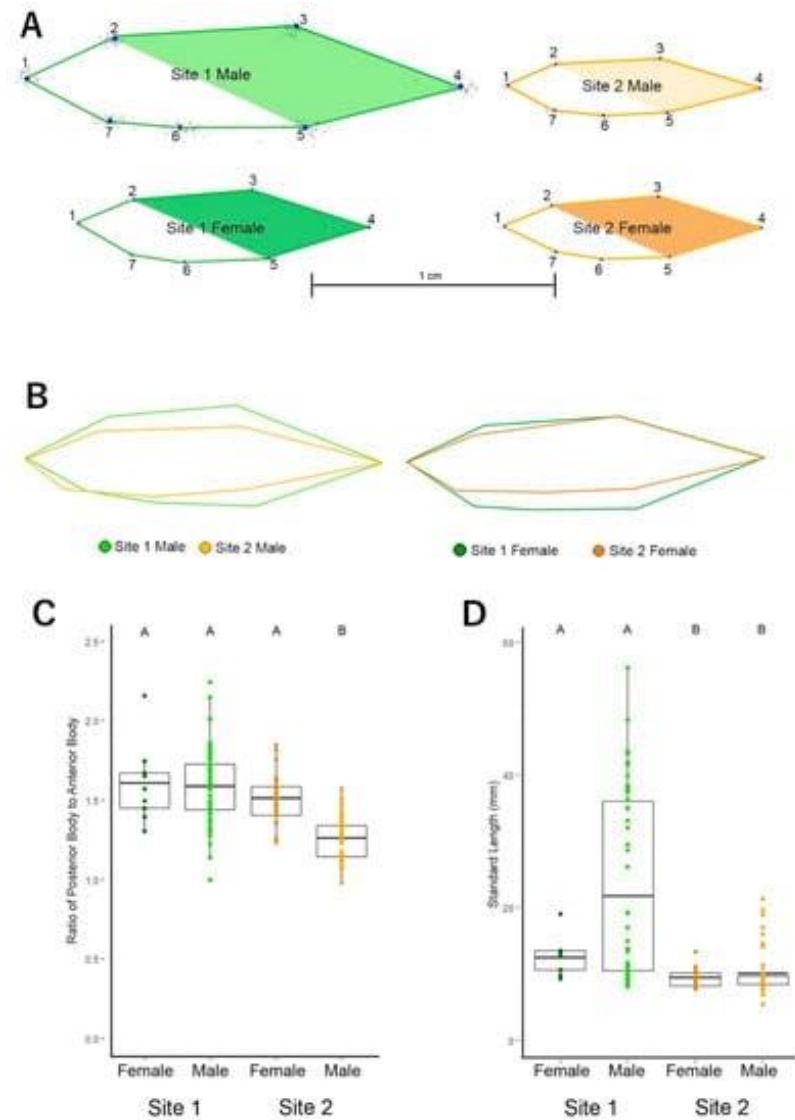


Fig 3. 3 Consensus shape differed significantly across Population, but not Sex. The greatest variance in shape between populations was among landmarks located on the posterior dorsal skull (LM 2), anterior dorsal fin (LM 3), caudal fin (LM 4), and anterior anal fin (LM 5). **(A)** Size differences in fish between levels of Population and Sex. Shaded regions denote the posterior body region identified by PCA as contributing to shaping variance, while the white region denotes the anterior body region not identified by PCA. **(B)** Overlaid shapes of the most morphometrically-extreme fish across levels of Population and Sex. **(C)** Fish from Site 1 and Site 2 female fish had a larger posterior region of their body relative to the anterior region compared to Site 2 male fish. Individual samples are overlaid onto boxplots. Data analyzed with a two-way ANOVA. **(D)** Fish from Site 1 had a greater standard length than fish from Site 2. Statistical analysis showed that Site 2 fish, not Site 1 fish, were sexually dimorphic. Individual samples are overlaid onto boxplots. Data analyzed with a Kruskal-Wallis test. Boxplots denote means and interquartile range. Error bars indicate the range of scores. Letter markings denote significant (different letters) and non-significant (same letters) differences ($p < 0.05$).

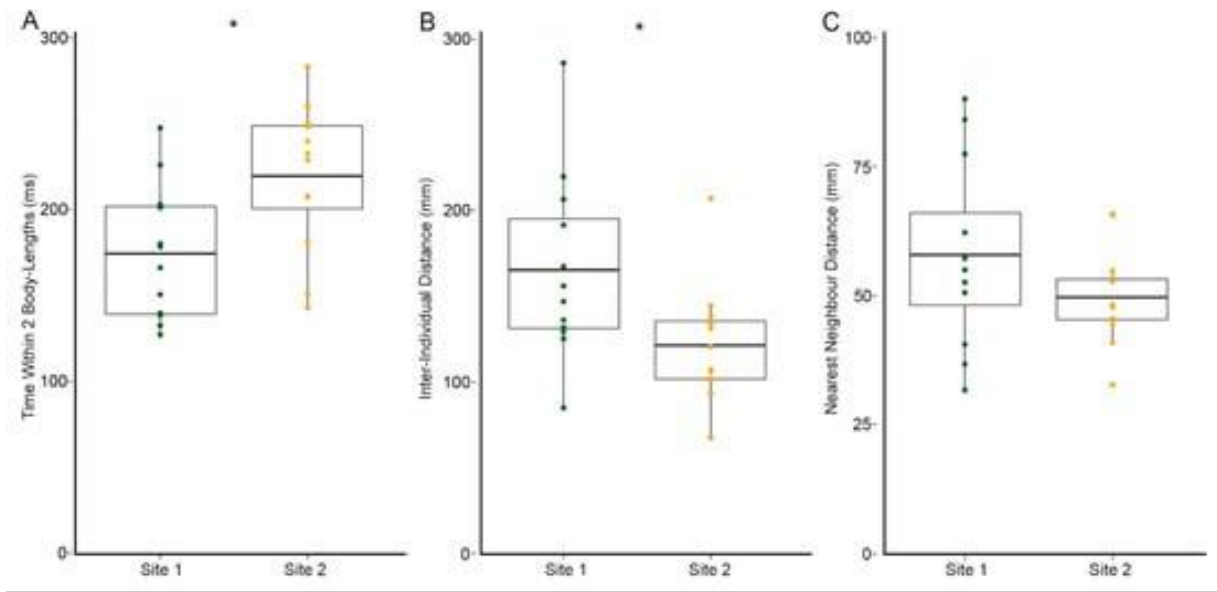


Fig 3. 4 We found that fish from Site 2 were more cohesive than fish from Site 1 across three social metrics. **(A)** Fish from Site 2 spent a greater amount of time within two body-lengths of conspecifics than fish from Site 1. **(B)** Fish from Site 2 maintained closer inter-individual distances between group members, while fish from Site 1 had greater spacing between individuals. **(C)** There were no significant differences in distances between individual fish and their nearest neighbors between populations. Individual samples are overlaid onto boxplots. Boxplots denote means and interquartile range. Error bars indicate the range of scores. * $p < 0.05$. All data analyzed with independent samples t -tests.

