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Social status mediates behavioral, endocrine, and neural responses to an intruder challenge in a social cichlid, *Astatotilapia burtoni*

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ABSTRACT

Most animals encounter social challenges throughout their lives as they compete for resources. Individual responses to such challenges can depend on social status, sex, and community-level attributes, yet most of our knowledge of the behavioral and physiological mechanisms by which individuals respond to challenges has come from dyadic interactions between a resource holder and a challenger (usually both males). To incorporate differences in individual behavior that are influenced by surrounding group members, we use naturalistic communities of the cichlid fish, *Astatotilapia burtoni,* and examine resident dominant male responses to a territorial intrusion within the social group. We measured behavior and steroid hormones (testosterone and cortisol), and neural activity in key brain regions implicated in regulating territorial and social dominance behavior. In response to a male intruder, resident dominant males shifted from border defense to overt attack behavior, accompanied by decreased basolateral amygdala activity. These differences were context dependent – resident dominant males only exhibited increased border defense when the intruder secured dominance. Neither subordinate males nor females changed their behavior in response to a territorial intrusion in their community. However, neural activity in both hippocampus and lateral septum of subordinates increased when the intruder failed to establish dominance. Our results demonstrate how a social challenge results in multi-faceted behavioral, hormonal, and neural changes, depending on social status, sex, and the outcome of an intruder challenge. Taken together, our work provides novel insights into the mechanisms through which individual group members display context- and status-appropriate challenge responses in dynamic social groups.

1. Introduction

Throughout their lives, most animals encounter social challenges and opportunities. An enduring question is how, and why, individuals respond to such situations with context-appropriate behavior (O'Connell and Hofmann, 2011). For example, aggressive responses to challenges, such as territorial intrusions, are often accompanied by an increase in circulating androgens (Goymann et al., 2019; Moore et al., 2020; Wingfield et al., 1990). However, most studies have focused on dyadic encounters, which fail to capture interactions across individual group members that can affect the response to a territorial intrusion (Nilsson et al., 2014). While focused mainly on behavior rather than responses to aggressive challenges, there has been a resurgence in studies that examine the effects of individual variation within social groups. This research demonstrates that heterogeneity in behavior or other phenotypes across group members can influence group-level processes such as collective decision making or movement (Couzin et al., 2002; Farine et al., 2015; Jolles et al., 2017; Rodriguez-Santiago et al., 2020; Schaerf et al., 2016). It is also well known that an individual behavioral trait may not be expressed in the social group depending on environmental conditions (Dussutour et al., 2008), the type of behavioral trait (Koski and Burkart, 2015), or the presence and composition of the group (McDonald et al., 2016). Finally, simply observing or being observed by other group members can also result in dramatic changes in individual behavior (Coppinger et al., 2017; Zajonc, 1965). For example, territorial males will adjust their response to an intruder based on the

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intruder identity (familiar vs. unfamiliar; Weitekamp and Hofmann, 2017) or the response of surrounding group members to the intruder (Christensen and Radford, 2018). To understand the causes and consequences of social challenges – such as territorial intrusions – in group-living species it is therefore critical to examine the neural, physiological, and behavioral responses to such dramatic stimuli across different members of a social group.

