Impacts of repeated social defeat on behavior and the brain in a cichlid fish

C. Rose Wayne¹, Avia M. Karam¹, Alora L. McInnis¹, Catherine M. Arms¹, Michael D. Kaller² and Karen P. Maruska¹,*

ABSTRACT
Social defeat is a powerful experience leading to drastic changes in physiology and behavior, many of which are negative. For example, repeated social defeat in vertebrates results in reduced reproductive success, sickness and behavioral abnormalities that threaten individual survival and species persistence. However, little is known about how neural mechanisms are involved in determining whether an individual is resilient or susceptible to repeated social defeat stress. It also remains unknown whether exclusive use of reactive behaviors after repeated social defeat is maintained over time and impacts future behaviors during subsequent contests. We used a resident–intruder experiment in the African cichlid fish *Astatotilapia burtoni* to investigate the behavior and neural correlates of these two opposing groups. Behavior was quantified by watching fish during defeat trials and used to distinguish resilient and susceptible individuals. Both resilient and susceptible fish started with searching and freezing behaviors, with searching decreasing and freezing increasing after repeated social defeat. After a 4 day break period, resilient fish used both searching and freezing behaviors during a social defeat encounter with a new resident, while susceptible fish almost exclusively used freezing behaviors. By quantifying neural activation using pS6 in socially relevant brain regions, we identified differential neural activation patterns associated with resilient and susceptible fish and found nuclei that co-varied and may represent functional networks. These data provide the first evidence of specific conserved brain networks underlying social stress resilience and susceptibility in fishes.

KEY WORDS: Status loss, PS6, Coping behavior, Social decision-making network, *Astatotilapia burtoni*, Teleost

INTRODUCTION
Group-living organisms, which comprise a diverse representation of organisms including invertebrates, fish, birds and mammals, have many social interactions with conspecifics, which can be of positive, neutral or negative valence. How individual group members respond to social stressors can have lasting effects on individual survival and species persistence. However, little is known about how neural mechanisms are involved in determining whether an individual is resilient or susceptible to repeated social defeat stress. It also remains unknown whether exclusive use of reactive behaviors after repeated social defeat is maintained over time and impacts future behaviors during subsequent contests. We used a resident–intruder experiment in the African cichlid fish *Astatotilapia burtoni* to investigate the behavior and neural correlates of these two opposing groups. Behavior was quantified by watching fish during defeat trials and used to distinguish resilient and susceptible individuals. Both resilient and susceptible fish started with searching and freezing behaviors, with searching decreasing and freezing increasing after repeated social defeat. After a 4 day break period, resilient fish used both searching and freezing behaviors during a social defeat encounter with a new resident, while susceptible fish almost exclusively used freezing behaviors. By quantifying neural activation using pS6 in socially relevant brain regions, we identified differential neural activation patterns associated with resilient and susceptible fish and found nuclei that co-varied and may represent functional networks. These data provide the first evidence of specific conserved brain networks underlying social stress resilience and susceptibility in fishes.

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Fernald, 2018). Because males can reversibly switch between dominant and subordinate status, their resilience to repeated social defeat while they are subordinate can have a profound impact on their future ability to gain a territory and rise in social rank. As such, they are an excellent system to understand which brain regions are involved in regulating resilience, susceptibility and future behavior after repeated social defeat.

Immediate early genes (IEGs) and other markers of neural activity are useful tools for measuring region-specific brain activation in neuroethological studies. Changes in the expression of IEGs, such as the transcription factors cfos and ergl, correspond to changes in the expression of many other neuronal genes caused by extracellular signals. They are commonly used to compare neural activation patterns in fishes and other vertebrates under different social or sensory conditions to evaluate where this information is processed in the brain (Butler and Maruska, 2016; Maruska et al., 2013). In contrast to more transient changes in IEGs, immunohistochemistry for phosphor-S6 ribosomal protein (pS6) stains ribosomal proteins that have been phosphorylated in the previous ∼1 h. This increased phosphorylation is tied to increased translation, and thus pS6 is emerging as another useful marker for the neural activation toolkit (Maruska et al., 2020; Ruvinsky and Meyuhas, 2006).

We used a resident–intruder experiment to expose male A. burtoni to repeated social defeat from the same aggressor, followed by a break period and defeat from a new aggressor. By allowing the intruders to be socially defeated for 4 consecutive days, we observed that most A. burtoni males initially performed a mix of searching and freezing coping behaviors, and that over 4 days, freezing behaviors increased while searching behaviors decreased. When re-exposed to social defeat after a break, fish could easily be classified as either resilient or susceptible depending on their coping behaviors: resilient fish displayed the mix of searching and freezing behaviors seen at the beginning of the experiment, while susceptible fish reduced searching and almost exclusively used freezing behaviors.

Our previous work identified a subset of brain nuclei with greater activation in fish displaying searching but not freezing behaviors after repeated social defeat, and activation in a single region (superior raphe nucleus) was associated with freezing but not searching behaviors (Butler et al., 2018). Our experiment expanded on this work by identifying which brain regions are involved in the retention of behavioral coping strategies in response to a new social defeat interaction with a novel aggressor. By comparing activation in 13 different brain regions in control, resilient and susceptible fish, we describe novel neural activation patterns that may mediate the behavioral differences seen among these groups. Additionally, in contrast to simple winner–loser effects, our experiment allowed us to gain insight into which brain regions are involved in recall of previous defeat interactions and the decisions involved in behavioral displays of future social contexts based on past experience that leads to susceptible and resilient phenotypes. Repeated social defeat can occur in all vertebrate groups, including humans, and studying the neural and molecular correlates of resilience in a social cichlid fish can provide valuable insights into the conservation of neural substrates regulating coping behaviors and the selective pressures shaping the evolution of social interactions across taxa.

