Behavioral and neural correlates of social hierarchy formation in a sex-changing fish

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Abstract

Social hierarchies in sex-changing fish determine which fish will change sex, yet little is known about how these hierarchies are formed at the neurobehavioral level. Here, we investigate the formation of social hierarchies within groups of the New Zealand spotty wrasse (spotty), integrating both behavioral observations of social activity with neural activation patterns upon social disruption. We find that dominance hierarchies form rapidly and linearly based on size, with larger fish displaying more aggressive or dominant behaviors (such as monopolizing the shelter) and smaller fish displaying more submissive behaviors (such as escape behavior). Disruption of the social hierarchy induced rapid behavioral changes, particularly in the second-ranked fish, highlighting that second ranked spotty will opportunistically adopt a dominant position in the hierarchy upon removal of the most dominant fish. Neural activation patterns reveal that the social decision-making network is deeply involved in the establishment of dominance, with the fish attaining dominance showing significant differences to all other ranked fish. This study underscores the complexity of social relationships and their neural underpinnings in the spotty wrasse, providing a foundation for further research into the cellular and molecular mechanisms of socially-controlled sex change. Overall, we demonstrate that disruption of social hierarchy triggers rapid changes in behavior and brain regions associated with social decision-making.

Introduction

Social dominance hierarchies are common across the animal kingdom and have important implications for survival and reproduction. Dominance within a group is associated with asymmetric aggression and priority access to physical or social commodities that ultimately increase fitness, such

as mating opportunities, food, shelter etc. (Kaufmann, 1983). One of the most extreme consequences of a dominance hierarchy is socially-controlled sex change that certain fishes undergo upon attaining dominance. In protogynous (changing from female to male) species, removal of the dominant male in a group will trigger sex change in the dominant female. These fish often have harem or lek-like mating systems (Gemmell et al., 2019; Hodge et al., 2020), where a single male maintains a territory or breeding ground with multiple females (Benvenuto et al., 2017). While it is the male that holds the most dominant position, the females can arrange themselves further in the hierarchy, as has been observed in other species (Chase, 1982; Mills et al., 2018; Pini-Fitzsimmons et al., 2021; Van Haeringen and Hemelrijk, 2022). Often, the dominant female will change her behavior within minutes to hours after the removal of the male, with gonadal and morphological changes following on a scale of days to months (Liu et al., 2017; Todd et al., 2019). The main hypothesis as to why sex change occurs is outlined in the size-advantage model, which suggests that body size influences reproductive or mating success differently between sexes, and that an individual can maximize their reproductive success by changing sex once a particular body size has been reached (Ghiselen, 1969; Warner, 1975). Therefore, individuals should first reproduce as a certain sex (e.g., female) early on and undergo sex change when they have increased to a size or age that they will benefit as the opposite sex (e.g., male). Thus, size clearly plays an important role in hierarchy formation, although this has scarcely been systematically tested in sex-changing fish (Bessa et al., 2021).

Although changes in social environment are known to be the primary triggers for sex change, most research on sex-changing fish considers only the behavior among the most dominant individuals, with little attention for the entire group (Benvenuto and Lorenzi, 2023). Understanding differences between subordinates could provide more detailed insight into the earlier changes that occur before the sex changing process among sequential hermaphrodites. It remains unknown whether there are factors that delineate high-ranked individuals predisposed to change sex from their conspecifics, and whether these differences prime or influence the molecular and neural systems that underlie sex change. If the second ranked individual in the hierarchy can ‘determine’ that they will be the most fit for sex change even before the removal of the male, this may initiate molecular changes that prime them for sex change. Although the cost and benefits of being dominant and subordinate are well-studied (Creel, 2001; Dantzer and Newman, 2022; Kustan et al., 2012; Sawada et al., 2017; Zizzari et al., 2017), little work has been done to observe differences between subordinates of different rank. Moreover, the majority of hierarchy studies focus solely on male hierarchies (De Moor et al., 2020; Williamson et al., 2016), leaving female hierarchy broadly neglected. Understanding the behavioral differences between females of different ranks among protogynous hermaphrodites will help us determine how female dominance hierarchies are formed, maintained and altered.

It is clear that socially-controlled sex change is independent of the gonad (Godwin et al., 1997), that visual and neuroendocrine components are crucial ((Elofsson et al., 1999; Godwin, 2010); and that gene expression in the brain differs between females and dominant males (Todd et al., 2018). Sex change has furthermore been shown to involve the preoptic area (Elofsson et al., 1997) and courtship behavior in the protogynous bluehead wrasse is controlled by a network of brain areas that associate

with the social behavior network (Luong et al., 2023). A brain-wide investigation of neural activity upon hierarchy formation is lacking to date, and the neural correlates of attaining dominance have not been studied. Phosphorylated S6 protein (pS6) can be employed as an indicator of neural activity (Knight et al., 2012). PS6 reveals cytoplasmic staining of ribosomal proteins that were phosphorylated in the preceding hour, a process associated with enhanced translation within the cell. The pS6 marker has been utilized in various fish species across different social or sensory contexts in the brain (Akinrinade et al., 2023; Tripp et al., 2020).

In this study, we investigate the group formation of the New Zealand spotty wrasse (*Notolabrus celidotus*; hereafter referred to as spotty), a protogynous sequential hermaphrodite. Spotty are ubiquitous across New Zealand and take well to being held in captivity (Goikoetxea et al., 2021; Jones, 1980). Spotty appear in three sexual phenotypic morphs: initial phase (IP) females, IP males, and terminal phase (TP) males. TP males and IP individuals can be easily differentiated from each other as they have different color morphs. All spotty first appear as the IP coloration, with most juveniles developing as females and a small minority developing as IP males (Jones, 1980). During sex or role change to a TP male, individuals will develop the TP coloration. TP males can typically be found in groups with 4-6 females (Jones, 1980). Here we allocated spotty into novel groups of 5 females and determined rank in the hierarchy based on submissive and aggressive behavior. Physical position in the tanks, subordinate behavior, and dominant behavior were analyzed over nine days. After 11 days, we removed the largest (most dominant) fish from each group and analyzed the behavior of the remaining four fish for 30 minutes after social disruption. One hour after social disruption, we removed and sampled the remaining fish to observe differences in the brain based on the observed rank and behavior.

Materials & Methods

Animals

Animal handling was performed in accordance with New Zealand National Animal Ethics Advisory Committee guidelines. New Zealand spotty wrasse were captured around high tide by hook and line off the coast of Tauranga, Bay of Plenty, New Zealand (37.6878° S, 176.1651° E) and subsequently maintained at the Aquaculture Centre at Te Pūkenga/Toi Ohomai Institute of Technology, Tauranga. Fish were housed for several months in a large 1600L recirculating holding tanks to acclimate prior to setting up the experiment. Prior to experimental tank allocation, standard length (SL; ranging from 108.87-179.91mm) and body weight (ranging from 22-122g) were measured. SL was measured by taking photos of each fish next to a metric ruler and using Image J; two measurements from the tip of the nose of the posterior margin of the caudal peduncle were taken in Image J and then averaged (Table S1). Floy tags (Fine Anchor T-bar 5 cm, Hallprint T-Bar tags) were attached to enable individual recognition. Fish were housed in 400-L recirculating seawater systems under a natural photoperiod. Fish were fed frozen greenshell mussels (*Perna canaliculus*) two to three times per week. At the conclusion of behavioural experiments, fish were anaesthetised through immersion in 600 ppm 2-phenoxyethanol and sacrificed by decapitation.

Group Formation

Groups of five fish of different sizes were placed in 400-L recirculating seawater systems and kept under a natural photoperiod. There were 11 groups in total. A PVC pipe (~100mm wide x ~200mm long) was placed in each tank to act as a shelter. Behavior was recorded 2-3 times per day between 9:00-18:00 for nine days after group initiation. Videos were taken using GoPro HERO Session and GoPro Hero 11 Black Mini cameras for a period of 20-30 minutes. Dummy cameras were placed in the tanks when recordings were not being taken to habituate the fish to the presence of a camera.

Social Disruption

After 11-12 days of group formation, the dominant fish was removed in n=7 treatment tanks (n=4 fish remaining in each tank). In the remaining four tanks, the dominant fish was removed and immediately reintroduced to act as a control for the disruption (n=5 fish in each tank). All tanks (control and treatment) were then recorded for one hour post disruption and then all remaining fish were removed and immediately euthanized for gonadal histology and immunohistochemistry analysis.

Behavioral Analysis

Videos were watched and scored using BORIS software (Friard and Gamba, 2016). All videos during the first three days of group formation, and then videos from every other day (days 5, 7, and 9) were analyzed. All individual observations were summed together for statistical analysis. At the start of recording, 10 minutes were left unanalyzed to allow for fish to recover after the introduction of the camera. The next 10 minutes were then observed to count the number of aggressive and submissive interactions. A rush (aggression) was defined as a directed movement or acceleration towards another individual and an escape (submission) as acceleration away from or change in direction to avoid another individual. Position and movement in the tank were also recorded. State behaviors were measured in seconds and were divided into three behavior types: Time spent in shelter, time spent on the floor, and time spent swimming around the tank (Table 1).