Individual responses to an intruder depend on underlying patterns of physiology and neural activity (e.g., Marler and Trainor, 2020). Across vertebrates, circulating androgens, such as testosterone, are key mediators of behavior during aggressive (e.g., Wingfield et al., 1990, revisited by Goymann et al., 2019), reproductive (e.g., reviewed by Marler and Trainor, 2020), and other social contexts (e.g., reviewed by Fuxjager and Schuppe, 2018; Oliveira, 2009). Testosterone can regulate behavior either directly or indirectly – after aromatization to estradiol – by binding to nuclear receptors that act as transcription factors throughout the brain (Davey and Grossmann, 2016; Eder et al., 2001). Many brain regions that are responsive to sex steroid hormones and have been implicated in different components of territorial behavior are part of an evolutionarily conserved interconnected social decision-making network (SDMN) that has been proposed to regulate and reinforce context-appropriate social behavior across vertebrates (O'Connell and Hofmann, 2011, 2012). These SDMN nodes include the *hippocampus*, which plays a critical role in spatial memory in mammals (see reviews by Hojo and Kawato, 2018; Murakami et al., 2018; Ophir, 2017) and birds (Colombo and Broadbent, 2000) and has functional equivalent putative homologs in non-avian reptiles (Butler, 2017) and in teleost fish (Elliott et al., 2017; Trinh et al., 2019; Vinepinsky et al., 2020). The *basolateral amygdala* differentially modulates anxiety-related behaviors and plays a key role in defensive (reviewed by Marler and Trainor, 2020) and sexual behaviors (reviewed by Petrulis, 2013) across taxa (reviewed by O'Connell and Hofmann, 2011). The *nucleus accumbens* differentially modulates social approach and social vigilance in mammals (Williams et al., 2020) and its putative teleost homolog responds to several odor types in dominant compared to subordinate males in cichlid fish (Nikonov and Maruska, 2019). The *lateral septum* is involved in regulating aggressive behavior across vertebrates (e.g., mammals: Blanchard et al., 1977, Lischinsky and Lin, 2020; birds: Goodson et al., 2005; reptiles: Font et al., 1998; teleosts: Oldfield et al., 2015). The *extended medial amygdala*, including the *bed nucleus of the stria terminalis*, is central to neural circuits underlying aggression across taxa (Lischinsky and Lin, 2020). It is selectively active based on the social status of the cue source in mice (Lee et al., 2021) and during social ascent to dominance status in cichlid fish (Maruska et al., 2013). Finally, the *preoptic area (POA)* plays a key role in social behaviors such as sexual and aggressive displays in all vertebrates studied to date (Goodson, 2005; Hull and Dominguez, 2006; Newman, 1999; O'Connell and Hofmann, 2012) and may mediate the establishment of a territory (Eastman et al., 2020; Hahn et al., 2019; Spool et al., 2016, 2019; Villavicencio et al., 2021; Zhao et al., 2020). Almost all studies that have investigated the role of these SDMN nodes in responses to an intruder challenge or other aggressive context have focused on dyadic interactions. Consequently, the neurobiology of an integrated response to territorial intrusions within social groups remains poorly understood.

Burton's Mouthbrooder cichlid, *Astatotilapia burtoni*, is a highly social teleost fish species characterized by greatly divergent patterns of behavior, space use, and physiology between socially dominant or subordinate males. Behaviorally, dominant males are characterized by high levels of aggression and territorial defense that allow them to secure reproductive opportunities with potential mates and suppress the maturation and chance for reproductive success of subordinate males (Maruska, 2014; Maruska and Fernald, 2018; O'Connell et al., 2013). Dominant males also exhibit higher levels of sex steroids than subordinates (Maruska et al., 2013; O'Connell et al., 2013), but these phenotypic and molecular differences are rapidly reversible, and males can ascend or descend in response to social or physiological changes many times during their life (Hofmann and Fernald, 2001; Maruska and Fernald, 2013). Differences in response to social defeat have also been classified across individuals and distinct measures of behavior and physiology have been characterized for two sub-types of dominant male (Butler et al., 2018). Proactive males attempt to escape the stressor, and have low stress-induced circulating cortisol levels, low brain serotonin levels, and high brain dopamine levels in response to social defeat. In contrast, reactive males do not attempt to escape the stressor, have high hypothalamic-pituitary-adrenal (HPA) axis reactivity and brain serotonin levels, and low brain dopamine levels. To which extent these differences in the response of dominant males to a social challenge are evident in more naturalistic group settings has not been examined. Male *A. burtoni* modify their behavior by observing other individuals (Grosenick et al., 2007; Desjardins et al., 2012; Alcazar et al., 2014) and can learn the implied hierarchy vicariously (as 'bystanders') by watching fights between rivals (Grosenick et al., 2007). Specifically, males will modify aggressive behavior based on the presence or absence (Desjardins et al., 2012), or the identity (familiar or unfamiliar: Weitekamp and Hofmann, 2017; Weitekamp et al., 2017) of other males. The complex social cognition exhibited by *A. burtoni*, along with remarkable social plasticity and established associations between neural activity and social dominance behavior, provide a unique opportunity to examine the response to an intruder challenge across all members of a naturalistic social group.

In the present study, we used naturalistic communities of *A. burtoni* to test the hypothesis that, depending on their sex and social status, individual group members respond to a territorial intrusion differently at the level of behavior, physiology, and neural activity in key nodes of the SDMN. We further hypothesized that these responses depend on the outcome of the intrusion (i.e., whether the intruder can successfully establish a territory or fail to do so). We first established eight communities in naturalistic enclosures, which allowed us to estimate space use along with well-studied territorial (aggressive and reproductive) and social displays for all resident dominant males. We then introduced a socially dominant male into each community and examined the social behavior and space use in territory-holding dominant males displayed in response to this intruder. Finally, we quantified hormone levels (testosterone and cortisol) and neural activity patterns in dominant and subordinate males as well as females. We used immunohistochemistry of the phosphorylated ribosomal protein S6 (pS6) as a neural activity marker in key nodes of the SDMN (for homology inference, see O'Connell and Hofmann, 2011): two subdivisions (granular and ventral) of the *lateral part of the dorsal telencephalon* (putative teleost homolog of the *hippocampus; Dlg, Dlv*); two subdivisions of the *medial part of the dorsal telencephalon* (putative teleost homolog of *basolateral amygdala; Dm1, Dm3*); the *dorsal* (putative homolog of *nucleus accumbens; Vd*), *ventral* (putative homolog of *lateral septum; Vv*), and *supracommissural* (putative homolog of the extended *medial amygdala/bed nucleus of the stria terminalis*; Vs) nuclei of the *ventral telencephalon*; and the preoptic area (*POA*). Finally, we used multivariate analyses to test our hypotheses and gain an integrative understanding of the social status and sex-dependent responses of group members to a social challenge.