**MATERIALS AND METHODS**

**Animals**

Adult laboratory-bred A. burtoni were originally derived from a wild-caught population from Lake Tanganyika, Africa, and kept in community aquaria with conditions similar to their natural environment: pH 7.8–8.0, 28–30°C, 12 h:12 h light:dark cycle, and constant aeration. Fish were fed cichlid flakes (AquaDine, Healdsburg, CA, USA) daily, supplemented with brine shrimp twice a week. Experimental male fish were selected shortly after the onset of adult-typical coloration and social behaviors (∼60 days of age). Only yellow-morph males were used as experimental animals. In addition, all fish were the largest fish in their community prior to selection and no other fish had challenged their dominance status (mean±s.e.m. standard length: 47.42±0.24 mm; body mass: 1.34±0.050 g; gonadosomatic index, GSI: 0.51±0.034 g). This ensured that experimental males had not previously experienced prolonged social conflict and that all animals had a similar social background. As such, subordinate individuals (based on behavior and appearance) were excluded, and all subject males were dominant prior to use in experiments. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA (#20-036).

**Experimental design**

To examine how A. burtoni males respond to repeated social defeat, we used a resident–intruder experiment. For experiments, a 37.85 l tank was divided into two equal-sized compartments by a clear acrylic barrier sealed to the sides and bottom of the tank that prevented transfer of any chemosensory signals. Each compartment was also visually isolated from the other using a removable opaque blue acrylic barrier inserted alongside the clear sealed barrier (Fig. 1). A single dominant male was placed in each compartment (one to serve as the resident and the other as the intruder) and allowed to acclimate for 1 day and establish a territory. Each compartment contained half of a small terracotta pot to serve as a shelter and dominant males were selected based on their display of dominance behaviors for several weeks prior to experiments as described above. Resident males were slightly larger (∼10%) than the intruder males to increase the chance of their victory over the intruder when placed together. There was no difference in standard length (ANOVA, F2,43=0.74; P=0.48) or body mass (ANOVA, F2,43=0.082; P=0.92) across conditions for resident males. A separate tank on the left (not shown in Fig. 1) containing non-gravid females and subordinate males was visible to each dominant male during acclimation and between trials to prevent social isolation stress.

To stimulate repeated social defeat, trials were conducted where intruders were exposed to the same resident once daily 4 days in a row between 14:00 h and 15:00 h. At the start of each trial, the intruder was quickly netted from his home territory, placed into the resident’s territory, and the two fish were allowed to interact for 30 min. There was no difference in standard length (ANOVA, F2,43=0.029; P=0.97), body mass (ANOVA, F2,43=1.07; P=0.35) or GSI (ANOVA, F2,43=1.54; P=0.23) among experimental intruder males (control, resilient, susceptible). After the 30 min trial, the intruder was placed back into his home territory until the subsequent day. This same sequence of moving the intruder into the resident’s territory was repeated for three more days. This design allowed for the initial territorial fight to occur on day 1. By separating trials by 1 day, the intruder could re-establish his dominance in his own compartment between successive daily trials. During trials on days 2–4, the intruder was immediately suppressed by the resident
without a true territorial fight occurring (fight criteria based on Butler and Maruska, 2015). This part of the experiment closely resembles the natural ecology of the Lake Tanganyikan lek system in which males can be exposed and suppressed by the same individuals on a regular basis (Maruska and Fernald, 2018). After 4 days of repeated social defeat by the same resident male, the resident was removed, and the intruder male given a consecutive 4 day break in his own territory. On the fourth day of this break, a new resident male was placed in the vacant territory and allowed to acclimate for 1 day before engaging in a single social defeat trial with the intruder male, as described above. This single social defeat trial with a new resident took place the day after the 4 day
break (i.e. day 9), between 14:00 h and 15:00 h. A 4 day break was chosen because it represents a biologically relevant time period for territorial interactions in this species (Fernald and Hirata, 1977; Maruska and Fernald, 2018). A 2 day break was tested but the intruder was still experiencing acute effects of defeat. A 4 day break was tested and allowed systems to return to behavioral baselines (e.g. not displaying anxiety) before experiencing a new social defeat encounter. After this final trial, the intruder was collected (see below).

For comparison with the experimental males that were exposed to 4 consecutive days of chronic social defeat as described above, we used control dominant males that were not placed into tanks with resident males but were exposed to the same holding conditions and movements as intruder males, followed by a single bout of social defeat. Control males were placed into their respective compartment and allowed to acclimate for 1 day and establish a territory before being moved into the resident’s compartment – sans resident – for the first four consecutive trials. The intruder male was then given a consecutive 4 day break in his own territory. On the fourth day of this break, a resident male was placed in his respective territory and given 1 day to acclimate before a single social defeat trial with the control male on day 9. Thus, the only difference between control males and experimental males (that resulted in resilient or susceptible phenotypes) was that they did not experience the initial 4 day repeated social defeat and just experienced a single bout of social defeat on day 9. This controlled for the stress associated with social isolation during acclimation, handling and transfer to a novel territory, and allowed us to ensure that behavior and brain differences were due to social defeat and not stress related to the experimental design itself. This also allowed us to determine whether chronic social defeat resulted in the retention of specific coping behaviors employed in future encounters with novel aggressors, or whether they were fixed before social defeat.