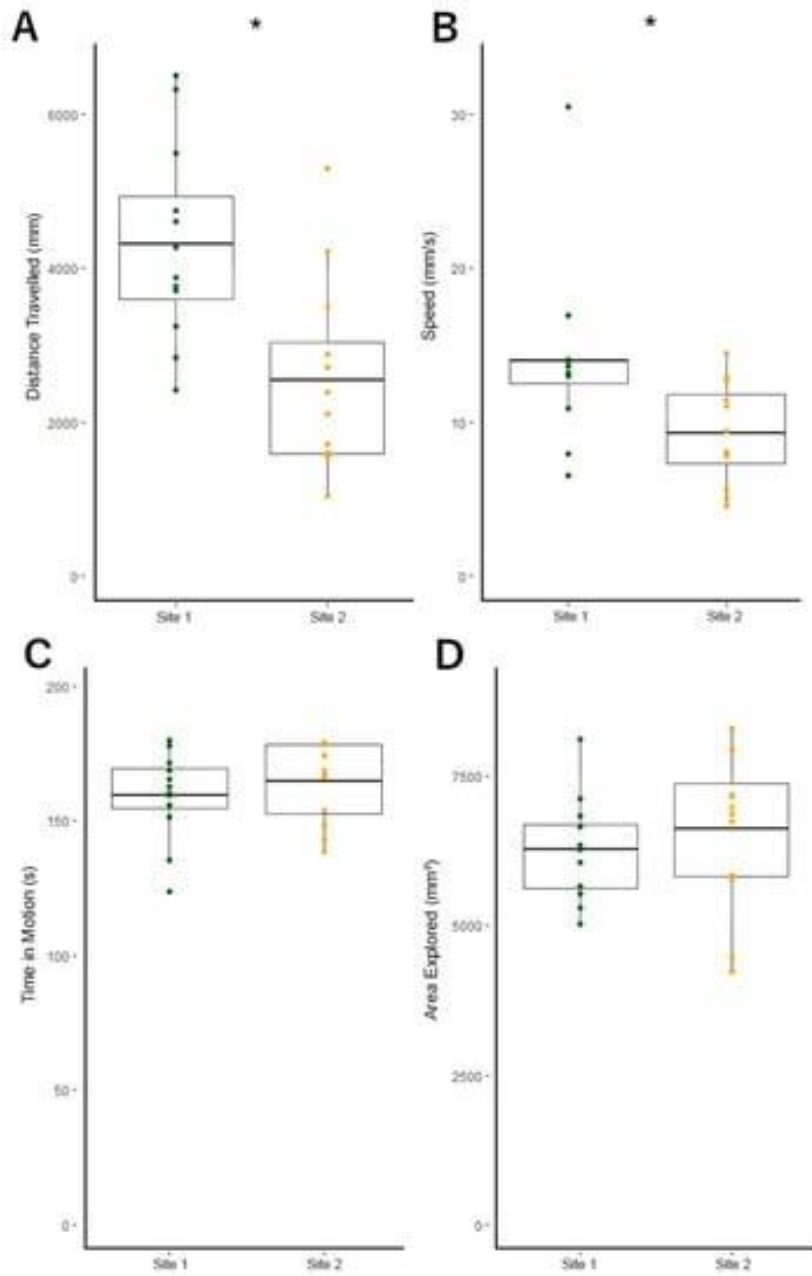


Fig 3. 5 Site 1 fish swam faster and further, but fish from both populations maintained similar time in motion and exploration. **(A)** Fish from Site 1 swam significantly greater distances than fish from Site 2. **(B)** Fish from Site 1 exhibited significantly greater average swimming speeds than Site 2. **(C)** Fish from each population spent a similar amount of time in motion throughout the sample period. **(D)** Fish from each population explored a similar number of areas within the testing arena and thus did not differ significantly in exploratory behavior. Individual samples are overlaid onto boxplots. Boxplots denote means and interquartile range. Error bars indicate the range of scores. * $p < 0.05$. All data analyzed with independent samples t -tests.

Chapter 4 Figures & Tables

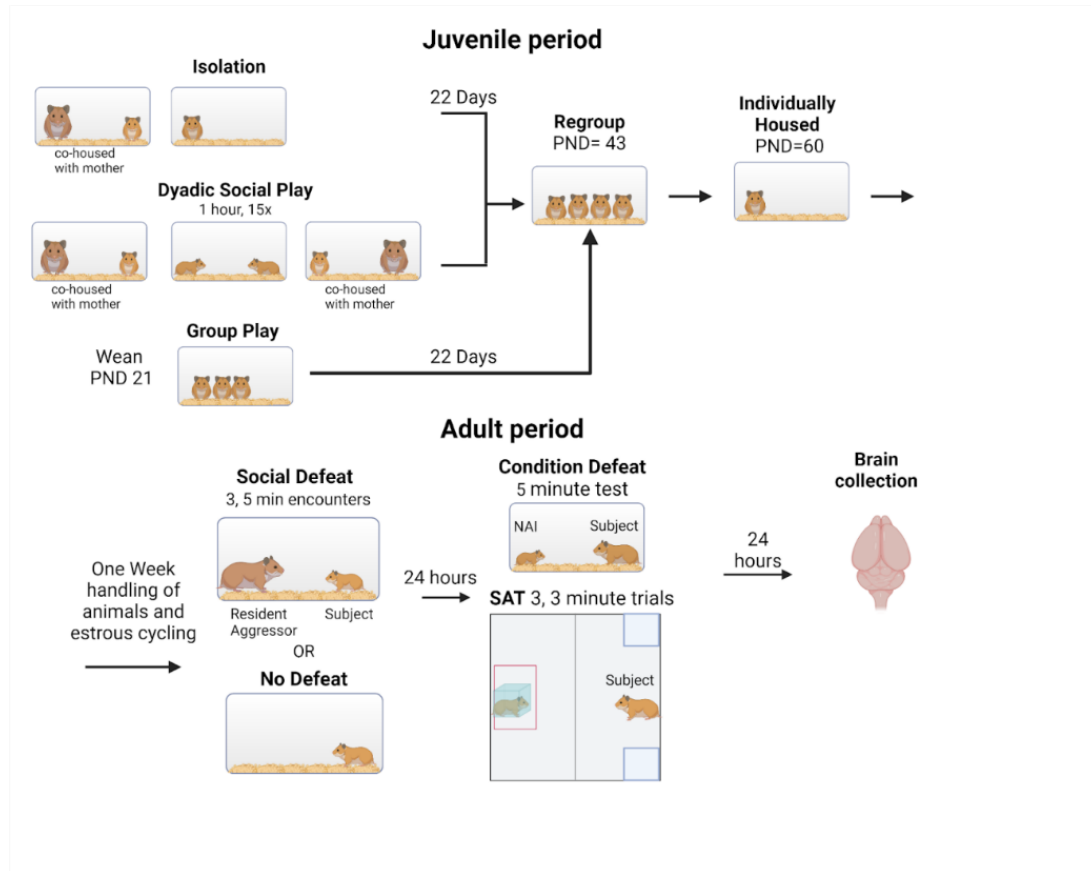


Fig 4. 1 Diagram of experimental design. After weaning (postnatal day 21), subjects were divided into four treatment groups. Peer-Isolated was raised with their dam and given 15, 1-hour isolated encounters in an empty neutral cage. Dyadic Play animals were similarly raised with their dam but given 15, 1-hour social play encounters with a sex-matched conspecific across PND 21-43. A Group Play condition included hamsters that were housed with 3 littermates after weaning. A Handle Control condition included hamsters that were housed with their littermates after weaning, just as the Group Play. After 22 days, Isolated animals and Dyadic Play animals were re-housed with Group Play littermates for an additional 3 weeks until adulthood (PND 60). After subjects were individually housed, subjects were handled for one week, and females were additionally evaluated for signs of Estrus. Subjects were exposed to social defeat stress in a series of 3, 5-minute encounters in which they were placed in the home cage of a larger, more aggressive hamster. Handle Controls were exposed to resident aggressors home cages without any aggression for 3, 5 minute trials. 24 hours after social defeat stress, subjects underwent a 5-minute condition defeat (CD) test followed by social avoidance (SA) testing. 24 hours after CD and SA testing, subjects were euthanized for brain collection. Condition defeat consisted of placing a smaller, younger non-aggressive intruder into the home cage of the test subject to measure territorial aggression. Social avoidance test consisted of a 3 minute habituation phase, followed by a 3 minute acclimation phase with an empty, perforated box and a 3 minute testing phase with a box containing a novel conspecific.

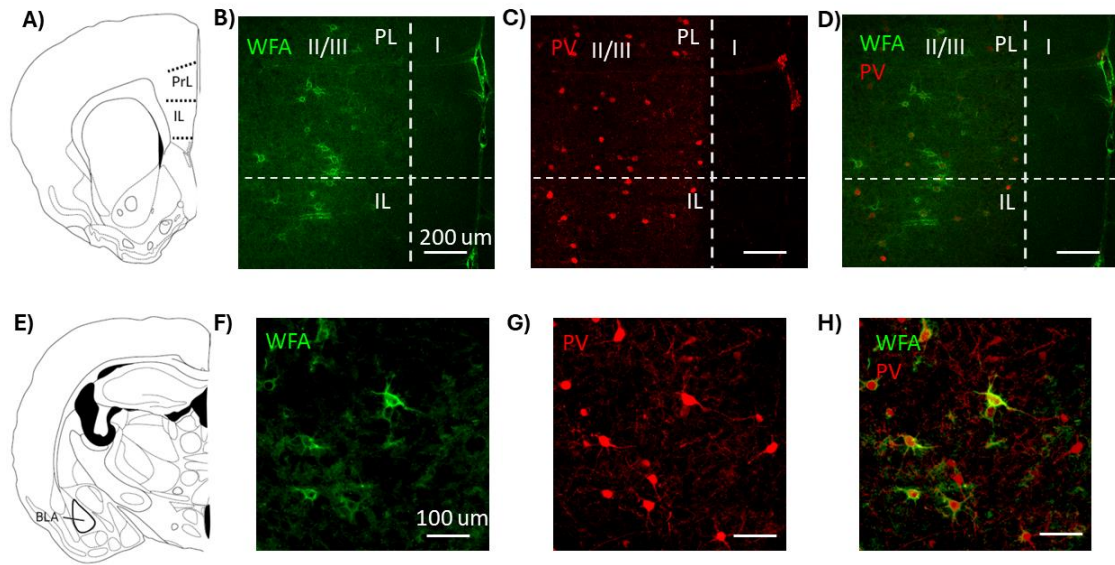


Fig 4. 2 Representation images of WFA and PV cells. **(A,E)** Coronal image showing the location of the prelimbic (PL), infralimbic (IL), and basal lateral amygdala (BLA). **(B-D)** Images were collected in layers II and III of the IL and PL of the prefrontal cortex. Majority of the WFA and PV expression was localized in layers II/III in the PL and IL. Images were taken at 20x with a 300 μm X 400 μm clip region that were used to quantify WFA cells, PV cells, and co-labelled cells in all subjects. **(F-H)** Representation of 20x magnification 500 μm X 500 μm images that were used to quantify WFA cells, PV cells, and co-labelled cells in all subjects.