2. Methods

2.1. Experimental design

Burton's Mouthbrooder cichlids (*Astatotilapia burtoni*) descended from a wild caught stock population and were maintained in stable communities. For this experiment, we created eight communities (two sets of four over four months) (Fig. 1). Each community contained eight males and eight females, and social hierarchies formed spontaneously such that one to five dominant males ascended to dominance status per community. All fish were tagged with colored beads attached to a plastic tag (Avery-Dennison, Pasadena, CA) for individual identification. The tag was inserted at least one week before the experiment began using a

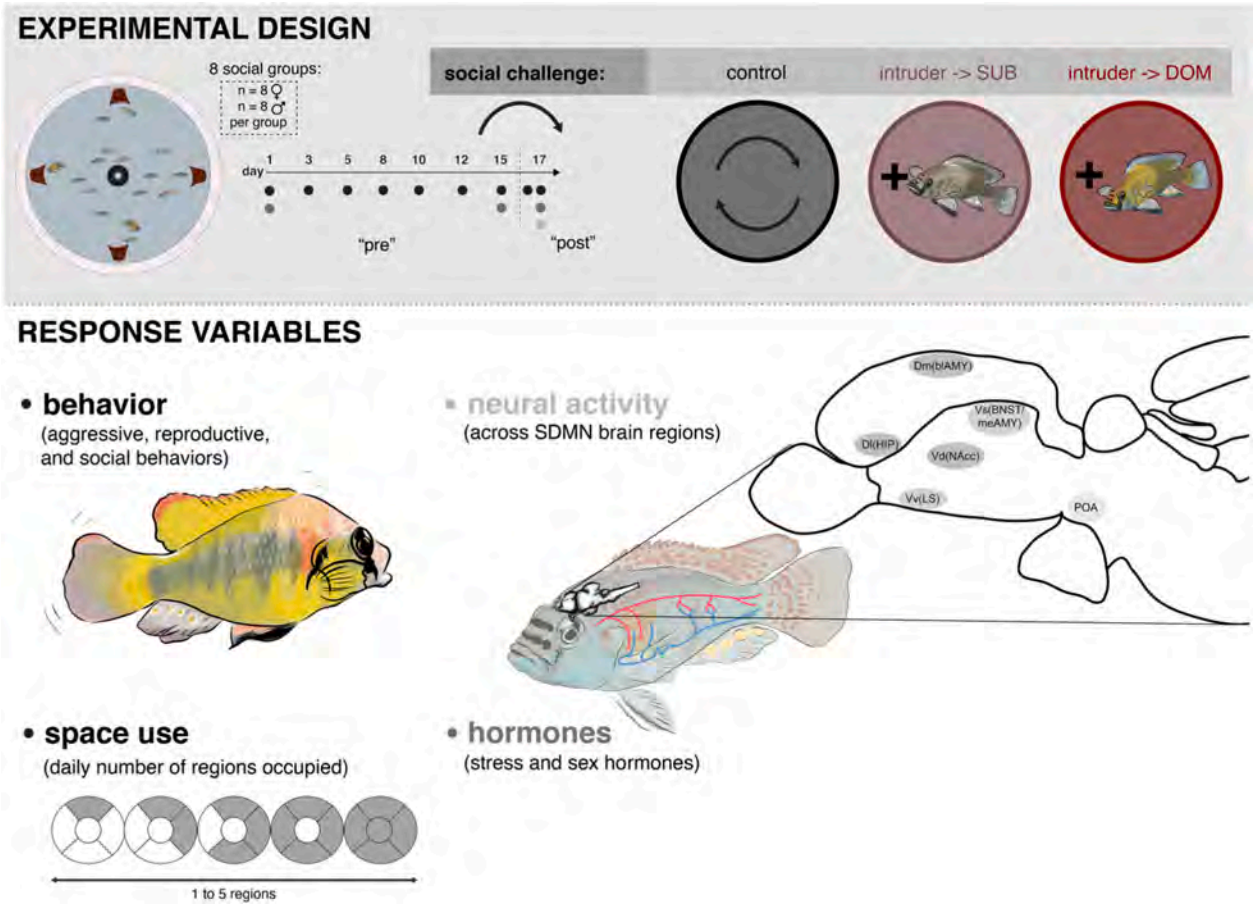

Fig. 1. Experimental design to investigate behavioral, endocrine, and neural responses to an intruder challenge across resident dominant males. To quantify how socially dominant male *A. burtoni* respond to an intruder in their social group, we used eight circular enclosures that each contained eight males and eight females. A two-week period was provided for social dominance hierarchies to form within each social group, and males were classified as socially dominant once they were able to successfully defend a territory for 2 or more consecutive days of observation. The remaining males were classified as socially subordinate and associated with other subordinate males and females rather than establishing a territory. After behavioral observation and hormone collection on day 15, every social group was removed from their enclosure and held in a bucket. In two control social groups, individuals were then returned to their enclosure; in six treatment groups, an intruder male was added to the enclosure before the social group returned and all groups were monitored for two days. For all social groups, space use patterns of the social group and individual dominant males, plus aggressive, reproductive, and social behavior of each dominant male was recorded every other day. Stress (cortisol) and sex (testosterone) hormones were collected on days 1, 15 and 17. Brains of every member of each social group were collected at the end of the experiment on day 17.

stainless-steel tagging tool (Avery-Dennison) through the skin just below the dorsal fin (left side on males, right side on females) and each individual of a given sex had one of eight colors (red, orange, yellow, green, light blue, lilac, brown, and black). Each social group was housed in a circular enclosure (diameter: 94 cm, height: 23 cm, area: ~0.7 m^2, volume: ~160 L) with an air filter in the center. Gravel was provided as substrate and four terracotta pots were equally spaced along the perimeter to facilitate the establishment and maintenance of territories necessary for reproduction. Fish were fed daily with cichlid flakes (Omega One Cichlid Flake Fish Food, Arcata Pet Supplies, Arcata, CA) and a 12:12 h light:dark cycle was maintained. All work was carried out in accordance with the Institutional Animal Care and Use Committee at the University of Texas at Austin.