Each individual was given a dominance score calculated by their overall rate of rushing behavior divided by their overall rate of rushing plus the overall rate of escaping behavior (rRush/(rRush+rEscape)). Therefore, highly dominant individuals will have a dominance score closer to 1 and highly subordinate individuals a score closer to 0. This dominance index is similar to one used by Oliveira and Almada (1996), but uses the rate of aggressive and submissive interactions rather than the number of behaviors. The proportions and rates of the behaviors were used, rather than the measure of time or total number of a point behavior by itself, as some fish were out of view for periods of filming, therefore, not all fish have the same total time of observation. Fish in each tank were given a rank from 1-5 (1 being the most dominant) based on their dominance scores.

Gonadal histology

Gonadal samples, which were collected upon finalization of the social disruption experiment (see subheading “Social Disruption”), were fixed in 4% paraformaldehyde in 1x PBS solution at 4□ for 72 hours. Gonadal samples gonadal were embedded in paraffin and subsequently cut at 3–4 µm on a Leica RM2125 RTS rotary microtome. Standard Mayer’s haematoxylin and eosin staining was performed as previously described (Kamstra et al., 2023) to assess tissue structure. Images were taken using an Aperio CS2 Digital Slide Scanning System (Otago University Histology Unit). Gonadal sex was classified according to Goikoetxea et al. (2020).

Immunohistochemistry

Following dissection and subsequent fixation in 4% paraformaldehyde in 1x PBS solution at 4□ for 72 hours, brains were cryoprotected in 30% sucrose solution until the tissue lost buoyancy. Brains were flash frozen in dry ice-cooled isopentane and stored at -80°C. Brains were embedded in optimal cutting temperature medium (TissueTek, Sakura, Torrance, CA, USA) and sectioned coronally at 50µm on a CM1950 Leica cryostat. Cryosections were collected on alternate sets of SuperFrost plus slides (Thermo Fisher Scientific NZ Ltd, North Shore City, New Zealand), air dried at room temperature overnight and stored at -80°C. Slides were thawed and cryosections were surrounded with a hydrophobic barrier. Slides were then washed 3 times for 10 minutes with 1× PBS. Non-specific binding was blocked (1% bovine serum albumin, 0.3% Triton-X and 5.0% normal goat serum made in 1× PBS) for 2 hours at room temperature, and slides were incubated with primary pS6 antibody (1:1500; Cell Signaling Technologies pS6 ribosomal protein S235/236 antibody #4858) overnight at 4°C. Slides were rinsed with 1× PBS (3×10min), and further processed using the Vectastain ABC-HRP Kit (PK-4001)) according to the manufacturer’s instructions. Finally, slides were dehydrated through an alcohol series (50%, 70% and 95% for 1 min each; 100% 2×2 min each), cleared with xylene (2×2 min) and cover slipped using dibutylphthalate polystyrene xylene. Antibody specificity was confirmed through absence of staining upon leaving out the primary antibody or secondary antibody, or upon preincubation with a pS6 blocking peptide (Cell Signaling #1220) (Supplementary Figure S4).

Brain image analysis

Photomicrographs were taken on a Nikon Ti2-E inverted bright field microscope with a 20x magnification objective. Composite images were taken with 15% overlap and stitched together using Nikon NIS-Elements software. Raw images were processed in Fiji/ImageJ and Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to correct background noise and minor tissue artefacts. Image segmentation was performed as described previously using the Trainable Weka Segmentation plugin (Arganda-Carreras et al., 2017). Following comparison of the images with the spotty wrasse brain atlas (Kamstra et al., 2023), cell counts were performed using the Automatic Cell Counting with Trainable Weka Segmentation tool (Kataras et al., 2023). The density of pS6-stained cells was calculated as the number of stained cells divided by the surface area of the regions of interest quantified. For each region (Table 2), 3–4 consecutive sections were quantified (dependent on region, but consistent across animals) at the same rostro-caudal location within the nucleus across animals and averaged together for each animal to obtain a mean pS6-stained cell density per brain region (# of cells per μm^2). Brain areas that were identified to have

significantly different pS6 cell counts between ranks were recounted manually by an experimenter blind to the experimental parameters.

Statistical analysis

Behavioral data was analyzed in R 4.1.1, using the R Studio Interface Version 2023.12.1+402. Linear mixed-effects models (LMMs) were used to determine how behavior variables were associated with dominance score, average SL, and the interaction between dominance score and SL. Tank was set as a random effect for all models. We then used models with rank as the fixed effect to get contrasts between all ranks. The *lmer* function was utilized from the *lme4* package. Normality was tested using the Shapiro-Wilk test. The proportion of time on floor (pFloor), rate or rushes per minute (rRush), and rate of escapes per minute (rEscape) variables were square root transformed to obtain a normal distribution. The best fit models (i.e., lowest AIC) were determined using the *aictab* function in the *AICcmodavg* package (Tables S4-8). Immunohistochemistry data analysis was performed using Prism (GraphPad Software, La Jolla, CA). Comparison of cell counts between brain areas was done by one-way ANOVA, followed by Dunnett's multiple comparison test. Results are presented as the mean ± SEM. $p < .05$ was considered statistically significant.

Brain-behavior correlation analysis

Behavioral and brain data obtained during social disruption were analyzed together to identify which regions of the brain correlate with which behaviors. Behaviors recorded during group formation were not used for these analyses. Outlier and/or confounding behaviors were first removed from analyses, and fish were only used if complete brain and behavior data was available (see Supplementary Table S11) for a total of 37 fish (four control tanks and six treatment tanks). No rank #1 fish (most dominant fish) were used in these analyses, as brain regions were not analyzed in these fish. The data were analyzed in R (v 4.3.1), using Rstudio (v 2023.12.1+402). PCA plots were generated using the *prcomp* function with scale=TRUE and plotted using *ggplot*, with ellipses added by rank. The loadings for each bbf (brain/behavior factor) from principal component 1 and 2 (PC1 and PC2) were extracted using the *hi_loadings* function in pcaExplorer (v 2.28.0). The sample correlation plot was generated using the *heatmaply_cor* function with na.rm=FALSE in heatmaply (v 1.5.0). Statistical differences between ranks (control versus treatment treated separately) were calculated using the *pairwiseAdonis* wrapper (v 0.4.1) with 999 permutations. As the PCA plot showed that 'rank 2 treatment' samples form a clear separate cluster, statistical differences between the 'rank 2 treatment' samples and all other samples were also calculated using *adonis2* function in vegan (v 2.6.4) with method='eu'.

Results

Dominance hierarchy in the New Zealand spotty wrasse is rapidly established based on size

A piecewise linear model was utilized for the relationship between dominance score and size (Standard length: SL) with a breakpoint at 140mm (Figure 1A). SL was significantly and positively associated with dominance score, with larger fish having higher dominance scores, both for fish above (β = 4.13e-3, df = 1,45.82, t = 2.02, p = 0.050) and below 140mm (β = 0.02, df = 1,41.58, t = 16.36, p < 0.001). The SLs were significantly different between all ranks, except for 3 and 4, with fish being significantly longer than fish ranked subordinate to them (Figure 1B; Table S9). As expected, with rank being given based on dominance score, all ranks had significantly different dominance scores from one another (Figure 1C; Table S10). A clear dominance hierarchy was apparent almost immediately, with only ranks 3 and 4 not being obvious in the first observation, but quickly differentiating after just a few observations (Figure S2). All raw group formation behavioral data can be found in Supplementary Table S2.

Group formation behaviors are dictated by dominance hierarchy

Dominance score was significantly and positively correlated with proportion of time spent in the shelter (pInShelter: β = 0.60, df = 1,53, t = 7.73, p < 0.001). However, when rank replaced dominance score as the fixed effect (Table S4), the model had a better fit and it demonstrated that the fish with the highest dominance score (i.e., Rank 1) spent significantly more time in the shelter than all other ranks (Table S11). The other ranks did not differ significantly from one another (Table S11). The best fit LMM for proportion of time spent swimming included only dominance score as a fixed effect (Table S5). Fish with a higher dominance score spent a significantly smaller proportion of their time swimming (β = -0.37, df, 1,53, t = -7.62, p <0.001). The model with rank as the fixed effect did not have a better fit and showed that Rank 1 spent significantly less time swimming than all other ranks and that Rank 2 spent significantly less time swimming than Rank 5. The rest of the ranks did not differ significantly from each other (Table S12).

The best fit LMM model for the proportion of time on floor (pFloor) also only had dominance score as a predictor variable (Table S6). Dominance score and pFloor were significantly and negatively correlated with each other (β = -0.25, df, 1,43.39, t = -4.83, p <0.001). Using rank as the only fixed effect did not have a better fit model, but it showed that Rank 1 spent significantly less time on the floor than all other ranks, and that all the other ranks did not significantly differ (Table S13).
Figure 2C-E illustrates the proportion of time each rank spent on each of the three major activities (proportion of time in shelter, proportion of time swimming and proportion of time on the floor), which shows that fish of decreasing rank spend more time swimming, and rank 1 fish spend the most time in the shelter.
Using rank as a fixed effect in the model testing the rate of rushes per minute (rRush) between ranks, we show that Rank 1 had a significantly higher rRush than ranks 3-5, but did not differ significantly from Rank 2. Rank 2 also had a significantly higher rRush than ranks 3-5. Rank 3 had a significantly higher rRush than Rank 5, but did not differ significantly from Rank 4. Rank 4 and 5 did not differ significantly from each other (Table S14). For rate of escapes per minutes (rEscape) between ranks, which was the best predictor variable , we show that Rank 1 had a significantly lower rEscape than all ranks and Rank 2 also had a significantly lower rEscape than ranks 3-5. Rank 3 had a significantly lower rEscape than Rank 5, but did not differ

significantly from Rank 4. Rank 4 and 5 did not differ significantly from each other for rEscape (Table S15). Figures 2A and 2B illustrate the rates of rush and escape behaviors across each rank, which clearly shows increasing submissive (escape) behavior as the ranks decrease, and inversely, decreasing aggressive (rush) behavior as the ranks decrease. All model AIC data for rRush and rEscape are in Tables S7-S8.