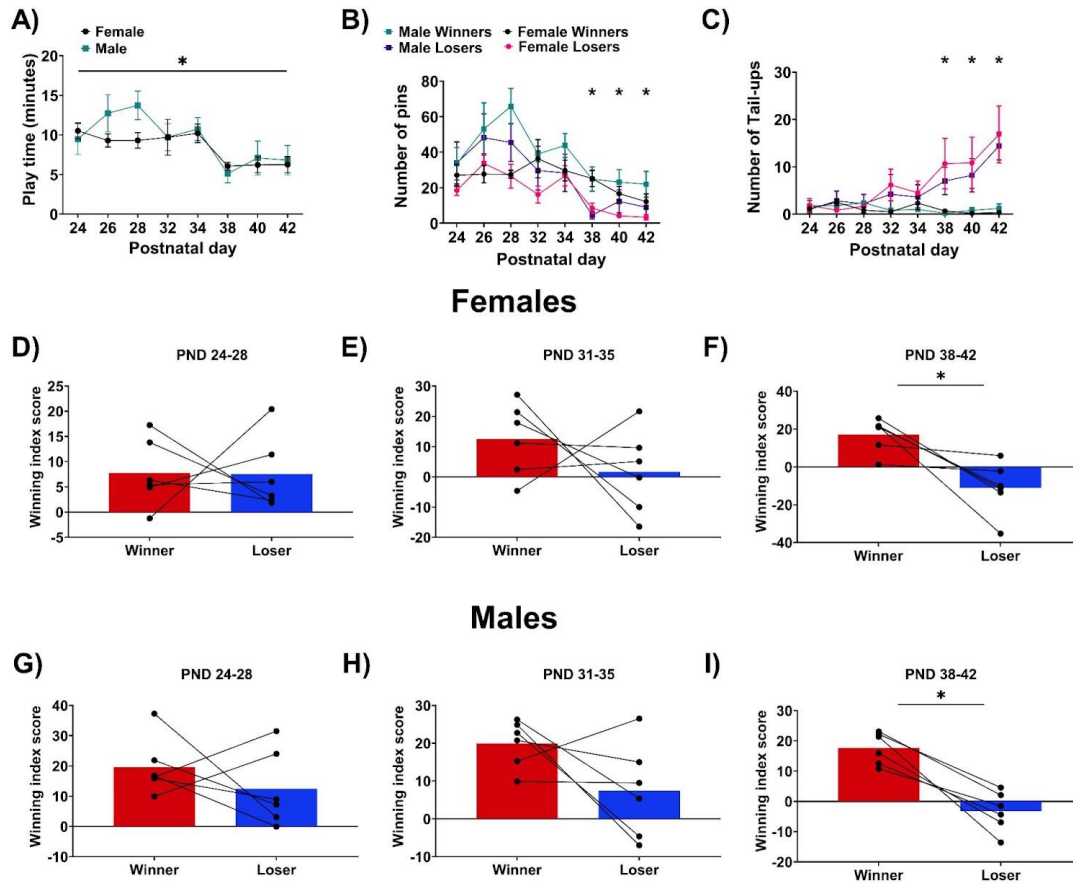


Fig 4. 3 Testing for the 50:50 ratio in dyadic play encounters. **(A)** Both males and females significantly change in the amount of time spent playing with one another. Specifically, social play decreased on PND 38-42 compared to PND 24-34. **(B)** Pins were reciprocated between winners and losers during the early PND (24-28) and mid phases (PND 31-35) of their juvenile development. However, during the late phases (PND 38-42) of juvenile development one hamster pinned their partner more often and winning became unilateral in both males and females. **(C)** Dominance relationships began to emerge as losers showed tail raise postures more often than winners in the late phase of development (PND 38-42). **(D,E,G,H)** Winning index score across the early and mid phases of juvenile stage, play partners did not differ in their winning index in both males and females. **(F,I)** However, in the late phases, one partner was winning significantly more than the other in both males and females. * indicates < 0.05 .

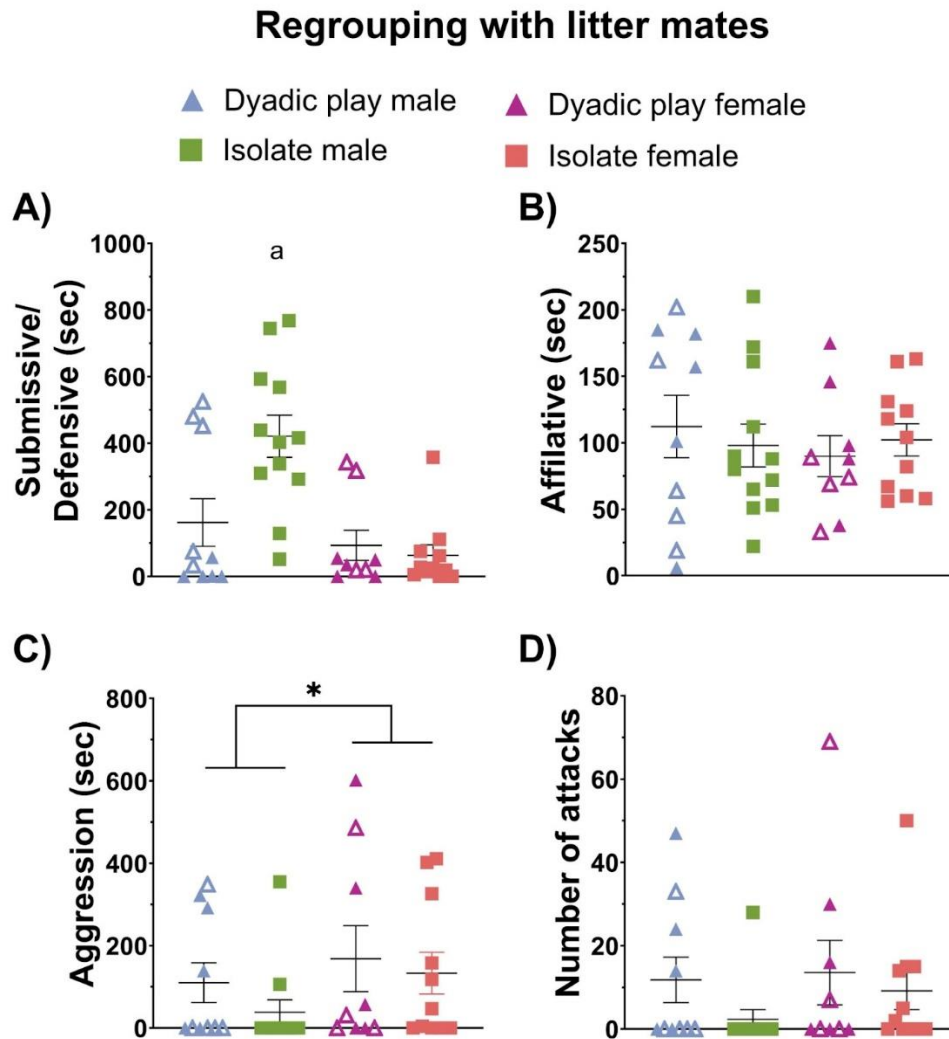


Fig 4. 4 Behaviors expressed during regrouping with littermates at PND 43. **(A)** Peer-Isolated males showed the greatest submissive & defensive behavior during regrouping. **(B)** There were no significant differences in affiliative social behavior between play treatment conditions. **(C)** While we did not find play-dependent effects on aggressive behavior during regrouping, female hamsters showed longer durations of aggressive behavior than males. **(D)** Groups did not statistically differ in the number of attacks against littermates. Graphs denote data spread and median values. Among Dyadic Play subjects, filled data points denote winners during dyadic play encounters, open data points denote losers. Error bars denote SEM. Statistical differences are denoted using bars to indicate a main effect of sex, and lower-case letters to denote significant differences from other conditions where letter “a” denotes a significant difference in Peer-Isolates compared to all other groups.