Each community was allowed to acclimate to the enclosure for one to two weeks before the 17-day experiment began. The first two weeks of the experiment provided time for social hierarchies to be established. On day 15, every individual within a community was captured by net and held individually in a 400 mL glass beaker for hormone collection (see below) for 30 min before being transferred to a bucket with other community members and held for approximately 15 min. During this time, a socially subordinate male was removed and a dominant intruder male was added to each experimental enclosure ($n = 6$), except for the control groups ($n = 2$), and individuals were then returned to their home enclosure. Individual and group-level responses were measured for two days following this social challenge. The total length (mm), standard length (mm), body mass (g), and condition (body mass [g]*100/ standard length $[mm]^3$) were measured for every individual on day 1 (start of the experiment), day 15 (social challenge), and day 17 (end of the experiment). Sizes ranged from 51 mm – 67 mm and 3.43 g – 8.04 g for males and 45 mm – 63 mm and 2.06 g – 6.91 g for females. Sizes of intruder males ranged from 55 mm – 75 mm and 4.09 g – 9.80 g. The relative size of dominant resident males compared with intruder males ranged from 84 % to 102 % (i.e., the intruder was the largest male in almost all communities, though not by much).

2.2. Behavior

We recorded each community for one hour starting at 10:00 h using overhead video surveillance cameras (Alibi) three days per week for the duration of the experiment. Each 1-h video recording was analyzed for 10 min (10:20–10:30 h). First, the community was surveyed daily, and males that successfully defended a territory for two or more consecutive days were considered socially dominant. Any males that did not successfully defend a territory for two or more consecutive recording periods were classified as subordinate. During the two weeks before we removed members of the social group for the sham perturbation ($n = 2$

control groups) or intruder challenge ($n = 6$ social groups) there were 23 dominant males and 40 subordinate males (Supplementary Table 1). One male was discovered to be a female at the end of the experiment, so was included in all analyses as a female rather than a male. We estimated space use for each dominant male daily by first subdividing each circular enclosure into four peripheral segments (each with a terracotta pot) and one central segment, followed by counting the number of segments an individual occupied over the 10 min observation period. We used the same approach for estimating space use of the shoal that is formed by subordinate males and females in this species. We scored the behavior of each dominant male using BORIS (Friard and Gamba, 2016), including aggressive displays (lateral display, border conflict, attack), reproductive displays (court, lead), and social approaches (approach) based on established ethograms for this species (Fernald and Hirata, 1977; Supplementary Table 2). Lateral displays (identified as a courting display when directed at a female), attacks, and approaches were quantified separately based on the sex of the target. When intruder males were added to six of the social groups on day 15, they were assessed as socially dominant (or subordinate) using the same criteria described above (i.e., dominant males must successfully defend a territory for $\geq$2 consecutive days). Note that a resident dominant male did not need to lose dominance status for an intruder male to become socially dominant. Immediately following the intruder challenge (or perturbation) on day 15, three resident dominant males lost dominance status ($n = 20$ dominant males remained), and two more resident dominant males lost dominance status by the end of the experiment ($n = 18$ dominant males at the end; Supplementary Table 1). In addition, six subordinate males died by the end of the experiment and were excluded from analyses.