Removal of the dominant fish induces a linear shift in hierarchy status

Socially-controlled sex change is initiated when an opportunity arises for a subordinate fish to attain dominance, usually due to the death or disappearance of the dominant fish. To investigate the behavioral and neuronal changes associated with this change in social environment, we removed the dominant fish from its home tank. As a control, we removed the dominant fish but immediately returned it to its home tank.

In treatment tanks, after the removal of the Rank 1 fish, Rank 2 experienced a significant increase in dominance score and rRush, with a significant decrease in rEscape (Figure 3). Rank 3 had a significant increase in rRush but no significant differences for dominance score and rEscape. Rank 4 experienced both a significant increase in rRush and rEscape, but no significant change in dominance score. Rank 5 had a significant decrease in dominance score, a marginally significant decrease in rRush, and a significant increase in rEscape. Rank 2, 3, and 4 had a significant decrease in pFloor, and Rank 5 a marginally significant decrease. Rank 3 and 5 also had a marginally significant increase in pSwimming (Table S16). Thus, fish retain their relative position in the social hierarchy, and all move up one spot.

In control tanks, after the Rank 1 fish was removed and immediately returned, Rank 1, 2, and 4 did not have a significant change in dominance score; however, Rank 3 had a significant decrease and Rank 5 a marginally significant decrease in dominance score (Figure 3A). No rank had a significant change in rRush (Figure 3B). Rank 5 had a marginally significant increase in rEscape, while no other ranks differed significantly (Figure 3C). Rank 1 had a significant decrease in pInShelter and a marginally significant increase in pSwimming (Table S16). All other ranks did not have significant changes in any of their state behaviors. All raw social disruption behavioral data can be found in Supplementary Table S3.

Dominance is confirmed by masculinization of the gonad in most, but not in all dominant fish

Upon conclusion of the behavioral observations all fish were sacrificed, and gonadal histology was analyzed to confirm sex. Remarkably, in 8 out of 12 tanks the largest and most dominant fish showed clear signs of gonadal masculinization (Supplementary Figure S3), despite external coloration and morphology indicating the fish were IP females. However, given that these fish were already the largest, it is most likely that they had began the sex changing process before the start of the experiment. Due to the identical external morphology of IP female and IP males, it is impossible to tell the two apart until gonadal morphology can be assessed. However, it is statistically unlikely that the gonad masculinization in the largest fish was because they were IP males (see discussion).

In tank 8, it was found that two fish had masculinized gonads (Rank 1 and Rank 3 by size; Supplementary Figure S3), leading us to believe that the 3rd-ranked fish in this tank was an IP male. While there is insufficient data here to statistically determine if IP males behave differently to IP females in the context of hierarchy formation, anecdotally, the behavior we observed of the IP male was more aggressive than was expected for its rank based on size. Tank 8 was therefore excluded from further analyses.

The social decision-making network is differentially activated in dominant vs subordinate fish

To investigate the neural correlates of social hierarchy and its formation, neural activation patterns were compared between differently ranked fish following removal of the dominant fish. Brain areas that were identified as having significantly different pS6 cell counts between the new dominant (= rank 2) vs subordinate fish (= rank 3-5) were: the medial part of the dorsal telencephalon (Dm; Figure 4A), the dorsal part of the ventral telencephalon (Vd; Figure 4B), the ventral part of the ventral telencephalon (Vv; Figure 4C), the supracommissural nucleus of the ventral telencephalon (Vs; Figure 4D), the central nucleus of the telencephalic are (Vc; Figure 4E), the preoptic area (POA; Figure 4F), the ventral tuberal region of the hypothalamus (vTn; Figure 4G), the periaqueductal gray (PAG; Figure 4H), the periventricular nucleus of the posterior tuberculum (TPp; Figure 4I) and the anterior tuberal nucleus (aTn; Figure 4J). Mammalian homologs of these brain areas are listed in Table 2. In each of these brain regions the number of pS6-stained cells was significantly different between the now largest 2nd ranked fish and any of the other fish ranked 3-5 (one-way ANOVA with Dunnett's multiple comparisons test, Table S17). No significant differences were observed between the fish ranked 3-5. Importantly, this difference is a result of the removal of the 1st-ranked fish specifically, as there were no significant differences between any of the ranked fish in the control group where the 1st ranked fish was caught but immediately returned, or between the fish ranked 3-5 in the experimental group and any of the fish in the control group. In 9 out of 10 of the identified brain areas we observed increased neural activity in the 2nd-ranked fish compared to the other fish. In the aTn, on the other hand, a decrease in pS6 staining was observed in the 2nd-ranked fish compared to the other ranks.

Behavior and brain activity correlate based on social rank and the social decision-making network

Finally, we analyzed the correlation between the behavioral observations following social disruption with the associated neural activation patterns. Principal component analysis of all behavioral observations and brain activity for each of the 37 individual fish (Table S18) revealed that samples are mainly organized by social rank (PC1: 43.6%; Figure 5A). Rank-based clustering of the data shows a hierarchical distribution, from left (rank 5) to right (rank 2). Within-rank variation increases with a rise in ranking (Figure 5A), reflecting the increased social instability or opportunities associated with higher ranking. The proximity of clusters 3 and 4 indicates a similarity in both behavior and social decision-making network activation that is explained by the absence of significant differences in SL between these groups ($p > 0.05$; Figure 1B; Table S9). Comparing treatment samples between ranks shows only rank 2 treatment vs rank 5 treatment are significantly

different (Pr > F = 0.002; Table S20). Comparing control samples between ranks shows only ranks 3 control vs rank 4 control are significantly different (Pr > F = 0.001; Table S21). Comparing treatment versus control samples are only significantly different within rank 2 (Pr > F = 0.008; Table S22). However, the rank 2 treatment group cluster is statistically distinct from all other samples (Pr > F = 0.001; Table S23), emphasizing the effect that removal of the dominant fish has on this group .

The individual loadings for PC1 (Figure 5Bi) show that there is no single specific behavior or brain area driving social rank. The rush and escape behaviors that were used to calculate dominance score are indeed among the factors contributing the most to social rank, in opposing directions, confirming that they are appropriate behaviors for this calculation. Among the brain areas found to be differentially activated upon disruption of the social hierarchy, little variation in loading score was found, but of these the PAG and POA have the highest positive loadings, and the aTn is the only brain region which had a negative rather than a positive loading.

PC2 explains only 12% of the data distribution, but its loadings have a much more distinct pattern (Figure 5Bii). The most significant positive factor contributing to PC2 is the proportion of time spent swimming, whereas the most significant negative factor is the proportion of time spent stationary on the floor. Given the vertical orientation of the rank-based clusters in figure 5A, within-cluster variation appears to depend mostly on how active fish are.

The correlation matrices of behavior and brain activity (Figure 5C-F; Table S19) show that the strongest correlations found between all factors are between the different brain areas, emphasizing the interconnectedness of the social decision-making network. Comparing ranks in figure 5C-F, both positive and negative correlations decrease in intensity as rank lowers. . Within the social decision-making network, the strongest positive correlations (above 0.75) across ranks are between the PAG and the POA, between the vTn (AH) and the PAG, between the Vv (LS) and the POA, and between the Vv (LS) and the Vs (BNST/meAMY). The aTn correlates negatively with all other social decision-making network nodes, most notably the vTn (AH), Vs (BNST/meAMY) and the PAG, which is particularly evident in rank 2 fish (Table S19). Standout brain-behavior correlations include a strong negative correlation between the PAG and escape behavior, as well as a strong positive correlation between POA activation and rushing behavior.

Discussion

In the current study, the behavioral and neural correlates of social hierarchy formation were investigated in a sex-changing fish. We hypothesized that size, as measured by standard length, is the main determinant of rank and dominance, with larger fish being more likely to score higher in both categories. Indeed, dominance hierarchies were rapidly established upon group formation in a size-dependent manner, and each rank was found to have a significantly different dominance score. Fish size significantly affected aggressive (i.e., rate of rush behavior) and submissive (i.e., rate of escape) behaviors, and therefore overall dominance score. These results confirm the importance of size in dominance hierarchies (Buston, 2003; Tibbetts et al., 2022).

Surprisingly, ranks 1 and 2 did not differ significantly in aggressive behaviors (Figure 2A). However, this is likely because rank 1 fish spent a large proportion of their time in the shelter. This is a great example of how being at the top of a hierarchy allows for priority access to certain resources. This furthermore explains why all other ranks spent significantly more time swimming and, on the floor, than the dominant individual. It is typical in many social species for the dominant individual to take possession of the shelter, as well as other resources before subordinates (Clifton, 1991; Dantzer and Newman, 2022; Kroon et al., 2000). Occupation of the shelter by the dominant fish also corresponds with spotty behavior in the wild (Jones, 1981). In line with this, time spent actively swimming increased as rank decreased, suggesting that locomotion could be a sign of subordination. The energetic cost that comes with actively swimming versus resting in the shelter further emphasizes the value of being a dominant individual. However, it should be noted that this experiment took place in a laboratory setting with only one shelter being present, potentially making the energy the dominant fish needs to surveil and maintain the territory much less intensive.