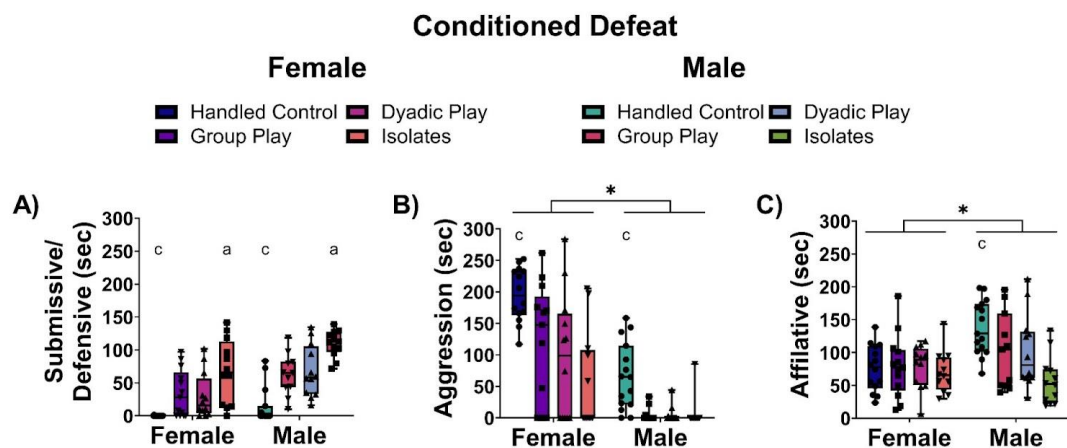


Fig 4. 5 Behavior scored during conditioned defeat testing. **(A)** Handled Control subjects showed less submissive and defensive behavior during conditioned defeat testing than other groups. Peer-Isolate subjects showed more submissive and defensive behavior than Group Play subjects. Dyadic Play subjects did not statistically differ from Peer-Isolated or Group Play hamsters. **(B)** Handled Control subjects were more aggressive than all other groups. Female subjects were also more aggressive than males. **(C)** Males showed more affiliation than females and among males Handled Controls showed more affiliation than other treatment groups. Graphs denote raw data and median values. Boxes represent standard deviations and error bars denote data range. Statistical differences are denoted using bars to indicate a main effect of sex, while lower-case letters are used to denote statistically differing groups. Letter “a” denotes significant differences in Peer-Isolates compared to Group Play and Handled Controls, and “c” denotes a main effect of defeat.

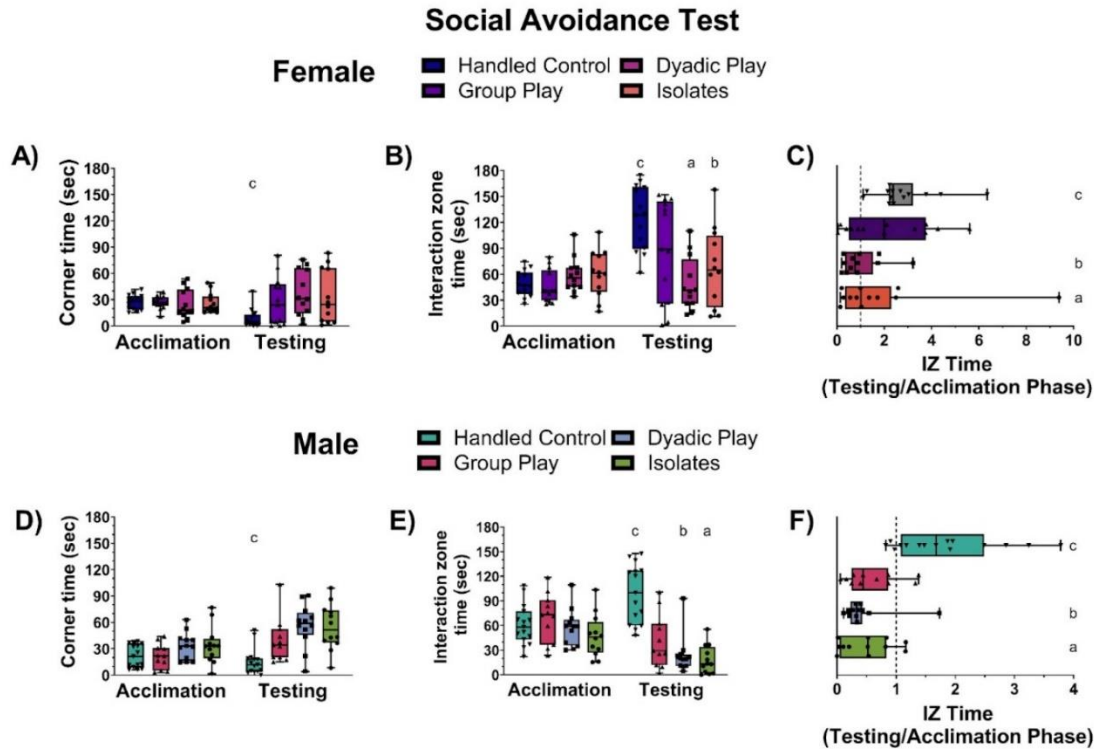


Fig 4. 6 Behavior quantified during social avoidance testing. **(A)** While Handled Controls spent less time in the corners than defeated groups during the testing phase, there were no significant differences among defeated groups. **(B)** During the testing phase, Dyadic Play and Peer-Isolated groups spent significantly less time in the interaction zone than Group Housed subjects and Handled Controls. Handled Controls also spent more time in the interaction zone than defeated groups. **(C)** Peer-Isolated and Dyadic Play Date females showed a smaller interaction ratio than Group Housed and Handled Control subjects. Handled Controls were also less avoidant than all defeated groups. **(D)** While Handled Control males spent less time in the corners than defeated groups during the testing phase, there were no significant differences among defeated groups. **(E)** During the testing phase, Dyadic Play and Peer-Isolated males spent significantly less time in the interaction zone than Group Housed and Handled Control males. Handled Controls also spent more time in the interaction zone than all defeated groups. **(F)** Peer-Isolated and Dyadic Play males showed a smaller interaction ratio than Group Housed and Handled Control subjects. Handled Controls were also significantly less avoidant than all defeated groups. Graphs denote raw data and median values. Boxes represent standard deviations and error bars denote data range. Statistical differences are denoted using bars to indicate a main effect of sex, while lower-case letters are used to denote statistically differing groups. Letter “a” denotes significant differences in Peer-Isolates compared to Group Play and Handled Controls, “b” denotes significant differences in Dyadic Play compared to Group Play and Handled Controls, and “c” denotes a main effect of defeat.