2.3. Hormone sampling and analysis

On experimental days 1, 15, and 17, each male was captured by net after behavioral observations and held in an autoclaved glass beaker with 300 ml of clean aquarium water for 30 min. All samples were collected between 11:00–13:00 h to minimize diurnal effects and stored at −20 °C until processing. Solid phase extraction (SPE) was used to extract hormones from holding water and Enzyme Immuno-assay (EIA) was used to determine concentrations of testosterone and estradiol according to previously established protocols (Friesen et al., 2012; Kidd et al., 2010). While 11-ketotestosterone (11-KT) is the major androgen controlling male reproduction in many teleost fish species (Borg, 1994), levels of circulating 11-KT are an order of magnitude lower and more variable than those of testosterone in male *A. burtoni* and other haplochromine cichlids (Dijkstra et al., 2012; Kidd et al., 2010), so we focused on testosterone instead. Briefly, holding water samples were thawed to room temperature overnight and extraction of steroid hormones was performed using a Sep-Pack Plus C18 cartridge (Waters #WAT020515) attached to a 12-sample vacuum manifold (VWR #CABJ9400) that was stored at −20 °C until elutions. Cartridges were thawed to room temperature approximately 30 min and free fractions of steroid hormones were eluted using ethyl acetate. The eluted solvent was immediately dried under a constant stream of nitrogen gas using an Evap-O-Rac drying rack (Cole-Parmer #01610–15) and the steroid pellet was stored at −20 °C until resuspension for enzyme immune-assay (EIA). Waterborne testosterone and cortisol were determined in separate assays using commercial EIA kits from Cayman Chemicals, Ann Arbor, Michigan (Testosterone #582701.1–96, Cortisol #582121.1–96) and EIA kits were run according to the manufacturer instructions. Hormone concentrations are presented as pg/ml according to the manufacturer instructions and normalized against body mass (in g) because smaller fish have a higher relative standard metabolic rate than larger fish and this can influence how much hormone is released in water (Killen et al., 2010). Waterborne hormone assays have been previously validated as representative for circulating hormone levels in this species (Kidd et al., 2010). The cross reactivity of the testosterone assay with another major androgen found in teleost fish, 11-ketotestosterone, is 2.2 %; the cross reactivity of the cortisol assay with another major steroid found in teleost fish, cortisone, was 0.13 % (Cayman Chemicals, Ann Arbor, Michigan). In our study, the intra-assay coefficient of variation (CV) was 4.65 % and the inter-assay CV was 4.62 % for testosterone. We found higher variation in the cortisol assay, with an intra assay CV of 11.72 % and an inter assay CV of 11.69 %.

2.4. Sample processing and immunohistochemistry (IHC) to examine neural activity

After behavioral observation and hormone sampling at the end of experimental day 17, animals were euthanized by rapid cervical transection, brains were removed and fixed overnight in 4 % paraformaldehyde at 4 °C, then washed in 1× phosphate-buffered saline (PBS) and cryo-protected overnight in 30 % sucrose at 4 °C, and finally embedded in O.C.T. compound (Tissue-Tek; Fisher Scientific Co., Pittsburgh, PA, USA) and stored at −80 °C. Brains were sectioned on a cryostat at 30 μm and thaw-mounted onto Super-Frost Plus slides (Fisher Scientific) in four series that were stored at −80 °C until further processing. We used immunohistochemistry (IHC) to visualize phosphorylated ribosomal protein S6 (pS6), a structural component of the ribosome that becomes phosphorylated when neurons are activated (Knight et al., 2012; Ruvinsky and Meyuhas, 2006). For brightfield detection of pS6, one series of sections was removed from −80 °C, dried on a slide warmer, and processed for IHC as described previously (Weitekamp and Hofmann, 2017) and stained with the neural activity marker, phospho-S6 ribosomal protein (pS6). This antibody has been previously validated *in A. burtoni* (Butler et al., 2018). Briefly, slides were fixed in 4 % PFA for 10 min, rinsed in 1× PBS, then quenched in H_20_2 for 20 min. Slides were then washed twice in 1× PBS and incubated in a mix of 2 % normal goat serum (NGS), 0.3 % Triton X-100 and 1:500 pS6 primary antibody (Cell Signaling pS6 ribosomal protein S235/236 antibody) overnight at 4 °C. Slides were rinsed in 1× PBS, incubated in 1:250 biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) for 2 h at room temperature, rinsed in 1× PBS, and incubated with Vectastain ABC (Vectastain Elite GRP ABC Kit, Fisher Scientific) for 1 h at room temperature then rinsed in 1× PBS. Stainaing was visualized by reaction with DAB for 2–3 min, rinsed in water, dehydrated in an alcohol series, cleared in xylene, and cover-slipped with Permount.