**Behavior analysis**

A total of 120 experimental animals were used to conduct behavioral trials: 74 were resident males and 46 were intruder males, 18 of which were control intruders (no social defeat until day 9). Behavioral data from all experimental intruder males were used to classify individuals into either susceptible (n=12) or resilient (n=16) phenotypes based on our previous study (Butler et al., 2018) (Fig. 2). Behaviors (see below) such as freezing, flee to freeze, and flinch identified resilient individuals. Conversely, behaviors such as searching, flee to search, and no response to resident aggression identified susceptible individuals. After one bout of social defeat on day 1 of the experiment, treatment fish displayed a mix of searching and freezing coping behaviors. After repeated social defeat on day 4 of the experiment, freezing behaviors increased while searching behaviors decreased. After a break period, when re-exposed to social defeat on day 9, resilient fish were classified as those that did not continue this trend, instead displaying a mix of searching and freezing coping behaviors similar to day 1 and control fish (Fig. 2A,B). Susceptible fish were classified as those that retained and continued to decrease the amount of searching behaviors displayed, resulting in almost exclusive use of freezing behaviors. To establish whether the difference in searching and freezing behaviors were sufficient for further investigation, we performed a generalized linear mixed model (logit link, binomial distribution with animal identity as a random effect) among groups. The difference between searching and freezing behaviors was significant in susceptible [LM means difference=1.01 (0.28 s.e.), \(F_{425}=3.55; P=0.0004\)], but not control [LM means difference=0.07 (0.22), \(F_{429}=0.34; P=0.732\)] and resilient [LM means difference=0.04 (0.24), \(F_{429}=0.20; P=0.843\)] fish, which were similar to one another on day 9 (Fig. 2C).

All trials were video recorded and behavioral analyses performed with BORIS software (Friard and Gamba, 2016) to quantify stereotypical aggressive behaviors of the resident and intruder (Butler et al., 2018). We quantified the number of aggressive behaviors (e.g. chases, lunges, nips, lateral displays) and time spent performing these behaviors (e.g. chase time, lateral display time), and calculated the percentage of total trial time spent performing the behaviors (e.g. % chase, % lateral display). Time was not recorded for lunges and nips because they are short duration behaviors (<1 s). Aggression score rate was calculated by dividing the total number of aggressive behaviors displayed by trial time.

For the intruder, we also quantified the number of coping behaviors (e.g. flee, freeze, search) and time spent performing these behaviors (e.g. flee time, freeze time, search time), and calculated the percentage of total trial time spent performing the behaviors (e.g. % flee, % freeze, % search). Fleeing was defined as actively trying to escape from a resident’s chase. Searching was defined as swimming perpendicular to the wall of the tank, often in the vertical plane, and had to last a minimum of 2 s. Freezing was defined as the...
intruder remaining stationary at the bottom of the tank or at the top of the water column and had to last a minimum of 2 s. To account for variation in the resident’s aggression across trials, how an intruder responded to an attack from the resident was also quantified. Intruder behaviors in response to resident attacks were divided into five categories: no response, flinch, flee to freeze, flee to search, and flee to other (e.g. regular swimming without disruption by the resident). For full definitions and criteria of all behaviors, see Table S1.

**Animal collections and tissue preparation**

After the final trial on day 9, all fish were anesthetized by gradual cooling in ice-cold chilid-system water and euthanized by rapid cervical transection. Animals were not collected following the initial territorial defeat because previous studies in *A. burtoni* have already characterized the neural correlates of social descent and territory loss (Maruska et al., 2013). Prior to euthanasia, blood was collected from the caudal vein with heparinized 100 μl capillary tubes, centrifuged at 8000 rpm for 10 min, and plasma collected and stored at −80°C until analysis. Brains were exposed and fixed in the head overnight at 4°C in 4% paraformaldehyde made in 1× phosphate buffered saline (1× PBS), rinsed for 24 h in 1× PBS, and cryoprotected overnight in 30% sucrose in 1× PBS. Brains were embedded in optimal cutting temperature (TissueTek OCT, Sakura Fine Tek, Torrance, CA, USA) media, sectioned in the transverse plane on a Thermo Scientific™ Cryostar NX50 cryostat at 20 μm, and collected onto two alternate sets of positively charged slides (VWR Superfrost plus, Chicago, IL, USA). Slides were dried at room temperature for 2 days before storage at −80°C until staining. Standard length (SL), body mass (BM) and gonad mass (GM) were measured at collection and GSI calculated as a measure of reproductive investment [GSI=(GM/BM)×100].

**Cortisol assays**

To measure plasma cortisol levels after the final trial on day 9, enzyme immunoassay (EIA) kits (500360, Cayman Chemical Company, Ann Arbor, MI, USA) were used. This EIA was previously validated and used to measure plasma cortisol levels in this species in other studies (Maruska et al., 2013; Maruska and Fernald, 2010). Plasma samples were diluted 1:45 in assay buffer without extraction. Instructions provided by the manufacturer were then strictly followed. All samples were assayed in duplicate, plates were read at 405 nm using a microplate reader (UVmax Microplate Reader, Molecular Devices, Ponway, CA, USA), and hormone levels determined based on a standard curve. All samples were run on a single plate and the mean intra-assay coefficient of variation (assessed using duplicate samples) was 13.7%.

**pS6 immunohistochemistry**

Sectioned brains of randomly selected fish previously used for behavior analysis were stained with an antibody generated against the neural activation marker phospho-S6 ribosomal protein (pS6). The pS6 marker is present in neurons that were activated within the previous ~1 h, and phosphorylation of pS6 is associated with increased translation (Knight et al., 2012; Ruvinsky and Meyuhas, 2006). In contrast to more transient IEGs, pS6 is better able to detect differences among more stable steady states of neural activity. In studies using neural activation markers such as pS6, it is also important to recognize the limitations their use may pose for interpretation. pS6 may not be expressed in all cell types, the cellular phenotype of activated neurons is not known, the relationship between pS6 expression and neural firing or changes in downstream translation are unclear, and the absence of pS6 staining is not necessarily indicative of an absence of activation (e.g. activation occurs but pS6 is not used for signaling). Many activation markers are most sensitive to novel or changing stimuli such that more stable states, as examined here, could reflect new baseline neural activity. However, while this transient response is typical for IEGs, pS6 is not an IEG and can better distinguish steady-state neural activity (Butler et al., 2018; Maruska et al., 2020). It is an informative and valuable approach to initially examine which brain regions and neurons are involved in processing salient stimuli in different contexts (York et al., 2019).