Although 1st-ranked- and 2nd –ranked fish did not differ significantly in aggression, they did differ in submission (i.e., rate of escape behavior). 1st-ranked fish showed little to no submission, which is expected in a stable hierarchy without competition and commonly observed in despotic hierarchies, where one individual is dominant over all others (Huang and Wu, 2022). Submissive behavior increased as the hierarchy descended but was not always significantly different between individuals. These observations show the importance of taking multiple behaviors into account when studying hierarchy formation. Future studies with spotty wrasse should aim to investigate hierarchy type and which individuals are interacting with each other (i.e., heterarchy) to gain further understanding on their social structure (Pini-Fitzsimmons et al., 2021). Overall, the current study suggests that spotty wrasse form a linear hierarchy, where the top individual dominates over all others, the 2nd-ranked fish over all but the dominant, and so on. However, further analyses would need to be conducted to confirm linearity (Albers and de Vries, 2001; Gammell et al., 2003; Pini-Fitzsimmons et al., 2021).

Disruption of the hierarchy by removing the dominant fish induced significant behavioral changes. Most notably, the 2nd-ranked fish displayed significantly increased aggressive behavior, as well as a significant decrease (in some cases, complete absence) of submissive behavior. This type of behavior has been investigated in many other sex changing species, with the removal of the dominant fish causing rapid changes in behavior (Reavis and Grober, 1999; Tamagawa et al., 2023; Todd et al., 2019). Most fish displayed increased activity over this period, with all ranks decreasing time resting on the floor and some a significant increase in swimming, demonstrating the disruption that has occurred. In the control treatment, where the dominant fish was removed and immediately returned, fish did not seem to have changes in their state behavior except for rank 1 (the displaced fish). Rank 1 showed a significant increase in its time swimming and decrease of time spent in the shelter. Rank 1 also showed an increase in its aggression with a significant increase in rushing behavior. This could be because rank 1 is trying to reaffirm its dominance and the stress caused by its removal. Behavior of rank 1 likely explains why some of the other fish experience a decrease in dominance score or increase in submissive behavior as well.

Hierarchical status was indicated not only by behavior and neural activation patterns, but also by gonadal histology. After only nine days of group formation, 8/12 dominant fish were found to have clearly masculinized gonads. A challenge working with New Zealand spotty wrasse is the potential to inadvertently introduce female-looking IP males in your experimental groups. Theoretically, the 8/12 fish that showed masculinized gonads, while also being the biggest and most dominant fish in their tank, could all be IP males. However, since the distribution of IP males in natural populations (~10% of fish) is random (e.g. not size-skewed), it would be expected that any IP males in our tanks would be distributed randomly as well. It is statistically not unlikely that 9 out of 60 fish are IP male, but the statistical likelihood of 8 out of those 9 fish being the biggest and most dominant fish in their tank is 0.032%. Therefore, it is more likely that larger IP fish had already begun gonad masculinization before groups were initiated.

It is possible that the gonads were masculinized because sex change was initiated following establishment of dominance during group formation, however, given the short time frame of this experiment (12 days), this seems less likely. The behavior of these fish during hierarchy formation also did not differ from other dominant fish and there was no statistical difference in fish size between masculinized fish and feminized fish. However, if differences in their behavior during social disruption exist there is insufficient statistical power to test this, with only two of the non-masculinized fish in the treatment condition and two in the control condition for social disruption. The potential for genetic, epigenetic, neuroanatomical and/or endocrine differences between the dominant fish with and without masculinized gonads is worthy of further investigation.

All animals exhibit adaptive responses to their social surroundings through a complex neural framework. This framework primarily consists of two neural circuits, described firstly in mammals but subsequently confirmed in all vertebrate lineages (O'Connell and Hofmann, 2012). These circuits are: (1) the social behavior network (Goodson, 2005; Newman, 1999), and (2) the mesolimbic reward system (Berridge, 2007; Wickens et al., 2007). The differentially activated brain areas in this study are all part of the mesolimbic reward system (Vv, Vs, Vd, TPp, Vc and Dm) and/or the social behavior network (aTn, vTn, PAG, POA, Vv and Vs). The remarkable social decision-making network-specificity of the difference in neural activation patterns is emphasized by the fact that the only missing node from this network is the ventral pallidum, for which no fish homolog has been identified to date. The one brain region with a specific decrease in neural activation upon attaining dominance was the aTn, which was negatively correlated with other regions of the SDMN (Figure 5C-F and Table S19) The mammalian homolog of the aTn, the ventromedial nucleus of the hypothalamus, drives the switch between feeding and defensive behaviors (Khodai and Luckman, 2021). The decreased activity in this area could therefore be an indication of active suppression of unhelpful behaviors during attainment of dominance. In accordance, Luong et al. (2023) found that in the closely related bluehead wrasse (*Thallasoma bifasciatum)* stimulation of brain areas including the Vs, Vv, Vd and POA induced changes in coloration associated with courtship behavior. In the same study pS6 and cfos immunohistochemistry revealed that the interpeduncular nucleus, red nucleus, and ventrolateral thalamus were more active in subordinate fish. These regions were not found to be significantly more active in subordinate fish in our study. The difference is likely due to the

differential brain sampling timeline used: whereas non-courting animals in the Luong et al. (2023) study were observed for at least four hours before sampling, we sacrificed our fish 1 hour after social disruption.

The current study provides further evidence for the role of the social decision-making network in the initiation of reproductive behavior changes. The social behavior component of this network is generally believed to regulate behaviors like reproduction, aggression, and parental care through interactions with various sex steroid and neuropeptide hormones (Goodson, 2005; Newman, 1999), whereas the mesolimbic reward system assesses the salience of stimuli through dopaminergic signaling (Berridge, 2007; Wickens et al., 2007). Specifically, we show that rank 2 fish have significantly more neural activation in both SDMN and mesolimbic regions of the brain compared to lower ranked fish (Figure 4), and that there is higher positive correlation between aggressive behaviors and the SDMN in rank 2 fish compared to the lower ranked fish (Figure 5). Conversely there is also a higher negative correlation between submissive (escape) behaviors and the SDMN in rank 2 fish, which together indicates that establishing dominance has a substantial effect on many regions of the brain of spotties. This ties in well with previous studies on the initiation of socially-controlled sex change. A multitude of neuroendocrine factors have been shown to be necessary, but not sufficient for sex change, including GnRH (Kramer et al., 1993), arginine-vasotocin (Semsar et al., 2004), and cortisol (Goikoetxea et al., 2022). Our data suggest that the onset of socially-controlled sex change does not depend one specific endocrine factor, produced in one specific brain area, but rather involves a highly interconnected network of brain areas and their associated signaling factors. Although pS6-immunohistochemstry is a reliable readout of neural activity, it does not distinguish cell type. We suggest that future work should focus on the transcriptional profile of specific cell populations within the social decision-making network.

In conclusion, our work shows that the New Zealand spotty wrasse rapidly form social hierarchies, based mainly on size. Disruption of this hierarchy induces rapid (1hr) and substantial changes in behavior, and leads to differential activation of the social decision-making network in the brain. This study confirms the importance of size in social group dynamics and provides the behavioral and neuroanatomical base for further investigation of social hierarchy formation, as well as the initiation of socially-controlled sex change.

Figures

Figure 1. Group formation average SL, dominance score, and social rank. (A) The average SL has a positive relationship with the dominance score. Each point represents an individual fish, with color signifying which tank in was in and the shape signifying the rank it held. (B) Each rank differs in average standard length from each other, except for ranks 3 and 4. (C) The dominance scores were significantly different between all ranks.

Figure 2. Group formation behaviors by social rank. (A) The rate of aggression (i.e., rushing behavior) decreases moving down the hierarchy. (B) Submission (i.e., escaping behavior) increased moving down the hierarchy. Rank 1 spent significantly more time in the shelter (C) than all other ranks and significantly less time swimming (D) and on the floor (E). Rank 2 also spent significantly less time swimming than Rank 5 (D).

Figure 3. Behavior during group formation and after social disruption. The tanks that received the control treatment are represented in red/pink with behavior during group formation (Before Control) in pink, and after social disruption (After Control) in dark red. The tanks in the experimental treatment are in blue, with light blue showing behavior during group formation (Before Treatment) and royal blue showing behavior after social disruption (After Treatment). Rank 1 does not have data for the experimental treatment as it was removed from the tank.

Figure 4. Neural activation patterns after social disruption. Representative images for each brain area are depicted on the left (I; rank 2) and middle (II; rank 3-5) in each panel. Boxplots of pS6 counts are depicted on the right of each panel (III). (A)The medial part of the dorsal telencephalon (Dm), with magnification of the insets to the right of each panel (B) The dorsal part of the ventral telencephalon (Vd). (C) The ventral part of the ventral telencephalon (Vv). (D) The supracommissural nucleus of the ventral telencephalon (Vs). (E) The central nucleus of the telencephalic are (Vc). (F) the preoptic area (POA). (G) the ventral tuberal region of the hypothalamus (vTn). (H) the periaqueductal gray (PAG). (I) the periventricular nucleus of the posterior tuberculum (TPp). (J) The anterior tuberal nucleus (aTn; Figure 4J). Scale bar = 100µm.