2.5. Quantification of activated neurons using phosphorylated ribosomal protein S6 (pS6)

Slides were coded such that the experimenter was blind to treatment. We quantified pS6 staining in eight brain regions implicated in social behavior (putative mammalian homologs according to O'Connell and Hofmann, 2011): two subdivisions (granular: *Dlg*; and ventral: *Dlv*) of the lateral part of the dorsal telencephalon (area *Dl*, putative *hippocampus* homolog, especially dentate gyrus: Elliott et al., 2017), two subdivisions (*Dm1*, *Dm3*) of the medial part of the dorsal telencephalon (area *Dm*: putative homolog of the *basolateral amygdala*), the dorsal part of the ventral telencephalon (area *Vd*: putative partial homolog of the *nucleus accumbens*), the ventral part of the ventral telencephalon (area *Vv*: putative partial homolog of the *lateral septum*), the supracommissural nucleus of the ventral telencephalon (area Vs: putative homolog of the extended *medial amygdala*, including the *bed nucleus of the stria terminalis*), and the preoptic area (*POA*). Brain regions were analyzed for all members of a given community (females, subordinate males, and dominant males; Table 1). For each brain region and individual, pS6-positive cells were counted across three sections using the Optical Fractionator workflow of the StereoInvestigator software (Microbrighfield, Williston, VT, USA). Briefly, a region of interest was defined using a 2× objective, then pS6-positive cells were counted using a 20× objective. The counting frame and sampling grid parameters varied for each brain region to account for differences in cell density and area of each region (Vv, Vd, POA: 25 × 25 counting frame, 75 × 75

Table 1
Brain sections collected for pS6 immunohistochemistry to quantify neural activity across eight nodes of the social decision-making network (SDMN): two subdivisions (granular and ventral) of the *lateral part of the dorsal telencephalon* (putative teleost homolog of the *hippocampus; Dlg, Dlv*); two subdivisions of the *medial part of the dorsal telencephalon* (putative teleost homolog of *basolateral amygdala; Dm1, Dm3*); the *dorsal* (putative homolog of *nucleus accumbens; Vd*), *ventral* (putative homolog of *lateral septum; Vv*), and *supracommissural* (putative homolog of the extended *medial amygdala/bed nucleus of the stria terminalis*; Vs) nuclei of the *ventral telencephalon*; and the preoptic area (*POA*).The numbers below reflect sample sizes for each brain region in females, subordinate males, and dominant males.

Putative mammalian homolog	*Teleost brain region* *Part* *Subdivision*	**females**	**males** **SUBs**	**DOMs**	**all**
	Dorsal telencephalon				
Hippocampus	*Lateral (Dl)*				
	Granular (Dlg)	47	24	21	45
	Ventral (Dlv)	44	25	21	46
Basolateral	*Medial (Dm)*				
amygdala	*Subdivsion 1 (Dm1)*	39	19	18	37
	Subdivsion 3 (Dm3)	44	22	20	42
	Ventral telencephalon				
Nucleus accumbens	*Dorsal (Vd)*	45	23	21	44
Lateral Septum	*Ventral (Vv)*	49	24	21	45
Extended Amygdala	*Supracommisural (Vs)*	49	23	21	44
Preoptic Area	*Preoptic Area*				
	Preoptic Area (POA)	42	25	20	45

sampling grid; Dlg, Vs: 30 × 30 counting frame, 100 × 100 sampling grid; Dm1, Dm3, Dlv: 50 × 50 counting frame, 150 × 150 sampling grid). For each brain region, data are presented as the estimated population of pS6-positive cells using number weighted section thickness divided by the area of the region. We confirmed that there was not a correlation between fish standard length and pS6-stained cell numbers in any brain region across both contexts (data not shown).