To identify pS6-labeled neurons, we performed immunohistochemistry as previously described for cfos staining (Butler et al., 2016; Butler and Maruska, 2016). Briefly, slides were brought to room temperature and tissue outlined with a hydrophobic barrier (Immedge pen; Vector Laboratories, Burlingame, CA, USA) before being rinsed in 1× PBS (3×10 min). Non-specific binding was blocked by incubating slides in 1× PBS containing 0.2% bovine serum albumin (BSA), 0.3% Triton X-100, and 5% normal goat serum (NGS) for 2 h prior to incubation in pS6 primary antibody (1:1500; prepared in blocking solution; Cell Signaling Technologies pS6 ribosomal protein S235/236 antibody 4858) overnight at 4°C. Slides were then rinsed in 1× PBS (3×10 min), incubated in biotinylated goat anti-rabbit secondary antibody (Vector Labs BA-1000; 1:277; prepared in 1× PBS with 5% NGS) for 2 h at room temperature, rinsed in 1× PBS (3×10 min), endogenous peroxidases quenched with 1.5% hydrogen peroxide for 8 min, rinsed in 1× PBS (3×10 min), incubated with Vectastain Avidin Biotin Complex (ABC Elite kit, Vector Laboratories) prepared in 1× PBS for 2 h, and rinsed in 1× PBS (3×10 min). Staining was visualized by reaction with diaminobenzidine (DAB Substrate kit, Vector Laboratories) for ~30 min. Slides were then rinsed in distilled water, dehydrated in an alcohol series, cleared in xylene, and coverslipped with Cytoseal-60 (Richard-Allan Scientific, San Diego, CA, USA). To verify antibody specificity, a pre-absorption control was run simultaneously on another set of sectioned brain slides and showed no reaction product.

**Imaging and analysis**

To quantify pS6 staining, slides were visualized on a Nikon Eclipse Ni microscope controlled by Nikon Elements software and images were taken at the highest magnification that encompassed the entire region of interest (ROI), and borders and gridlines were applied. Boxes were randomly selected (3–6 depending on ROI size) and the number of stained cells within those boxes was quantified. The density of pS6-stained cells was calculated as the number of stained cells divided by the total area of the boxes quantified. For reach region, 3–4 consecutive sections were quantified (dependent on region, but consistent across animals) at the same rostral–caudal location within the nucleus across animals and averaged together for each animal to obtain a mean pS6-stained cell density per brain region (number of cells per mm²). Brightfield and phase contrast was used to visualize cytoarchitecture and brain nuclei in relation to pS6-labeled cells. A Cresyl Violet stained *A. burtoni* reference brain, *A. burtoni* brain atlas and other relevant papers (Fernald and Shelton, 1985; Maruska et al., 2017; Maruska and Fernald, 2010; Munchrath and Hofmann, 2010) were used for identification of neuroanatomical structures. Quantification was performed by individuals blind to experimental group. We quantified activation in 13 brain regions, including but not limited to the social decision-making network: two subdivisions...
(rostral and caudal) of the ventral part of the ventral telencephalon (Vv-r, Vv-c), supramissomissural nucleus of the ventral telencephalon (Vv), central part of the ventral telencephalon (Vc), dorsal part of the ventral telencephalon (Vd), lateral part of the dorsal telencephalon (Dl-v2), medial part of the central part of the dorsal telencephalon (Dc-4, Dc-5), anterior tuberal nucleus (Atn), periventricular nucleus of the posterior tuberculum (TPp), parvocellular preoptic nucleus, anterior part (nPpa) and the superior raphe nucleus (SR).

**Statistical analysis**

All analyses were performed in SigmaPlot 12.3, SAS 9.4 and SPSS 25. Measures of physiology (e.g. standard length, body mass and GSI) were analyzed using one-way ANOVA followed by Tukey’s post hoc testing. Assumptions of ANOVA were evaluated by examination of residuals. A linear general mixed model (GLMM; log link, negative binomial probability) was used to compare the interaction of treatment and behavior for all intruder males. Behavior was used as a within-subject fixed effect, treatment group as a between-subject fixed effect, resident aggression score as a fixed covariate, and animal identity as a random effect. Resident aggression score, while not significantly different across treatment groups, was used as a covariate in the model because there was natural variation in the levels of aggression displayed by different resident males, which could influence intruder behaviors. We also used a discriminant function analysis (DFA); in SPSS on all coping behaviors for intruder males (the behavior ‘flee to other’ was excluded from statistical analysis because of the absence of variance within the dataset) to examine whether they could be classified into their respective control, susceptible or resilient groups based on behavior alone.

A GLMM (log link, negative binomial probability) was used to compare the interaction of group (control, resilient, susceptible) and brain region on pS6 expression in SAS. We also used a DFA on pS6 cell densities in all 13 quantified brain regions to examine whether males could be classified into their respective control, susceptible or resilient groups based on neural activation patterns alone. Pearson correlation coefficients were used to generate heatmaps of coactivation across brain regions for each treatment group, with significant correlations. K-means cluster analysis was performed to examine whether pS6 expression overlapped among treatment groups using SAS. The k-means cluster analysis examined the best supported number of groups based on individual fish pS6 expression across regions. The hypothesis that pS6 differed as a result of treatment would be supported by evidence that individual fish could be assigned by the analysis to their treatment group. For all pS6 analyses, we chose not to correct P-values for multiple tests because these tests can lead to misleading biological interpretations as a result of increases in Type II errors and reduced statistical power for pS6 expression data (Midway et al., 2020; Nakagawa, 2004). All raw data and detailed protocols are available upon request from the corresponding author.