Figure 5. Brain-behavior correlation analysis after social disruption. (A) PCA plot showing the distribution of each fish based on both proportional time spent on each behavior and neural activation. Ellipses are shown for each rank (control and treatment combined). (B) Loadings for each brain/behavior factor (bbf) for PC1 (Bi) and PC2 (Bii). (C) Sample correlation matrix for each bbf for rank 2 fish. (D) Sample correlation matrix for each bbf for rank 3 fish. (E) Sample correlation matrix for each bbf for rank 4 fish. (F) Sample correlation matrix for each bbf for rank 5 fish. All correlation values are in Table S19.

Tables

Table 1. Behavior definitions. All proportions and rates are adjusted for time spent in view of the camera. State behaviors were measured in seconds and point behaviors were measured in instances (counts).

Behavior	**Definition/explanation**	**Behavior Type**	**Behavior Category**
pSwimming	Proportion of time spent swimming	Active	State

pInShelter	Proportion of time spent in shelter	Stationary	State
pFloor	Proportion of time spent on the floor	Stationary	State
rRush	Rate of rushes per minute	Aggression	Point
rEscape	Rate of escapes per minute	Submission	Point
pOutofview	Proportion of time spent out of view of the camera (used to adjust proportions and rates of behaviors)	N/A	State

Table 2. Brain definitions for regions of the brain with significantly different neural activation between differentially ranked fish.

Teleost Brain Region		**Mammalian Homolog**		**System/network**
aTn	anterior tuberal nucleus	VMH	ventromedial hypothalamus	Social behavior network
Dm	medial part of dorsal telencephalon	blAMY	basolateral amygdala	Mesolimbic reward system
PAG	periaqueductal gray	PAG	periaqueductual gray	Social behavior network
POA	preoptic area	POA	preoptic area	Social behavior network
TPp	periventricular nucleus of the posterior tuberculum	VTA	ventral tegmental area	Mesolimbic reward system
Vc	central nucleus of telencephalic area	STR	striatum	Mesolimbic reward system
Vd	dorsal part of ventral telencephalon	Nacc	nucleus accumbens	Mesolimbic reward system
Vs	supracommissural nucleus of the ventral telencephalon	BNST/ meAMY	bed nucleus of the stria terminalis/medial amygdala	Both
vTn	ventral tuberal region of hypothalamus	AH	anterior hypothalamus	Social behavior network
Vv	ventral part of ventral telencephalon	LS	lateral septum	Both

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Experimental design was constructed by H.Q., C.v.d.B., K.K, N.G., C.B., S.M., E.T. and C.J. Behavioral experiments were carried out by H.Q., C.v.d.B, K.K. and S.M. Behavioral data were watched and generated by H.Q. Brain region data were generated by K.K. Brain and behavior data were analyzed by H.Q., C.v.d.B and K.K., with statistical and methodological input from C.B. and N.G. Figures were generated by H.Q., C.v.d.B. and K.K. The manuscript was written by H.Q., C.v.d.B and K.K. and edited and approved by all authors.

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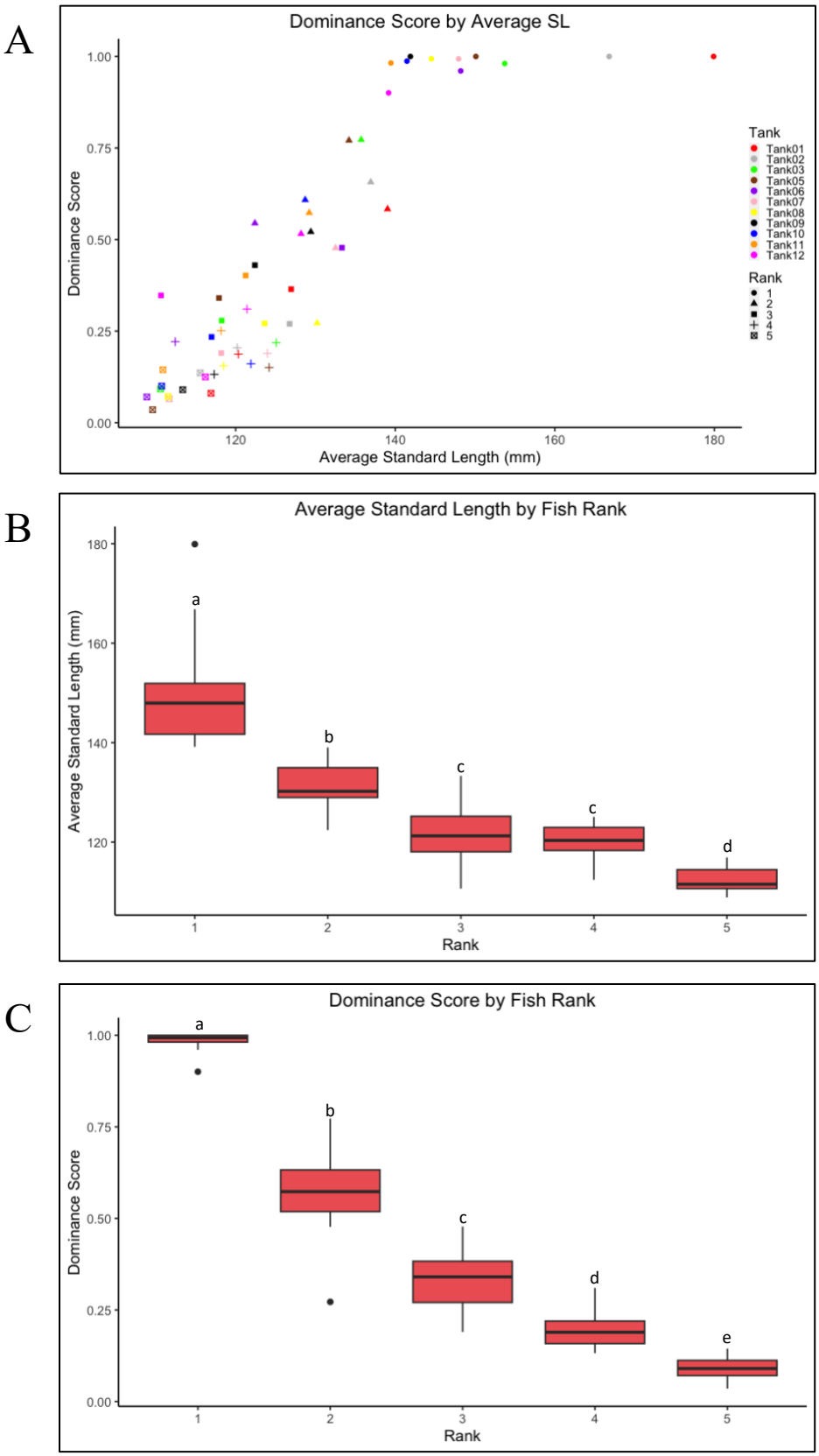
Data Availability Statement

The consolidated data for the behavioral datasets generated for this study can be found in Supplementary Table S2 (Group formation) and Table S3 (Social disruption). Raw data files from BORIS can be found at https://figshare.com/s/54488470957b773b34f5.



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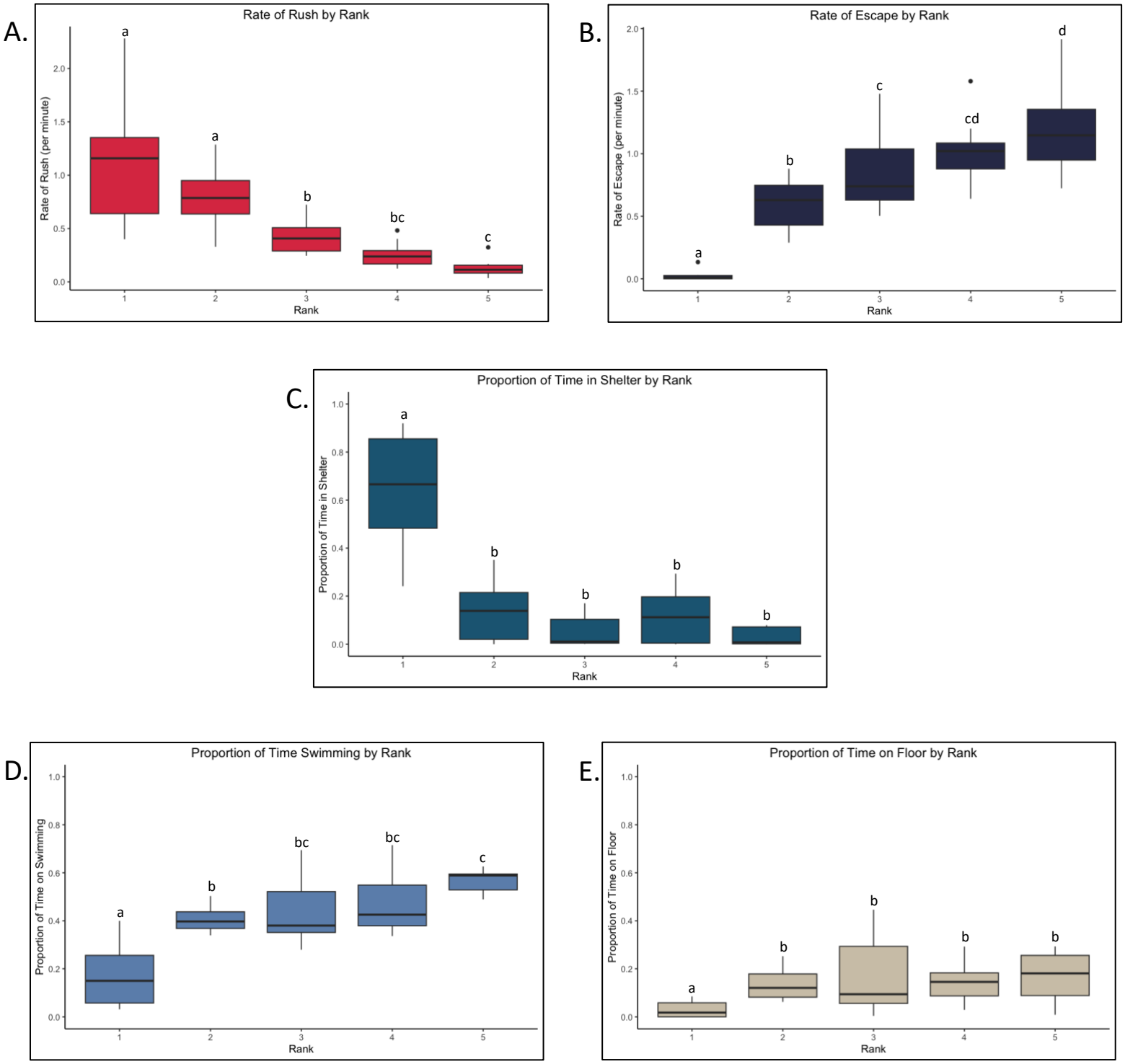