2.6. Data analysis

All data were analyzed in R studio ("Double Marigold", Version 1.2.5042) and statistical tests were conducted using the "stats" package (Version 3.6.2). To test for expected differences between subordinate and dominant males, we used a multivariate regression to test the effect of status (subordinate or dominant) on patterns of hormones (cortisol, testosterone) and morphology (standard length, condition) before (pre-) and after (post-) the social challenge. General linear mixed effects models were also used to look for multivariate differences across subordinate and dominant males over time with individual as a random factor (to account for repeated measures) and social group as a random factor (to account for the effect of the social group) using the "lme4" and "afex" packages. All other analyses reported were restricted to the post-challenge time point.

To examine whether dominant male traits were correlated we used Spearman's rank correlation test. To examine differences across dominant males depending on a) treatment (challenge vs. control), and b) challenge outcome (intruders that were successful or unsuccessful in establishing dominance status) we employed multivariate regression analyses that included hormone levels, size and condition, behavior, space use, and neural activity patterns across the eight brain regions examined. Analyses of neural activity patterns were also conducted separately. Note that analyses focused on challenge outcome were restricted to individuals from social groups that experienced an intruder. Finally, to examine the multivariate response of dominant males based on a) treatment and b) challenge outcome, a principal component analysis (PCA) was conducted. We carried out separate PCAs on i) behavior, space use, and hormone levels, ii) neural activity only, and iii) behavior, space use, hormones, and neural gene activity for dominant males both pre- and post-challenge. All PCAs presented as figures include all the traits described above (iii). We then performed one-way ANOVA to determine whether dominant males separated along either the first or second principal component axis due to either a) treatment (challenge vs. control) or b) challenge outcome (intruders that were successful or unsuccessful in establishing dominance status). General linear mixed effects models were also used to look for multivariate differences across resident dominant males over time with individual as a random factor (to account for repeated measures) and social group as a random factor (to account for the effect of the social group) using the "lme4" and "afex" packages.

To examine neural differences across community members that did not directly participate in the behavioral response to the challenge (i.e., the "audience") depending on a) treatment, and b) challenge outcome we employed multivariate regression analyses that focused on neural activity patterns across the eight brain regions examined.

3. Results

3.1. Social behavior and androgen levels vary with social status

We first asked whether patterns of behavior, body length and condition, and circulating steroid hormones (cortisol and testosterone) across subordinate ($n = 40$) and dominant ($n = 23$) males in our naturalistic communities prior to the social perturbation would replicate the differences often observed in this species in smaller enclosures. Note that one male was discovered to be a female at the end of the experiment, so was included in all analyses as a female rather than a male. As expected, subordinate males rarely, if ever, engaged in any aggressive or reproductive behaviors, so these behaviors were quantified only in dominant males. Before the social challenge, there were no status differences in standard length ($R^2_{adj} = -0.004$, $F(1,57) = 0.78$, $p_{adj} = 0.380$), condition ($R^2_{adj} = 0.031$, $F(1,57) = 2.86$, $p_{adj} = 0.096$) or cortisol levels ($R^2_{adj} = -0.004$, $F(1,57) = 0.75$, $p_{adj} = 0.390$), but dominant males had significantly higher testosterone levels ($R^2_{adj} = 0.105$, $F(1,57) = 7.80$, $p_{adj} = 0.007$) than subordinates, as expected (Table 2).

Since testosterone differed between subordinate and dominant males, we used a general linear mixed effects model and Akaike Information Criterion (AIC) scores to understand which variables affected this difference while accounting for the effect of the social group that individual reside in. We used an iterative process and compared AIC scores to select a model that best represented the variables that influenced testosterone levels in subordinate and dominant males (Supplementary Table 3), The addition of individual as a random effect (to account for repeated measures over time) and the social group as a

Table 2
Patterns of morphology and hormones in subordinate and dominant males pre- and post-perturbation. Values presented represent mean ± standard error for standard length (mm), condition (body mass [g]*100/standard length [mm]^3), testosterone per body mass (pg/ml/g), and cortisol per body mass (pg/ml/g) over the course of the experiment. All post-perturbation values, including the gonadosomatic index (GSI), were collected at the end of the experiment.

	Subordinate males		Dominant males	
Trait	Pre (n = 40)	Post (*n* = 39)	Pre (n = 23)	Post (n = 18)
Standard length (mm)	59.33 ± 3.70	59.38 ± 0.64	60.13 ± 0.80	60.11 ± 0.86
Condition ([body mass in g] *100/[standard length in mm]^3	2.52e-03 ± 0.04e-03	2.53e-03 ± 0.04e-03	2.60e-03 ± 0.03e-03	2.62e-03 ± 0.06e-03
Testosterone/body mass (pg/ml/g)	38.85 ± 8.68	36.93 ± 6.14	104.62 ± 21.84	88.42 ± 13.93
Cortisol/body mass (pg/ml/g)	73.81 ± 8.75	78.90 ± 10.10	95.79 ± 30.14	57.35 ± 8.83
Gonadosomatic Index, GSI (gonad mass in g/body mass in g)	NA	0.67 ± 0.04	NA	0.75 ± 0.06

random effect (to account for the influence of the social group) had no significant effect, and subordinate and dominant males still exhibited significantly different levels of testosterone when accounting for individual differences and the effect of the social group (Supplementary Table 3).