**RESULTS**

**Behavior**

The use of coping behaviors changed over the course of repeated social defeat and differed between control, resilient and susceptible groups. On day 1, there was a significant relationship between behavior (GLMM, $F_{0,259}=52.77; P<0.0001$) and the interaction of group with behavior (GLMM, $F_{0,259}=3.93; P<0.0001$). One behavior, no response to resident aggression [GLMM, LM means difference=$-3.05$ (0.52 s.e.), $F_{259}=5.80; P=0.0001$] showed a significant difference between resilient and susceptible males (Table 1; Table S2). There were no significant differences among groups for any other coping behaviors on day 1.

On Day 4, there was a significant relationship between behavior (GLMM, $F_{0,259}=46.98; P<0.0001$) but not the interaction of group with behavior (GLMM, $F_{0,259}=0.78; P=0.634$). One behavior, number of searches [GLMM, LM means difference=$5.47$ (0.70 s.e.), $F_{259}=2.20; P<0.029$] showed a significant difference between resilient and susceptible males (Table 1, Table S2). There were no significant differences among groups for any other coping behaviors on Day 4.

On day 9, there was a significant relationship between behavior (GLMM, $F_{0,259}=88.45; P<0.0001$) and the interaction of group with behavior (GLMM, $F_{18,429}=4.09; P<0.0001$). Three behaviors, number of searches [GLMM, LS means difference=$0.73$ (0.26 s.e.), $F_{259}=2.79; P=0.0054$], flee to searches [GLMM, LS means difference=$0.98$ (0.29 s.e.), $F_{259}=3.33; P=0.0099$] and no response to resident aggression [GLMM, LS means difference=$-1.60$ (0.31 s.e.), $F_{259}=-5.11; P<0.0001$], showed significant differences between control and susceptible males. These same behaviors, number of searches [GLMM, LS means difference=$0.93$ (0.26), $F_{259}=3.51; P=0.0005$], flee to searches [GLMM, LS means difference=$0.71$ (0.30 s.e.), $F_{259}=2.34; P=0.020$] and no response to resident aggression (GLMM, LM means difference=$-1.85$ (0.35 s.e.) $F_{259}=-5.24; P<0.0001$), also showed significant differences between resilient and susceptible males (Table 1; Table S2).

To explore the timing of three coping behaviors that differed between susceptible males versus control and resilient males, we used raster plots to depict individual displays of behaviors including searches, freezes and no response to resident aggression over the course of the day 9 trial (Fig. 3A). Susceptible males displayed flee to search behavior significantly less than control and resilient males (Fig. 3B). Susceptible males displayed flee to search behavior significantly less than control and resilient males (Fig. 3C). Susceptible males displayed no response to resident aggression behavior significantly more than control and resilient males (Fig. 3D). There were no significant differences among groups for any other coping behaviors on day 9.

Intruder aggressive behaviors were excluded from statistical analysis because of the absence of sufficient variance within the dataset. Intruder aggression score (number of aggressive behaviors divided by trial time) did not significantly differ across treatment groups (ANOVA, $F_{2,43}=1.18; P=0.32$). Resident aggression score (number of aggressive behaviors divided by trial time) did not significantly differ across treatment groups (ANOVA, $F_{2,43}=0.16; P=0.85$), and resident aggression did not predict which intruders were classified as susceptible or resilient.

On day 1, the behavior DFA produced two significant functions. Function 1 explained 97.7% of the variance in the data (Eigenvalue=$40.18$; Chi-square=$160.13; P<0.001$), and clearly separated control males from resilient and susceptible males. Function 1 was most positively correlated with the number of searches. Function 2 explained the remaining 2.3% of the variation in the data (Eigenvalue=$0.95$; Chi-square=$24.24; P=0.027$) and separated resilient from susceptible males. Function 2 was strongly negatively correlated with the no response to opponent bites behavior. The DFA for day 1 correctly classified 89.1% of fish into their respective groups, indicating that coping behaviors were different among the three groups and each group could be predicted by behavior (Fig. 4A).
On day 4, the behavior DFA produced two significant functions. Function 1 explained 99.3% of the variance in the data (Eigenvalue=140.31; Chi-square=207.93; \( P=0.001 \)), and clearly separated control males from resilient and susceptible males. Function 1 was most positively correlated with the number of freezes. Function 2 explained the remaining 0.7% of the variation in the data (Eigenvalue=0.95; Chi-square=24.75; \( P=0.016 \)) and separated resilient from susceptible males. Function 2 was strongly positively correlated with flee time, amount of flee to freeze, and amount of flinching. The DFA for day 4 correctly classified 89.1% of fish into their respective groups, indicating that, as on day 1, coping behaviors were different among the three conditions and each condition could be predicted by behavior (Fig. 4B).

On day 9, the behavior DFA produced one significant function. Function 1 explained 78.4% of the variance in the data (Eigenvalue=2.23; Chi-square=59.41; \( P=0.001 \)), and clearly separated susceptible males from control and resilient males, suggesting that social defeat has a lasting impact on the coping behaviors of the susceptible phenotype. Function 1 was strongly positively correlated with freeze time and negatively correlated with search time and number of searches. Because susceptible and resilient groups were defined by day 9 behavior, this result was somewhat expected. Although not reaching significance, function 2 (Eigenvalue=0.61; Chi-square=17.21; \( P=0.24 \)) explained the remaining 21.6% of the variation in the data and separated control males from resilient males. Function 2 was strongly positively correlated with search time and number of searches, and negatively correlated with number of lateral displays. The DFA for day 9 correctly classified 82.6% of fish into their respective groups, clearly distinguishing the coping behavior of susceptible fish as different from that of resilient fish and controls after repeated social defeat (Fig. 4C). There were no differences in circulating cortisol levels between control, resilient and susceptible males after collection on day 9 (ANOVA, \( F_{2,31}=2.09; P=0.14 \) (Table S3)).