Figure 2. Group formation behaviors by social rank. (A) The rate of aggression (i.e., rushing behavior) decreases moving down the hierarchy. (B) Submission (i.e., escaping behavior) increased moving down the hierarchy. Rank 1 spent significantly more time in the shelter (C) than all other ranks and significantly less time swimming (D) and on the floor (E). Rank 2 also spent significantly less time swimming than Rank 5 (D).

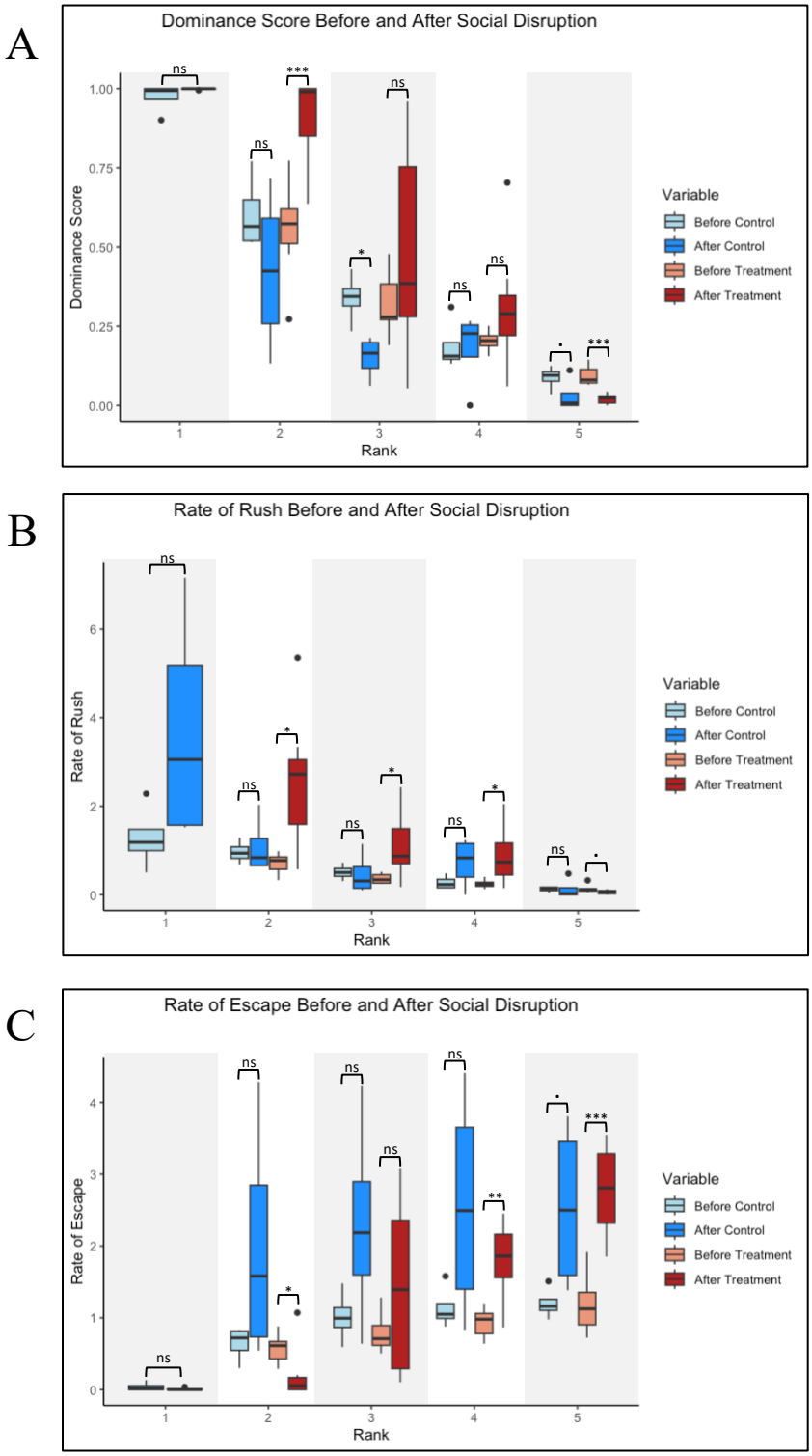
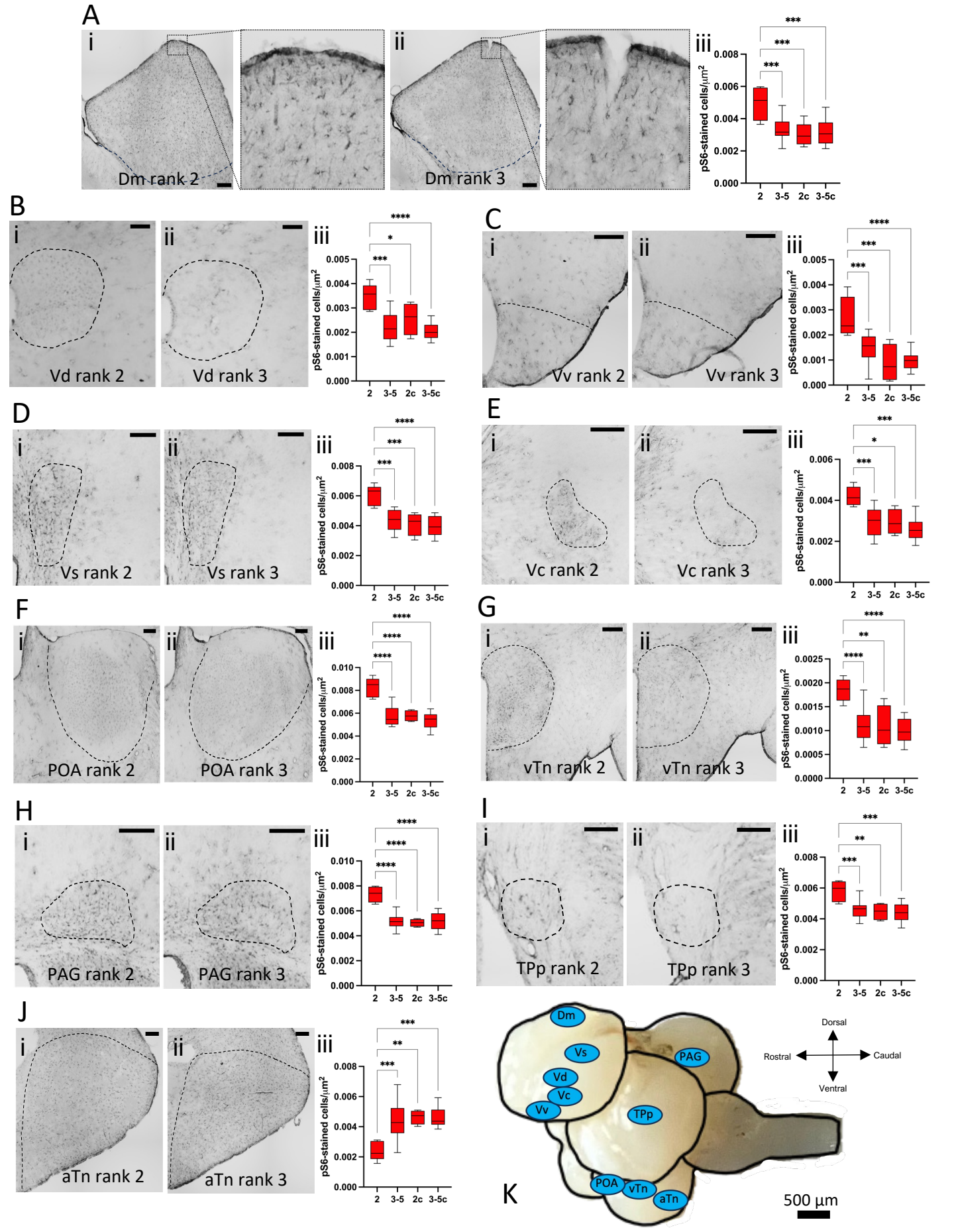
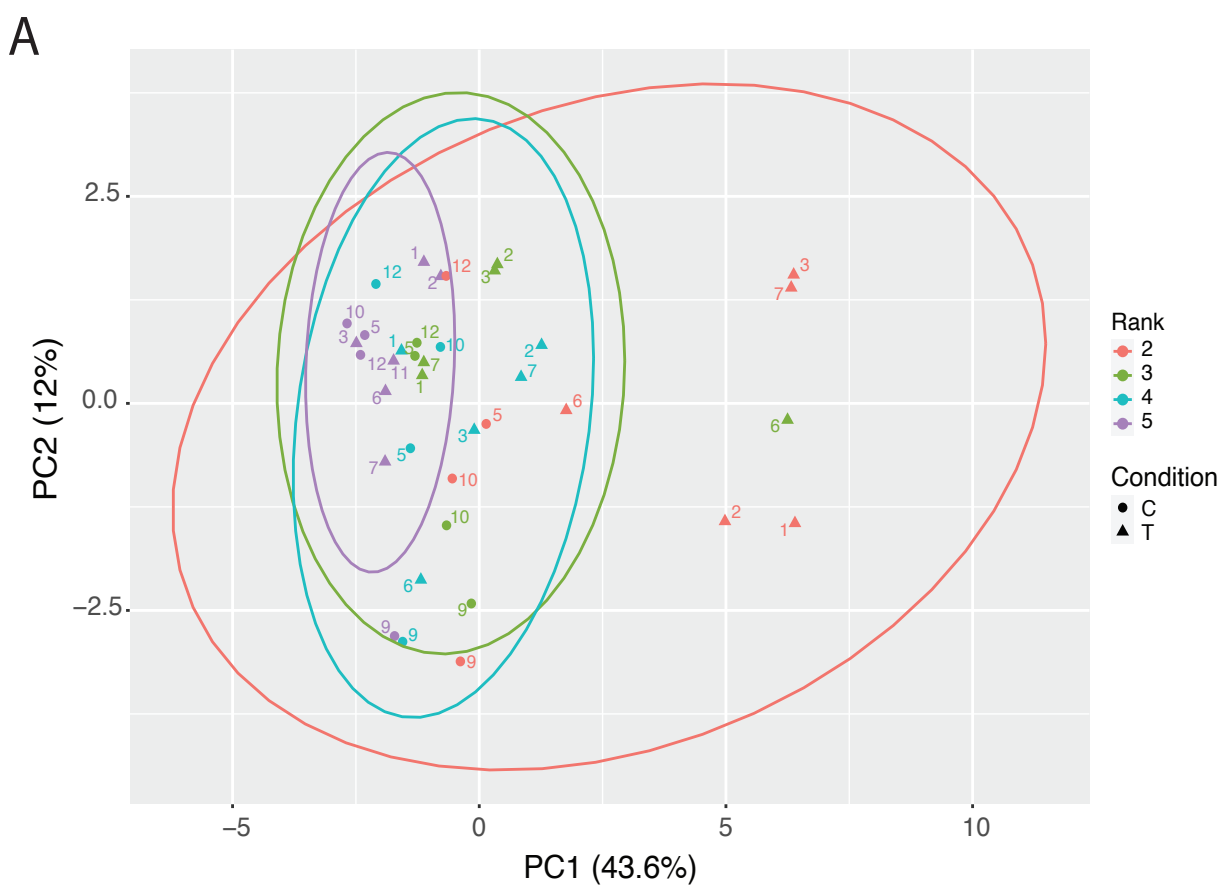
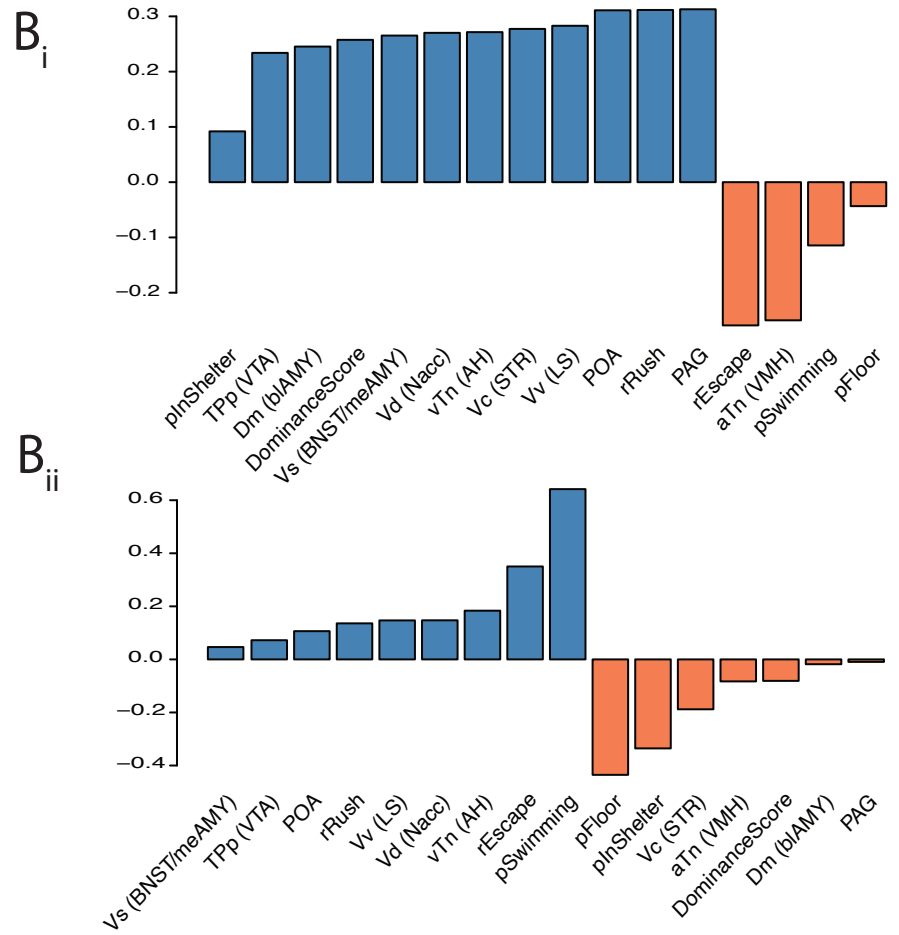
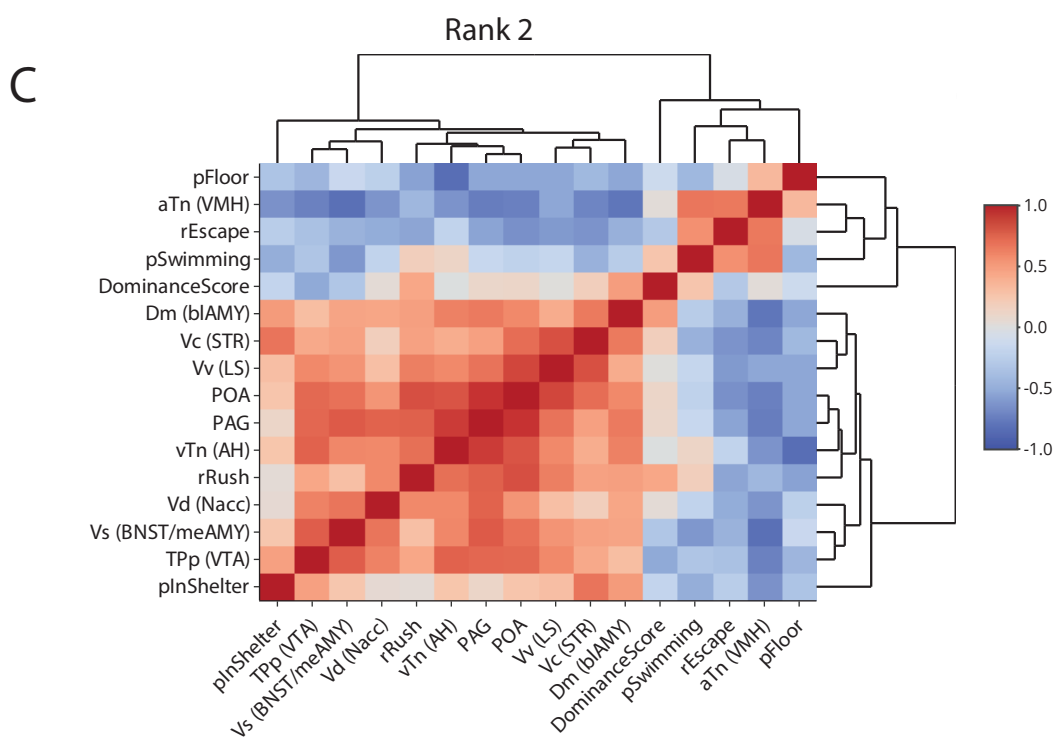