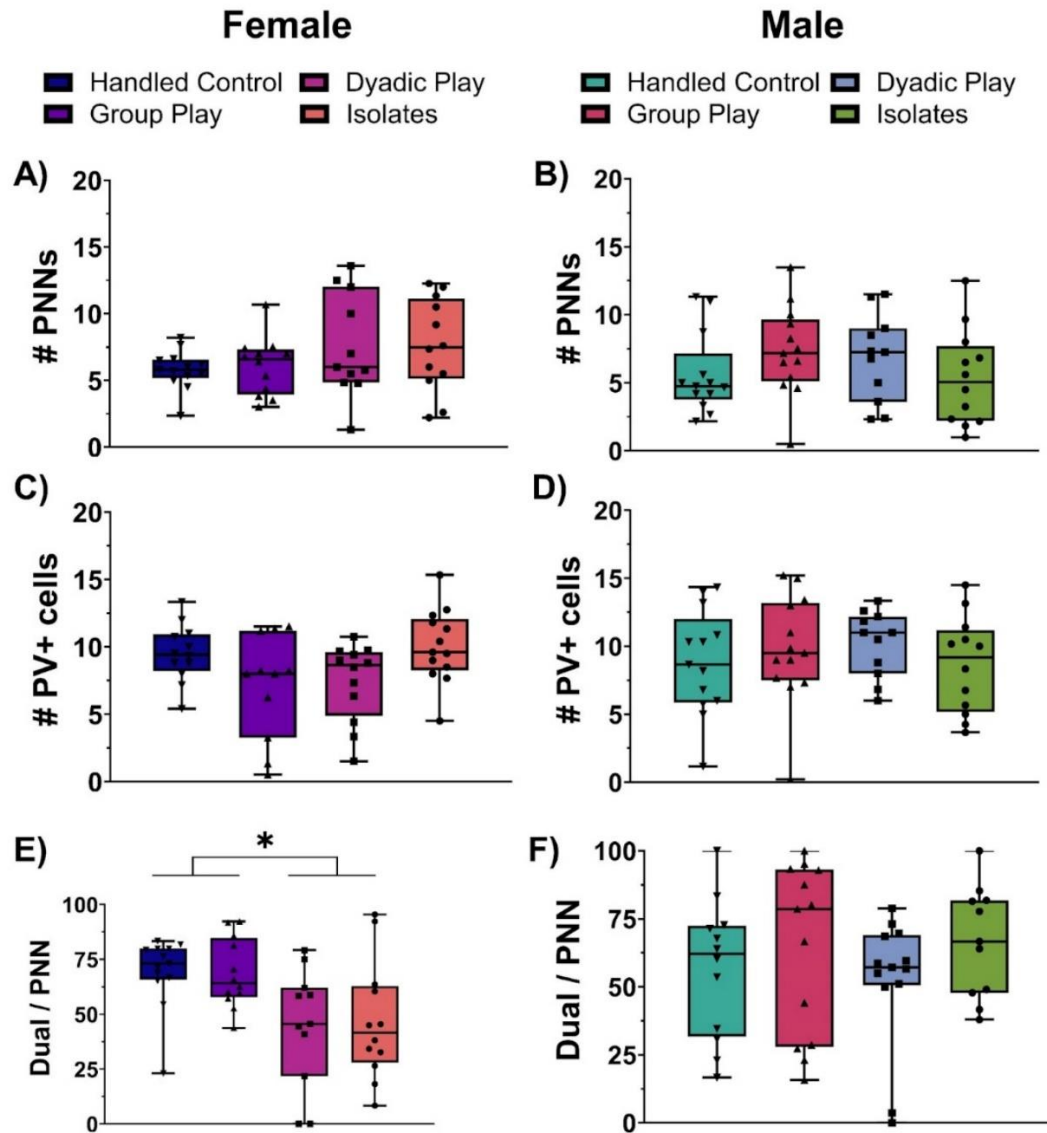


Fig 4. 7 Expression of PNNs, PV+ neurons, and the proportion of PNNs that enwrapped PV+ neurons in the PL. **(A)** Female subjects did not differ significantly in the number of PNNs in the PL. **(B)** Male subjects did not differ significantly in the number of PNNs in the PL. **(C)** Females did not differ significantly in their number of PV+ neurons in the PL. **(D)** Males did not differ significantly in their number of PV+ neurons in the PL. **(E)** Peer-Isolated and Dyadic Play females showed a significantly lower proportion of PNNs surrounding PV+ neurons in the PL. **(F)** Males did not differ significantly in the proportion of PNNs surrounding PV+ neurons in the PL. Graphs denote raw data and median values. Boxes represent standard deviations and error bars denote data range. Statistical differences are denoted using lower-case letters where letter “a” denotes significant differences in Peer-Isolates compared to Group Play and Handled Controls, “b” denotes significant differences in Dyadic Play compared to Group Play and Handled Controls.

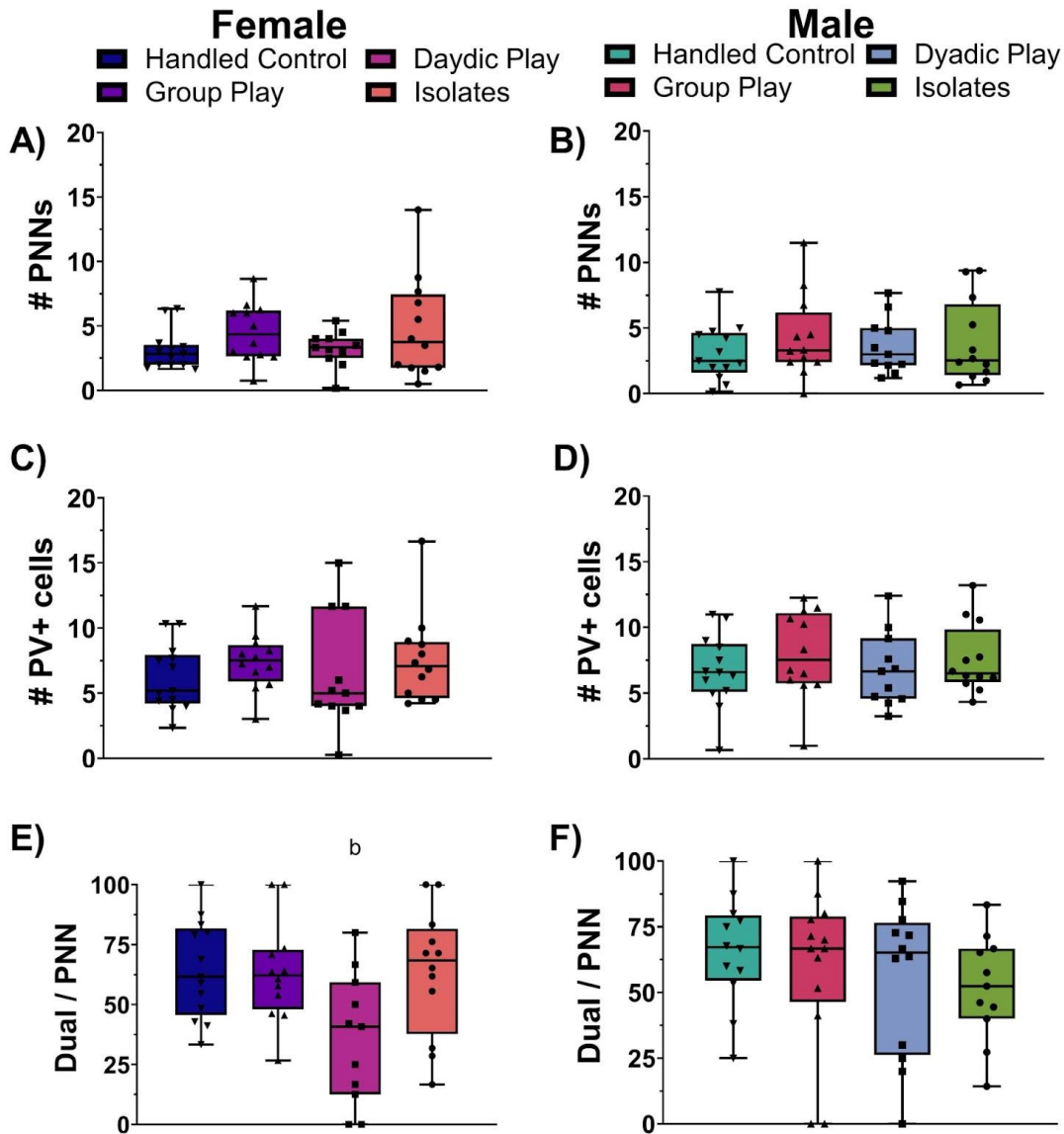


Fig 4. 8 Expression of PNNs, PV+ neurons, and the proportion of PNNs that enwrapped PV+ neurons in the IL. **(A)** Female subjects did not differ significantly in the number of PNNs in the IL. **(B)** Male subjects did not differ significantly in the number of PNNs in the IL. **(C)** Female subjects did not differ significantly in the number of PV+ neurons in the IL. **(D)** Male subjects did not differ significantly in the number of PV+ neurons in the IL. **(E)** Dyadic Play females showed a significantly lower proportion of PNNs surrounding PV+ neurons in the IL compared to other treatment groups. **(F)** Males did not differ significantly in the proportion of PNNs surrounding PV+ neurons in the IL. Graphs denote raw data and median values. Boxes represent standard deviations and error bars denote data range. Statistical differences are denoted using lower-case letters where letter “b” denotes significant differences in Dyadic Play compared to all other groups.