**Neural activation**

We analyzed pS6 activation in 13 brain regions in control (\( n=7 \)), resilient (\( n=8 \)) and susceptible (\( n=6 \)) intruder males after day 9 of the experiments. There was no difference in standard length (ANOVA, \( F_{2,18}=1.82; P=0.19 \)), body mass (ANOVA, \( F_{2,18}=2.75; P=0.091 \)) or GSI (ANOVA, \( F_{2,18}=1.63; P=0.21 \)) among experimental intruder males (control, resilient, susceptible) stained for pS6. There was also no difference in standard length (ANOVA, \( F_{2,18}=2.34; P=0.13 \)) or body mass (ANOVA, \( F_{2,18}=0.22; P=0.81 \)) across conditions for resident males. Animals used for brain analysis were verified to match the same behavior patterns described above (Fig. S1, Table S4).

The density of pS6-expressing cells differed by brain region (GLMM, \( F_{12,229}=65.32; P=0.0001 \)) and group (GLMM, \( F_{2,2}=3.29; P=0.043 \)). There were no specific brain regions that showed differential expression among control, resilient or susceptible groups (Table S5). To further examine how neural activation differed across groups, we performed a DFA with pS6 densities in all 13 quantified brain regions. The DFA produced one significant function (Eigenvalue=17.12; Chi-square=49.99; \( P=0.003 \)), explaining 87% of the variance in the data. Function 1 clearly separated control males from resilient and susceptible males and was most positively loaded by the periventricular nucleus of the posterior tuberculum (TPp). Although not reaching significance, function 2 (Eigenvalue=5.26; Chi-square=15.22; \( P=0.23 \)) explained the remaining 13% of the variation and separated resilient males from susceptible males. Function 2 was strongly positively loaded by the rostral part of the ventral telencephalon (Vv-r). The DFA correctly classified 100% of all animals. Together, these data indicate that brain activation patterns were different among the three conditions and each condition could be predicted by overall neural activation patterns across the 13 nuclei examined (Fig. 5).

To examine the connectivity of each brain region in control, resilient and susceptible males after repeated social defeat, we generated a heatmap based on Pearson correlation coefficients of pS6 cell densities (Fig. 6; see Table S6 for statistical values). Overall, we found that each heatmap revealed a unique functional network of co-activation, which was absent in other treatment groups. Each group had two significant clusters. The first consisted of the nPPa and Vv-r. The second consisted of the remaining SDMN nuclei (Dm-3, Dl-v2, Dc-4, Dc-5, Vv-c, ATn, TPp, SR, Vc, Vd, Vs). However, there was little overlap among significantly correlated regions across control, resilient and susceptible males. For example, in control fish, the nPPa, Dc-4, Dc-5, Vv-c and Vs were positively correlated (Fig. 6A). In resilient males, the nPPa, Dc-4, ATn, Vd and Vs were positively correlated (Fig. 6B). In susceptible males, the nPPa, Dl-v2, Dc-4, Dc-5, Vv-c, ATn, Vd, Vc, and Vs were positively correlated (Fig. 6C). This emphasizes the complexity of regulating coping behaviors after repeated social defeat in *A. burtoni* males.

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**Table 1. Effects of social defeat on intruder male coping behaviors**

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Day 1: Resilient versus susceptible</th>
<th>Day 4: Resilient versus susceptible</th>
<th>Day 9: Control versus resilient</th>
<th>Day 9: Control versus susceptible</th>
<th>Day 9: Resilient versus susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flee to freeze</td>
<td>259</td>
<td>-0.35</td>
<td>0.7253</td>
<td>259</td>
<td>-0.64</td>
</tr>
<tr>
<td>Flee to search</td>
<td>259</td>
<td>-0.69</td>
<td>0.4924</td>
<td>259</td>
<td>0.56</td>
</tr>
<tr>
<td>Flee</td>
<td>259</td>
<td>-0.60</td>
<td>0.5489</td>
<td>259</td>
<td>-0.40</td>
</tr>
<tr>
<td>Flee time (%)</td>
<td>259</td>
<td>-0.47</td>
<td>0.6764</td>
<td>259</td>
<td>-0.16</td>
</tr>
<tr>
<td>Flinch</td>
<td>259</td>
<td>-0.90</td>
<td>0.3666</td>
<td>259</td>
<td>0.07</td>
</tr>
<tr>
<td>Freeze</td>
<td>259</td>
<td>0.99</td>
<td>0.3224</td>
<td>259</td>
<td>1.20</td>
</tr>
<tr>
<td>Freeze time (%)</td>
<td>259</td>
<td>0.08</td>
<td>0.9328</td>
<td>259</td>
<td>0.34</td>
</tr>
<tr>
<td>No response</td>
<td>259</td>
<td>0.10</td>
<td>0.9773</td>
<td>259</td>
<td>0.32</td>
</tr>
<tr>
<td>Search</td>
<td>259</td>
<td>0.92</td>
<td>0.3594</td>
<td>259</td>
<td>2.20</td>
</tr>
<tr>
<td>Search time (%)</td>
<td>259</td>
<td>0.58</td>
<td>0.5635</td>
<td>259</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Bold indicates statistical significance at \( P<0.05 \). The behavior 'flee to other' was excluded from statistical analysis because of the absence of variance within the dataset. See Materials and Methods and Table S1 for explanations of behaviors. Sample sizes: \( n=18 \) control; \( n=16 \) resilient; \( n=12 \) susceptible.**
In this study, we found that individual male *A. burtoni* exposed to repeated social defeat could be classified as either resilient or susceptible, defined by their use of different coping behaviors. We identified differential neural activation patterns associated with each of these groups and found nuclei that co-varied and may represent functional networks. Discriminant function analysis revealed a significant function that separated resilient, susceptible and control fish from one another based on neural activation alone, suggesting different neural circuitry may be involved for different coping styles.