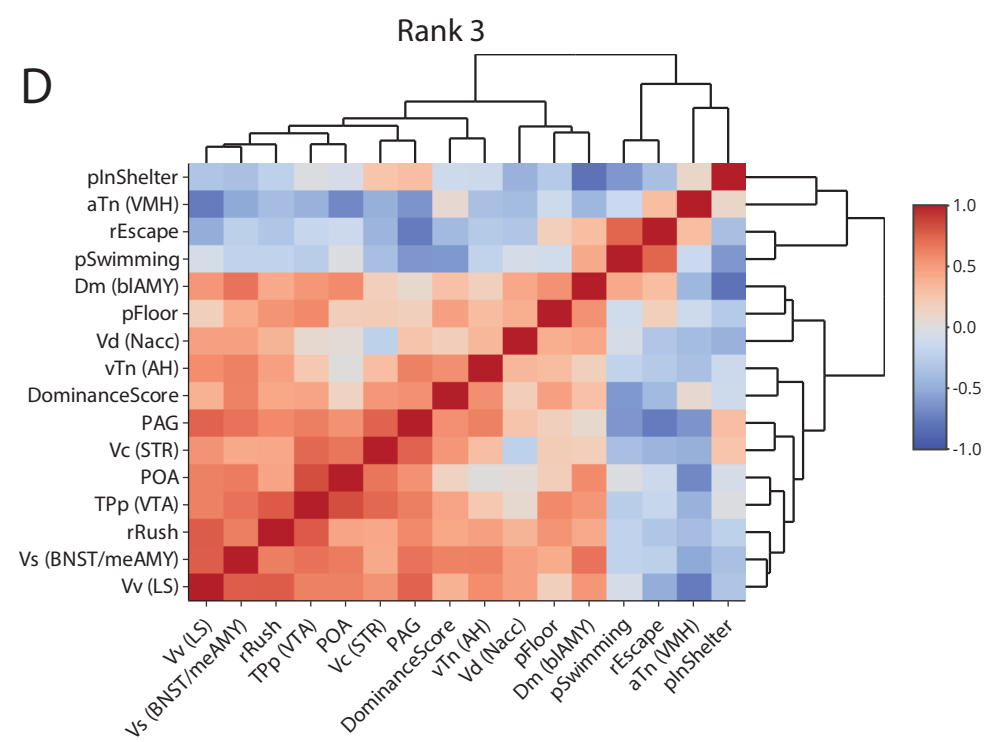
Figure 3. Behavior during group formation and after social disruption. The tanks that received the control treatment are represented in blue with behavior during group formation (Before Control) in light blue, and after social disruption (After Control) in royal blue. The tanks in the experimental treatment are in pink/red, with pink showing behavior during group formation (Before Treatment) and red showing behavior after social disruption (After Treatment). Rank 1 does not have data for the experimental treatment as it was removed from the tank. On the significance bars, a period represents marginal significance ($p > 0.1$).

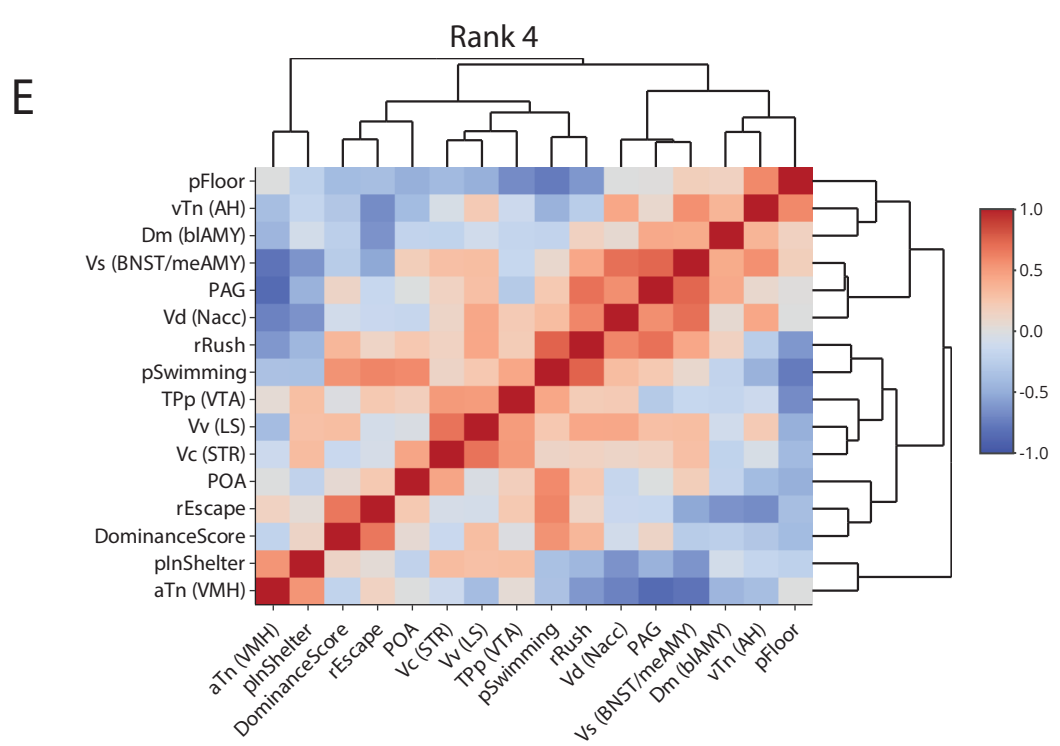

A
i
Dm rank 2
ii
Dm rank 3
iii
pS6-stained cells/μm²
2
3-5
2c
3-5c
B
Vd rank 2
Vd rank 3
C
Vv rank 2
Vv rank 3
D
Vs rank 2
Vs rank 3
E
Vc rank 2
Vc rank 3
F
POA rank 2
POA rank 3
G
vTn rank 2
vTn rank 3
H
PAG rank 2
PAG rank 3
I
TPp rank 2
TPp rank 3
J
aTn rank 2
aTn rank 3
K
Dm
Vs
Vd
Vc
Vv
PAG
TPp
POA
vTn
aTn
Dorsal
Rostral
Caudal
Ventral
500 μm

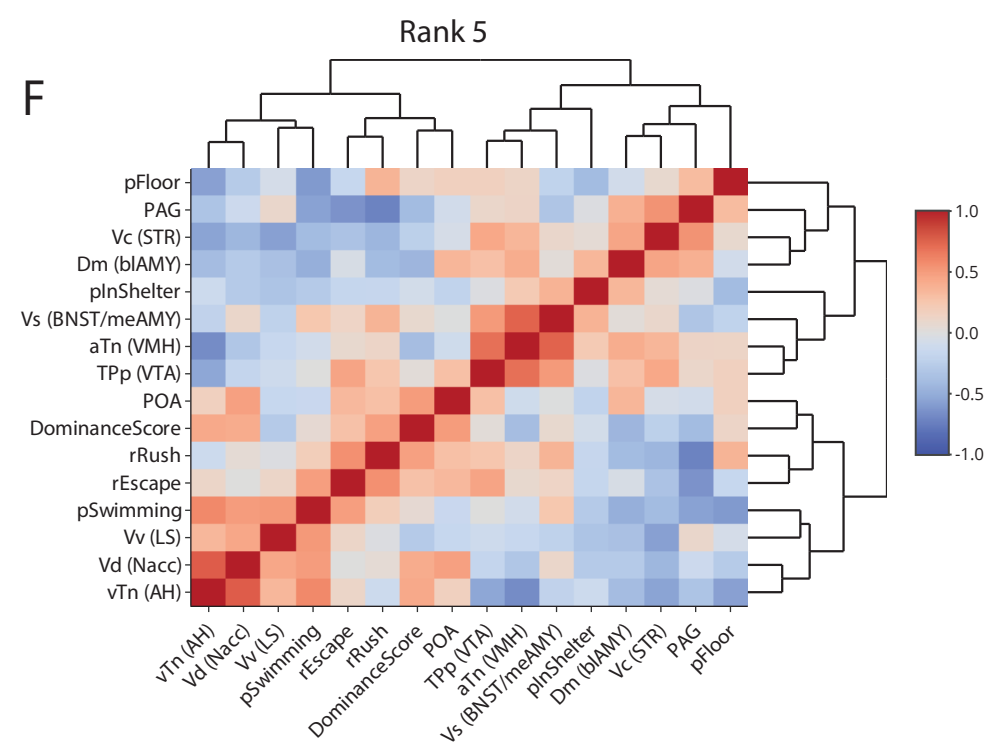

A
PC2 (12%)
PC1 (43.6%)
Rank
2
3
4
5
Condition
C
T


B_i
pInShelter
TPp (VTA)
Dm (blAMY)
DominanceScore
Vs (BNST/meAMY)
Vd (Nacc)
vTn (AH)
Vc (STR)
Vv (LS)
POA
rRush
PAG
rEscape
aTn (VMH)
pSwimming
pFloor
B_ii
Vs (BNST/meAMY)
TPp (VTA)
POA
rRush
Vv (LS)
Vd (Nacc)
vTn (AH)
rEscape
pSwimming
pFloor
pInShelter
Vc (STR)
aTn (VMH)
DominanceScore
Dm (blAMY)
PAG


C
Rank 2
pFloor
aTn (VMH)
rEscape
pSwimming
DominanceScore
Dm (blAMY)
Vc (STR)
Vv (LS)
POA
PAG
vTn (AH)
rRush
Vd (Nacc)
Vs (BNST/meAMY)
TPp (VTA)
pInShelter


D
Rank 3
pInShelter
aTn (VMH)
rEscape
pSwimming
Dm (blAMY)
pFloor
Vd (Nacc)
vTn (AH)
DominanceScore
PAG
Vc (STR)
POA
TPp (VTA)
rRush
Vs (BNST/meAMY)
Vv (LS)


E
Rank 4
pFloor
vTn (AH)
Dm (blAMY)
Vs (BNST/meAMY)
PAG
Vd (Nacc)
rRush
pSwimming
TPp (VTA)
Vv (LS)
Vc (STR)
POA
rEscape
DominanceScore
pInShelter
aTn (VMH)


F
Rank 5
pFloor
PAG
Vc (STR)
Dm (blAMY)
pInShelter
Vs (BNST/meAMY)
aTn (VMH)
TPp (VTA)
POA
DominanceScore
rRush
rEscape
pSwimming
Vv (LS)
Vd (Nacc)
vTn (AH)