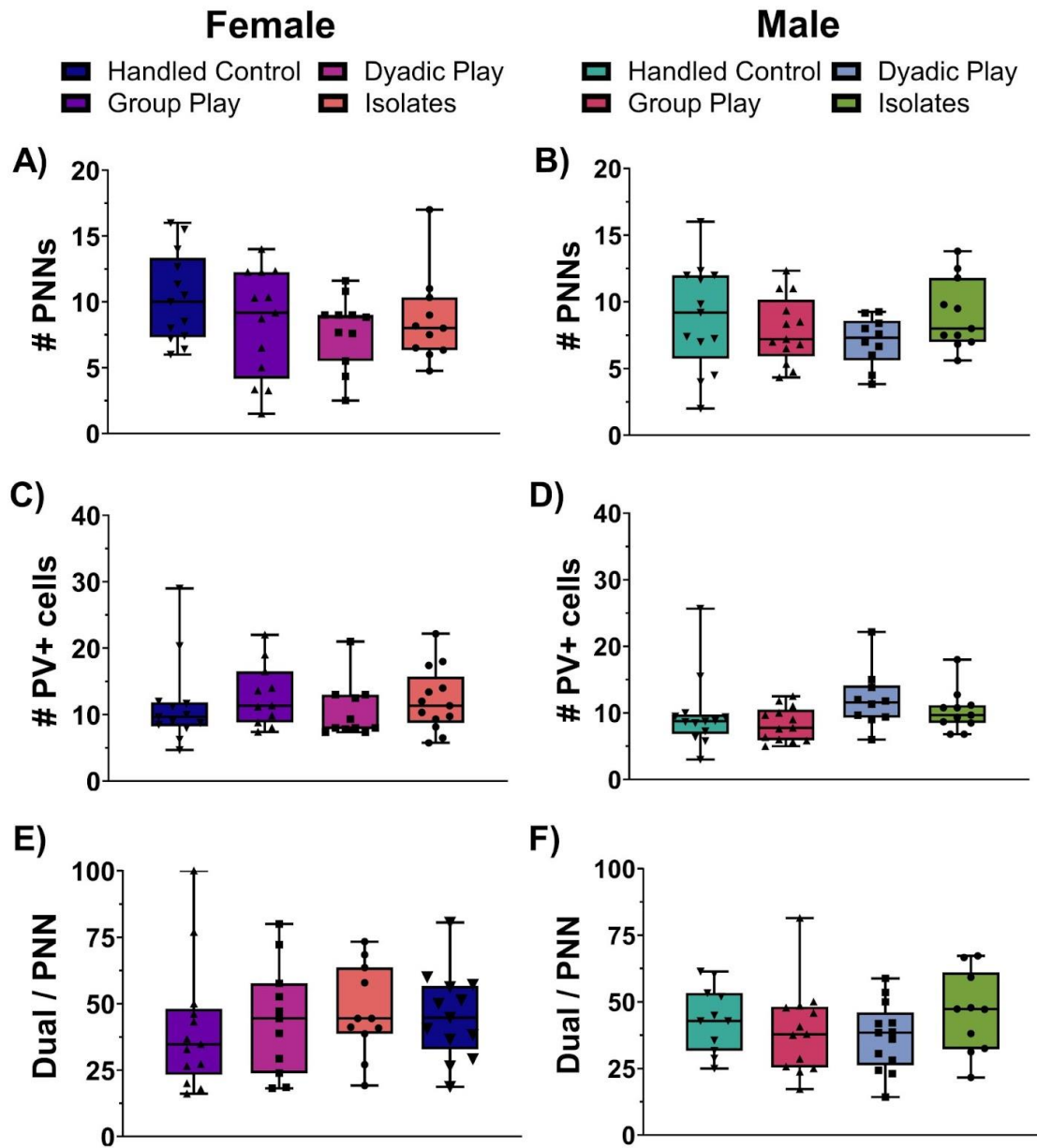


Fig 4. 9 Expression of PNNs, PV+ neurons, and the proportion of PNNs that enwrapped PV+ neurons in the BLA. **(A)** Female subjects did not differ significantly in the number of PNNs in the BLA. **(B)** Male subjects did not differ significantly in the number of PNNs in the BLA. **(C)** Female subjects did not differ significantly in the number of PV+ neurons in the BLA. **(D)** Male subjects did not differ significantly in the number of PV+ neurons in the BLA. **(E)** Females did not differ significantly in the proportion of PNN surrounding PV+ neurons in the BLA. **(F)** Males did not differ significantly in the proportion of PNNs surrounding PV+ neurons in the BLA. Graphs denote raw data and median values. Boxes represent standard deviations and error bars denote data range.

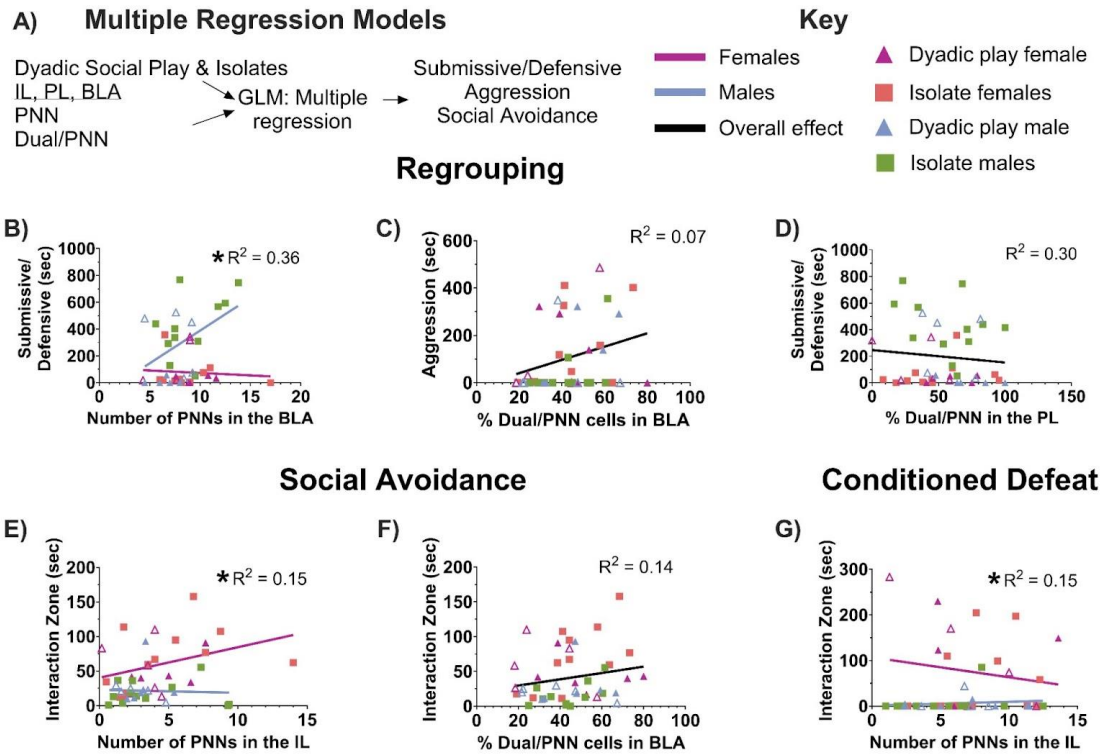


Fig 4. 10 Significant correlations between PNN expression and behavior during behavioral testing. **(A)** All data were analyzed using general linear models for multiple regression. We correlated PNN count and the proportion of PNN and PV+ co-expression in the PL, IL, and BLA with behaviors at regrouping, conditioned defeat, and social avoidance testing. Significant and trending correlations are presented here. **(B)** Among males, PNN count in the BLA was positively correlated with the duration of submissive & defensive behavior during regrouping ($R^2=0.36$). **(C)** The duration of aggressive behavior at regrouping tended to be positively correlated with the proportion of PNN and PV+ co-expression in the BLA in both males and females, though this trend did not reach statistical significance ($p=0.061$, $R^2=0.07$). **(D)** In both males and females the duration of submissive & defensive behavior during regrouping tended to be negatively correlated with PNN and PV+ co-expression in the PL, though this trend did not reach statistical significance ($p=0.073$, $R^2=0.30$). **(E)** Among females, PNN count in the IL was positively correlated with interaction zone ratio during social avoidance testing ($R^2=0.15$). **(F)** The proportion of PNN and PV+ co-expression in the BLA tended to be positively correlated with interaction zone ratio during social avoidance testing in both males, though this trend did not reach statistical significance ($p=0.068$, $R^2=0.14$). All lines denote lines of best fit, while data scatter shows individual raw data points. Among Dyadic Play subjects, filled data points denote winners during dyadic play encounters, open data points denote losers. Single lines of best fit are drawn for main effects of group, while lines for male and female data are drawn independently where interactions across levels of sex were found. Statistically significant interactions are denoted with asterisks while non-significant trends are shown without asterisks. Adjusted R^2 values are shown for each interaction.

Chapter 5 Figures & Tables

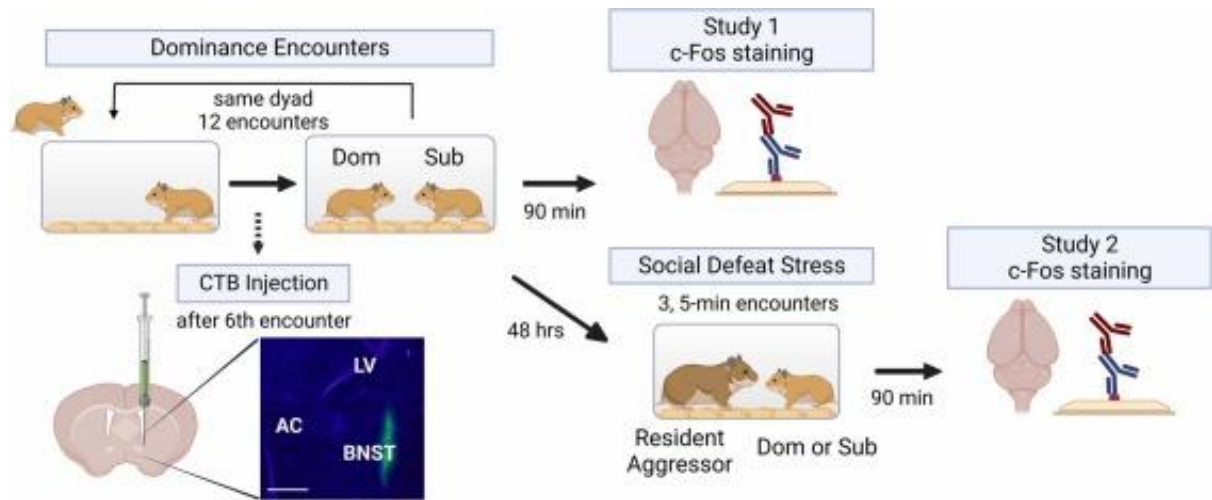


Fig 5. 1 Schematic of the experimental design. Using a resident-intruder format, hamsters were paired in 12 daily encounters in which they acquired a dominant or subordinate status. Following the 6th encounter, animals received a unilateral microinjection of CTB into the BNST. In study 1, brains were collected following the 12th dominant encounter for c-Fos staining in the MeA. In study 2, animals were exposed to acute social defeat stress prior to brain collection and c-Fos staining in the MeA. AC: anterior commissure; LV: lateral ventricle. The diagram was made in BioRender.