To better understand the behavioral and neural underpinnings of chronic social defeat across the animal kingdom, and its evolution, it is important to examine diverse species that experience defeat in natural contexts. In *A. burtoni*, individuals are constantly exposed to social rank dynamics and encounter the same individuals on a regular basis. Animals of lower rank may be chronically (continually, with no chance to rise in rank) or repeatedly (intermittently, with the potential to rise in rank between each defeat) defeated. Dominance is the default state in isolated males, and if a dominant male is absent or removed from a population, a subordinate male will rapidly transition to the dominant phenotype within minutes (Maruska and Fernald, 2013). If a dominant male is subjected to social defeat, often by a larger, more dominant fish, it will rapidly lose its coloration and transition into the subordinate phenotype (Maruska and Fernald, 2010). Subordinate males have smaller GnRh1 neurons (Davis and Fernald, 1990), lower plasma levels of gonadotropins and sex-steroid hormones (Maruska et al., 2011), and smaller testes (Davis and Fernald, 1990; Maruska and Fernald, 2011) compared with dominant males. The behavioral and neural data from this experiment, therefore, may only generalize to dominant males that experience chronic social defeat. Fish in our experiment were exposed to repeated social defeat by the same resident aggressor during days 1–4 and a new resident aggressor after a 4 day break on day 9, but were not suppressed into maintaining a true subordinate phenotype, and returned to

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**DISCUSSION**

In this study, we found that individual male *A. burtoni* exposed to repeated social defeat could be classified as either resilient or susceptible, defined by their use of different coping behaviors. We identified differential neural activation patterns associated with each of these groups and found nuclei that co-varied and may represent functional networks. Discriminant function analysis revealed a significant function that separated resilient, susceptible and control fish from one another based on neural activation alone, suggesting different neural circuitry may be involved for different coping styles.

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territoriosity in their home compartments between daily trials. This provided an opportunity to study the neural correlates of social defeat in an ecologically relevant setting and provides insight into how individuals within a social hierarchy might behave in future social contexts that impact their well being and reproductive fitness.

After repeated social defeat, intruder fish were classified as either resilient or susceptible based on their coping styles. Coping styles are a set of individual behavioral and physiological responses to stress that are consistent over time (Koolhaas, 2008; Koolhaas et al., 2007; Overli et al., 2007). As they are shaped by evolution to form general adaptive response patterns to everyday challenges such as social defeat, coping styles are an important factor in determining how populations and individuals respond to stress (Bell, 2007a; Sih et al., 2004). Two distinct styles are typically recognized (proactive and reactive) and have been demonstrated in a wide variety of species, including primates, rodents, birds, fishes and insects (Bell, 2007b; Réale et al., 2007; Sih et al., 2004). Behaviorally, proactive animals are bolder and more aggressive, and are characterized by low HPA axis reactivity, low brain serotonergic activity, high brain dopaminergic activity and low post-stress cortisol levels. Reactive animals have the opposite behavioral and physiological profile (Koolhaas, 2008; Koolhaas et al., 2007; Korte et al., 1996; Veenema et al., 2007).

Fish that experienced social defeat on day 1 initially employed a combination of searching and freezing coping behaviors (a proactive and a reactive behavior, respectively). When exposed to repeated social defeat, male behavior changed over time, where searching behaviors decreased and freezing behaviors increased by day 4. Similar results were found in a previous social defeat study in *A. burtoni*, where defeated individuals were also found to switch from a mix of coping behaviors to an almost exclusive use of freezing behaviors (Butler et al., 2018). What remained unknown was whether the exclusive use of freezing behaviors would be maintained over time and impact future behavior during subsequent contests. We observed that defeat by a new resident aggressor after a break period resulted in individuals that could easily be classified as resilient or susceptible based on whether or not they retained the same coping behaviors seen on day 4. Resilient fish returned to a mix of searching and freezing behaviors, while susceptible fish exclusively used freezing behaviors.

Approximately one-third of intruder fish were classified as susceptible. Across taxa, including humans, not every individual who experiences trauma or stress will develop psychological disorders such as depression, anxiety or post-traumatic stress disorder (PTSD), and we found the percentage of individuals classified as either resilient or susceptible to be similar to previous studies (Flandreau and Toth, 2018; Richter-Levin et al., 2019). Susceptible male *A. burtoni* significantly reduced the number of searches and flee to searches, and significantly increased the number of no responses to resident aggression compared with resilient and control males. Similar results have been found in rodents, where susceptible individuals exhibit depression-like symptoms such as anhedonia, social avoidance, locomotor changes and metabolic changes (Bondar et al., 2018; Hollis and Kabbaj, 2014; Huhman et al., 2003). As the social and physical environment of *A. burtoni* is dynamic, resiliency after social defeat is advantageous when an individual must encounter frequent loss and gain of territory ownership. When bitten by a resident aggressor, fish normally display a physical response in the form of aggression, which can be detrimental to their well-being.

**Fig. 4.** Discriminant function analysis (DFA) of intruder male coping behaviors on multiple days of the experiment clearly separated control, resilient and susceptible fish. Data from day 1 (A), day 4 (B) and day 9 (C) indicate that each treatment group was associated with specific styles of coping behaviors after repeated social defeat. On day 9, the DFA correctly classified >80% of fish into their respective groups solely on behavior responses, and clearly distinguished susceptible fish as different from resilient and control fish along function 1. Sample sizes: n=18 control; n=16 resilient; n=12 susceptible. Stars indicate mean response.

**Fig. 5.** Male fish could also be categorized into their treatment groups based on neural activation patterns alone. Discriminant function analysis of pS6 staining in all brain regions clearly separated control, resilient and susceptible fish, indicating distinct neural activation patterns associated with each group. The DFA correctly classified 100% of fish into their respective groups solely on neural activation. Sample sizes: n=7 control; n=8 resilient; n=6 susceptible. Stars indicate mean response.
of flinching. However, most susceptible fish did not flinch or react at all, instead remaining frozen and allowing the resident to continue to attack them without trying to escape. Therefore, this coping style may impair the susceptible individual’s ability to respond to social defeat, and negatively impact their well-being and reproductive fitness.

It is possible that behavioral differences between resilient and susceptible fish are due to differences in physiology. In this study, however, we found no difference in plasma cortisol levels between control, resilient and susceptible fish. This result was unexpected as dysregulation of the HPA axis, which controls the amount of circulating cortisol, is found across taxa after chronic social defeat (Huhman, 2006; Keeney et al., 2006). However, individual stress responses and HPA reactivity are highly variable in A. burtoni (Clement et al., 2005). In addition, recent research suggests that there are several types of dominant males, which may have different behavioral and hormonal profiles (Alcazar et al., 2016). We found individual variation in plasma cortisol within each group, suggesting that variation in HPA activity and differences in hormonal profiles do not strongly predict which fish are classified as either resilient or susceptible in A. burtoni.

To investigate the neural mechanisms governing behavioral responses to social defeat that might differ between resilient and susceptible fish, we used pS6 as a marker of neural activation in the nPPa of the SDMN. Based on previous work, we expected to see differences in neural activation in specific regions such as the superior raphe nucleus (SR); however, none were found (Butler et al., 2018). Although we did not find differences among groups in pS6 expression in any individual brain region, we revealed that neural co-activation patterns differed in socially relevant regions among control, resilient and susceptible males. Correlation heat maps identified two distinct networks within each group. The first network consisted of the nPPa and Vv-r. The nPPa is a subregion of the preoptic area (POA) that modulates social behaviors including aggression, reproduction and parental care (Forlano and Bass, 2011). The nPPa expresses various neuromodulators such as gonadotropin-releasing hormone, arginine vasotocin and galanin that can impact the activity of different circuits and neurons (Butler et al., 2021; King et al., 2022). This region likely functions as an integrative center that processes different types of sensory information and may help evaluate social signals based on an individual’s internal physiological state. Activity in the POA could also be related to activation of the stress axis, as is commonly seen in rodents following acute social defeat (Martinez et al., 2002). The Vv-r has been implicated in social defeat in A. burtoni, with males descending in social status having higher levels of IEG activation (cfos, egrl) than non-descending males (Maruska et al., 2013). In rodents, the lateral septum (mammalian homolog of Vv, in part) plays a role in modulating cognitive signals regarding social stimuli, which can consequently alter behavioral responses (Menon et al., 2022). The second network consisted of the remaining SDMN nuclei (Dm-3, Dl-v2, Dc-4, Dc-5, Vv-c, ATn, Tpp, SR, Vd, Vc, Vs). These brain nuclei are implicated in social behavior and sensory processing (O’Connell and Hofmann, 2011, 2012). In the context of social defeat, it is possible that the first network is closely tied to initial evaluation of an encounter with a resident opponent, while the second network is more closely related to which behaviors are subsequently employed.

As these regions play a role in multiple social contexts, it is not surprising that interconnected neural networks rather than individual nuclei are more likely to be involved in shaping complex social behaviors such as coping styles after repeated
social defeat. Previous studies have shown that *A. burtoni* males dropping in social rank after a single defeat rapidly activate specific socially relevant brain nuclei in a pattern that is different from males rising to a dominant position. Levels of *cfos* in the POA and ATn were most important for distinguishing IEG profiles of descending males from non-descending and control groups (Maruska et al., 2013). In this study, heatmaps of pS6 cell densities showed that the nPPa was active and significantly correlated with other regions across control, resilient and susceptible males after repeated social defeat. The ATn was active and significantly correlated with other regions only across resilient and susceptible males. Susceptible males exhibited the greatest amount of co-activation in nine nuclei while control and resilient males only exhibited co-activation in five nuclei (which differed between the two groups). The high co-activation of nuclei in the SDMN of susceptible males suggests an important role in regulating vulnerability to repeated social defeat stress. As a result, it is likely that components of the neural circuitry regulating the coping behaviors of susceptible males are shared by the stress-coping system [hypothalamic-pituitary–interrenal (HP1) axis] and should be investigated. Networks within the SDMN may also play an important role in other relevant behaviors such as opponent assessment. Male–male assessment and territorial interactions in a *A. burtoni* involve visual, chemosensory and mechanosensory signaling (Butler and Maruska, 2015; Chen and Fernald, 2011; Maruska and Fernald, 2012), so central processing regions for these senses may also be linked to social defeat phenotypes. During social defeat, it is likely that assessment behaviors are dependent on a network of males working together to integrate sensory cues with an individual’s own physiology, driving decision making regarding which coping style an individual will employ.

Further research is needed to fully understand the neural circuitry that underlies behavioral differences between resilient and susceptible fish, and the neurotransmitters and hormones involved in regulating these circuits. Fish are increasingly recognized and used as valuable biomedical models to understand human mental health disorders such as anxiety, depression and PTSD (Bozi et al., 2021; Cueto-Escobedo et al., 2022; Lima et al., 2016; Stewart et al., 2014). To improve animal and human models of neurological disorders, it is important to understand the evolution and conservation of neural networks underlying the behaviors displayed in those conditions. While some brain homologies between teleost fishes and tetrapods remain controversial or unknown, particularly in the forebrain, studying the neural mechanisms of repeated social defeat in a cichlid fish can help resolve some of these homologies (Maruska and Fernald, 2018). With this work, we have established a new social defeat model and investigated which brain regions may be involved in regulating social defeat behavior in resilient and susceptible fish. By comparing our results in cichlids with studies in mammals, we provide crucial information on the function of homologous nuclei in this defeat context to reveal which components of the neural circuitry may be conserved. We hope that these data will spur more functional studies to understand how these regions may regulate resilience or susceptibility to repeated social defeat across vertebrates.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Data availability**

All relevant data can be found within the article and its supplementary information.

**References**


Department of Experimental Biology