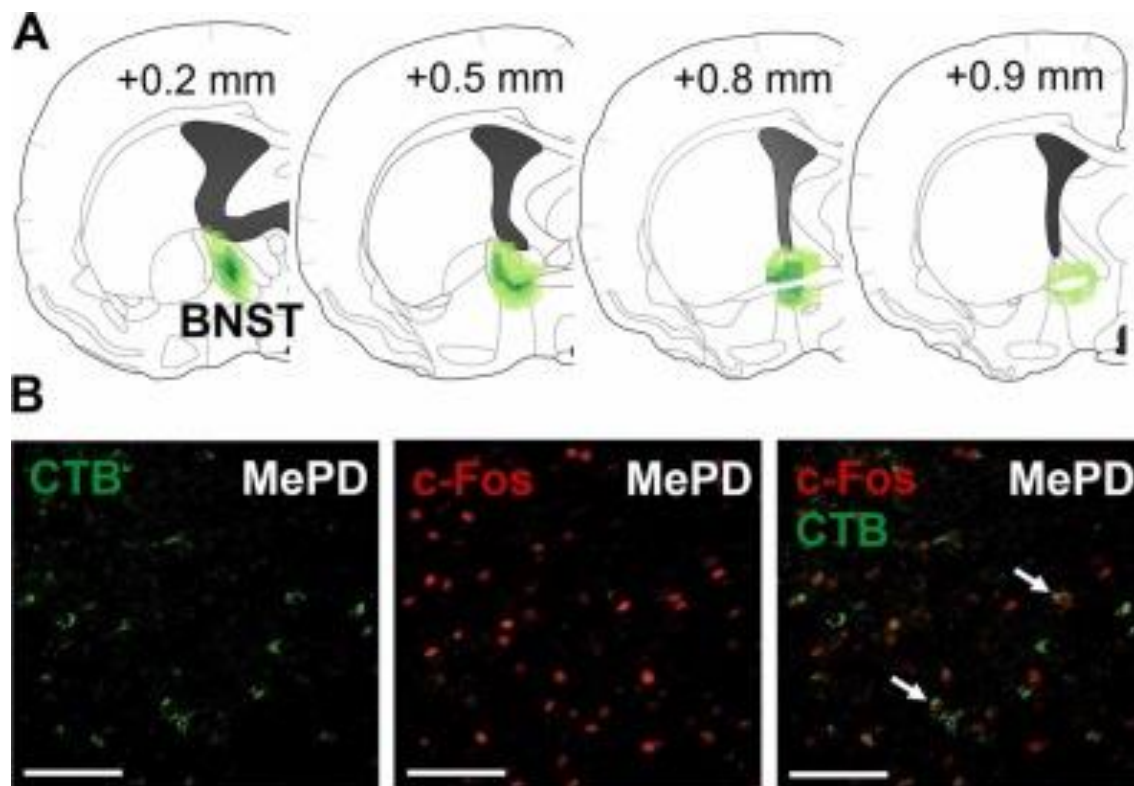


Fig 5. 2 Functional tracing of MeA afferents targeting the BNST. **(A)** Stereotaxic atlas plates showing the approximate location of the largest (light green) and smallest (dark green) spread of CTB in the BNST for animals included in statistical analysis. Coordinates on atlas plates reflect distance from bregma. **(B)** Sample photomicrographs (10x) showing CTB+ cells (green) and c-Fos+ nuclei (red) in a coronal section (40 μ m) of the MePD. Arrows denote double-labeled cells. Scale bar = 100 μ m.

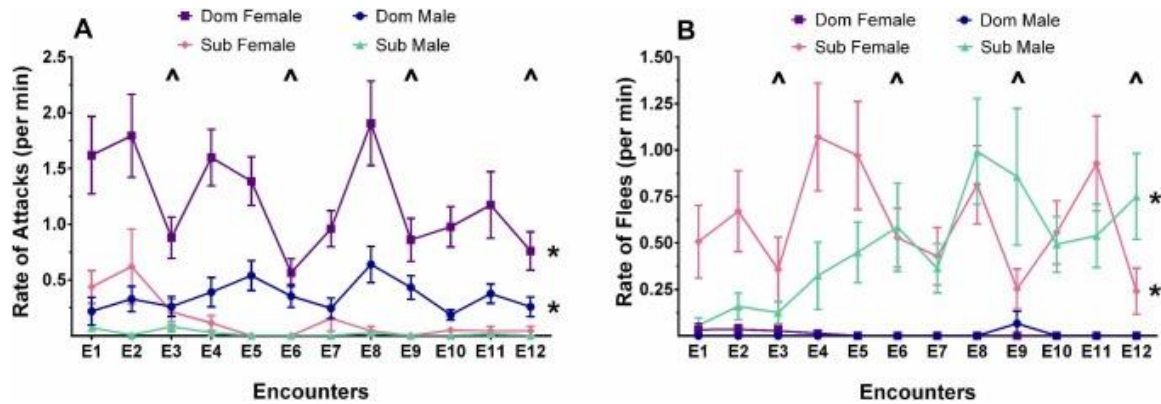


Fig 5. 3 Male and female hamsters readily form dominance relationships with same-sex partners. Animals were tested daily in 12 aggressive encounters (E). **(A)** In both males and females, dominant (Dom) animals showed a higher rate of attacks compared to their subordinate counterparts (* indicates difference compared to same-sex subordinates, $p < .05$). Female subordinates showed aggression during the first 3 encounters, while subordinate males rarely did. **(B)** In both males and females, subordinate (Sub) animals showed a higher rate of flees compared to their dominant counterparts (* indicates difference compared to same-sex dominants, $p < .05$). In males, the rate of flee increased gradually over the first 6 encounters. In females, agonistic behavior varied over the estrous cycle with the least amount of aggression and submission occurring during proestrus on E3, E6, E9, and E12 (^ indicates encounters on proestrus, $p < .05$). Dom female ($n = 15$), Sub female ($n = 15$), Dom male ($n = 15$), Sub male ($n = 15$).

Social Dominance Encounter

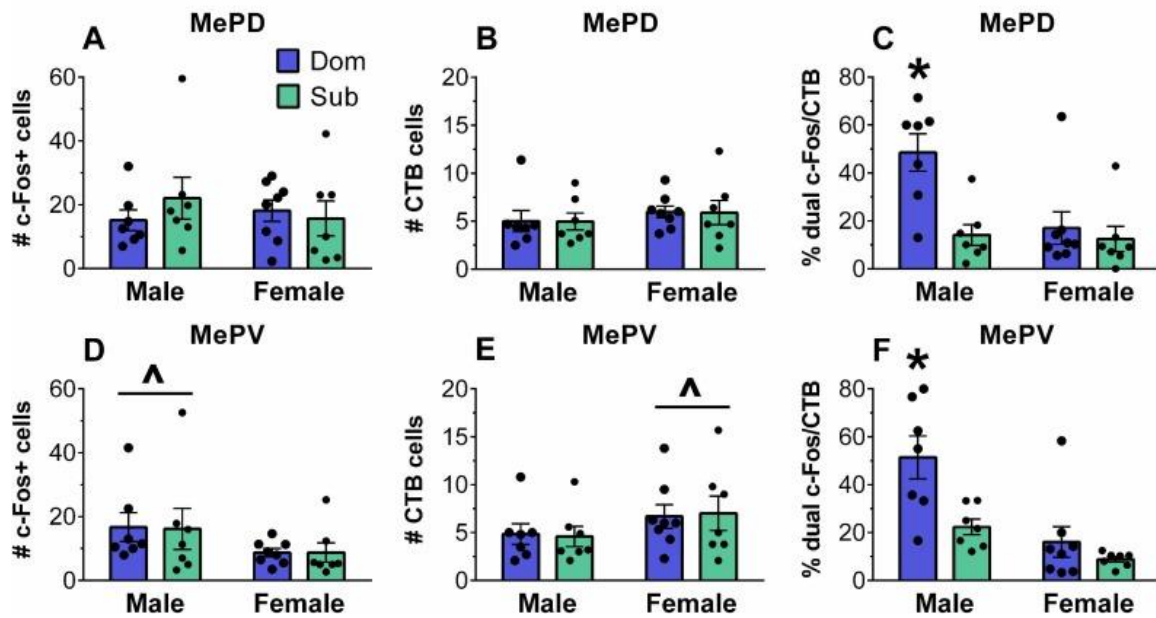


Fig 5. 4 Dominant hamsters show elevated c-Fos immunoreactivity in CTB+ cells within the MePD and MePV during the 12th dominance encounter, which occurred during proestrus for females. Dominant and subordinate animals did not differ in the number of **(A)** c-Fos+ cells or **(B)** CTB+ cells in the MePD. **(C)** Dominant males showed greater double-labeling of c-Fos+ and CTB+ cells in the MePD following their 12th dominance encounter compared to subordinate males (* indicates $p < .05$), and females show a similar trend. **(D)** Males showed a greater number of c-Fos+ cells in the MePV compared to females (^ indicates main effect of sex, $p < .05$). **(E)** Females showed a greater number of CTB+ cells in the MePV compared to males (^ indicates main effect of sex, $p < .05$). **(F)** Dominant males show greater double-labeling of c-Fos+ and CTB+ cells in the MePV after their 12th dominance encounter compared to subordinate males (* indicates $p < .05$), and females show a similar trend. c-Fos immunoreactivity was quantified during proestrus Dom female ($n = 8$), Sub female ($n = 7$), Dom male ($n = 7$), Sub male ($n = 7$).

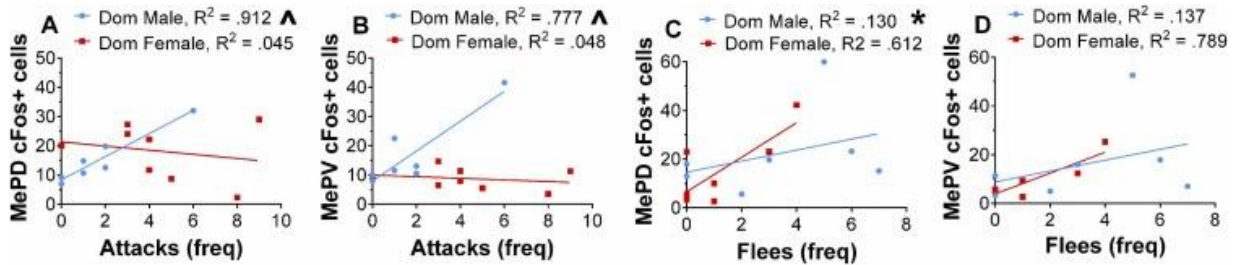


Fig 5. c-Fos immunoreactivity in the MePD and MePV was associated with both aggressive and submissive behavior during dominance encounters. **(A)** The number of c-Fos+ cells in the MePD predicted the number of attacks in dominant males but not dominant females (^ indicates interaction with sex, $p < .05$). **(B)** Similarly, the number of c-Fos+ cells in the MePV predicted the number of attacks in dominant males but not dominant females (^ indicates interaction with sex, $p < .05$). **(C)** The number of c-Fos+ cells in the MePD predicted the number of flees in both subordinate males and females (* indicates main effect, $p < .05$). **(D)** The number of c-Fos+ cells in the MePV did not significantly predict the number of flees ($p = .089$). R^2 values are shown to indicate that percentage of variance accounted for in each treatment group. Dom female ($n = 8$), Sub female ($n = 7$), Dom male ($n = 7$), Sub male ($n = 7$).

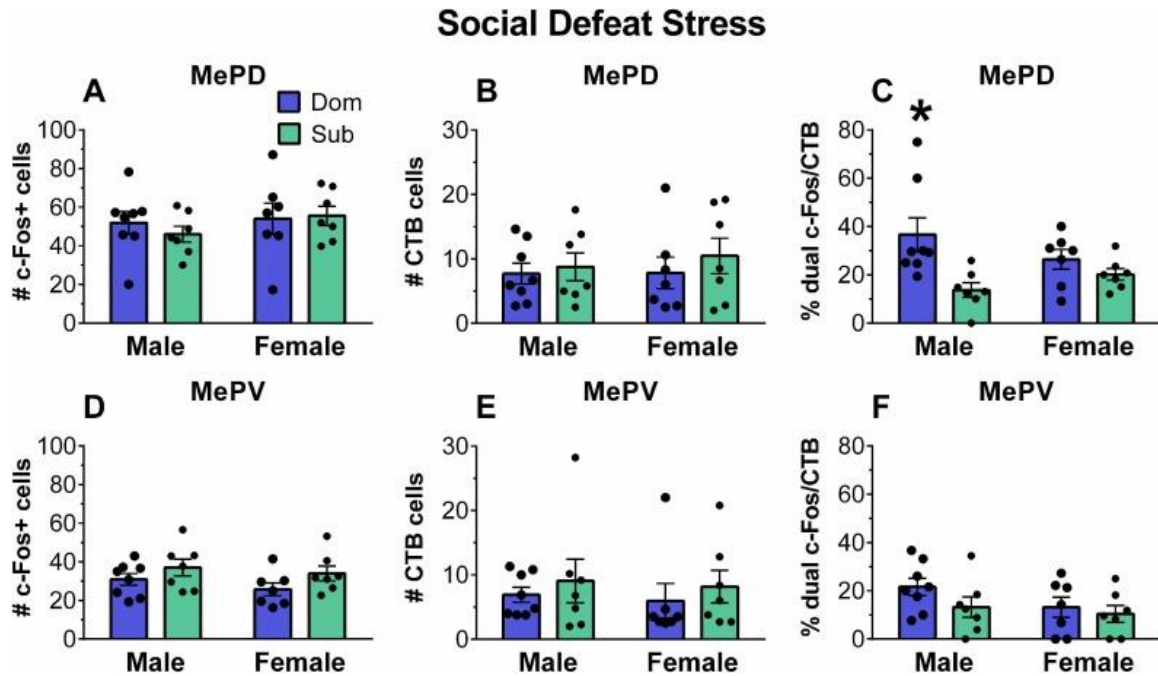


Fig 5. 6 Dominant hamsters show elevated c-Fos immunoreactivity in CTB+ cells within the MePD during social defeat stress, which occurred during Diestrus I for females. Dominant and subordinate animals did not differ in the number of **(A)** c-Fos+ cells or **(B)** CTB+ cells in the MePD. **(C)** However, dominant males showed greater double-labeling of c-Fos+ and CTB+ cells in the MePD after social defeat stress compared to subordinate males (* indicates $p < .05$), and females show a similar trend. Dominant and subordinate animals did not differ in the number of **(D)** c-Fos+ cells or **(E)** CTB+ cells in the MePV. **(F)** Dominant and subordinate animals did not differ in the proportion of double-labeled c-Fos+ /CTB+ cells in the MePV after social defeat stress. Dom female (n = 7), Sub female (n = 7), Dom male (n = 8), Sub male (n = 7).

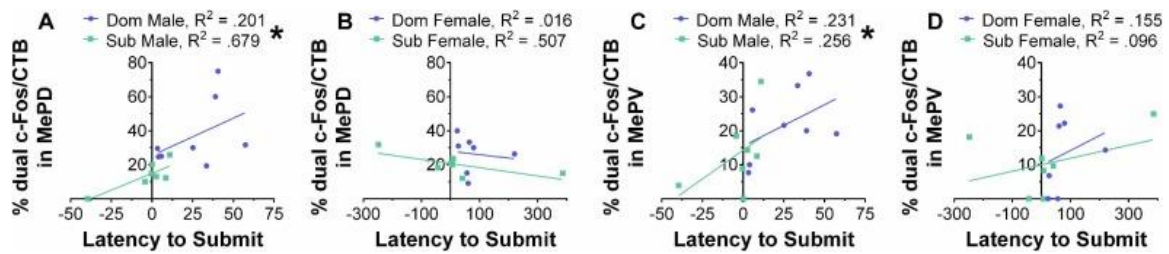


Fig 5. 7 The proportion of double-labeled c-Fos⁺/CTB⁺ cells in the MePD and MePV is associated with the relative latency to submit during social defeat in male, but not female, hamsters. The proportion of double-labeled cells in the MePD predicts the relative latency to submit in **(A)** males but not **(B)** females (* indicates main effect, $p < .05$). Similarly, the proportion of double-labeled cells in the MePV also predicts the relative latency to submit in **(C)** males but not **(D)** females (* indicates main effect, $p < .05$). R2 values are shown to indicate that percentage of variance accounted for in each treatment group. Dom female (n = 7), Sub female (n = 7), Dom male (n = 8), Sub male (n = 7).

VITA

Jeff Kelly was born in Seattle Washington and still misses the rain. Jeff's confusing and striated undergraduate career took place at North Seattle College, Western Washington University, University College Dublin, and University of Tromsø before concluding in a Bachelor of the Arts in Experimental Psychology from the University of Montana in 2016. Subsequently Jeff completed a Master of Philosophy in Behavioural Neuroscience at Liverpool John Moores University in 2019. Since 2019 Jeff has been doing their best against the best efforts of some in a PhD program at the University of Tennessee, Knoxville. Jeff will begin a position as a tenure-track assistant professor at Maryville College in August 2024.