3.2. Dominant males increase aggressive displays but reduce territorial defense and neural activity in Dm1 in response to an intruder challenge

To understand how dominant males respond to an intruder challenge, we first examined the effect of an intruding male within the social group on the behavior and circulating hormone levels of resident dominant males after the social perturbation. We found that across all

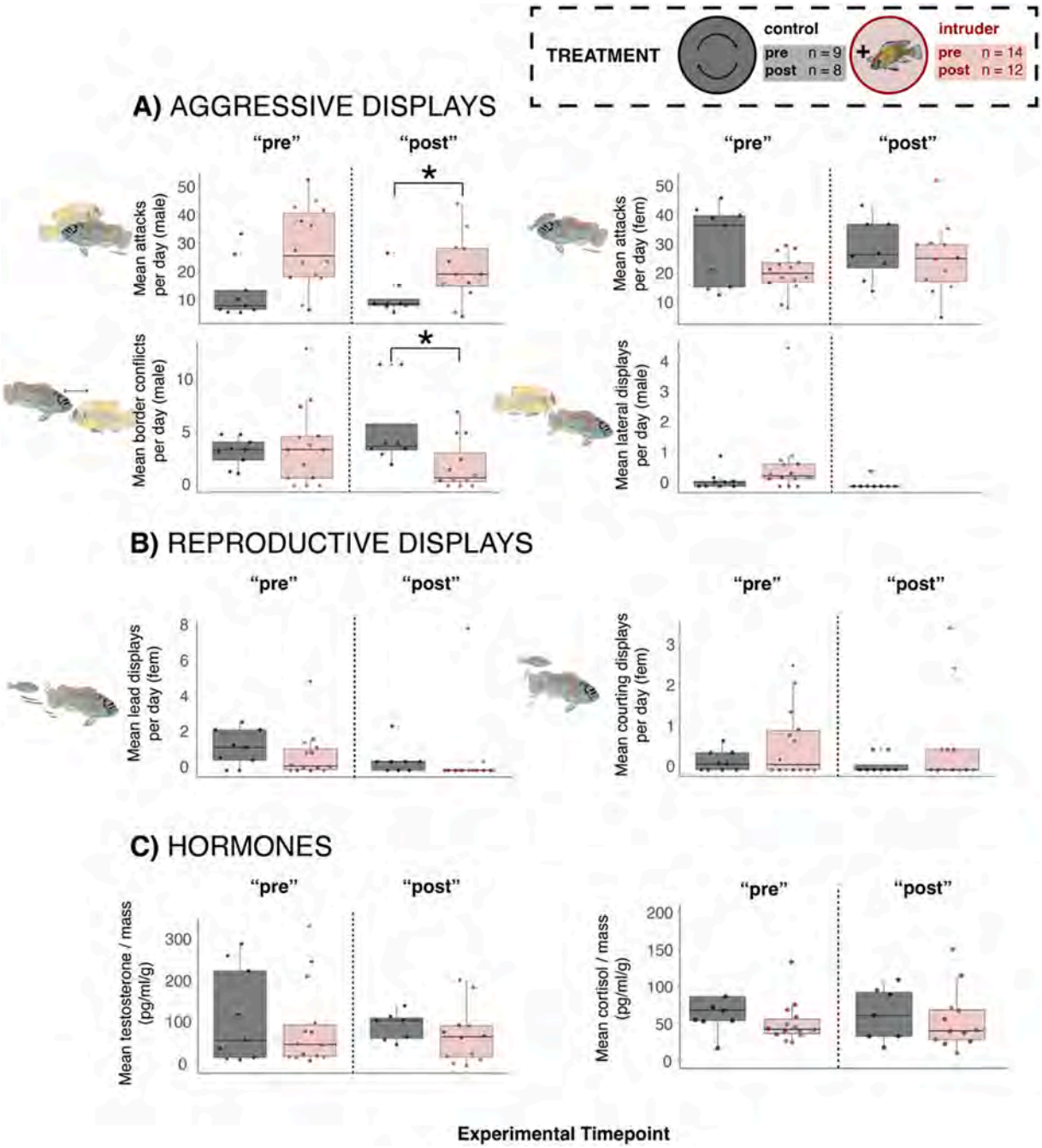

Fig. 2. Patterns of behavior and hormones in resident dominant males in social groups before ("pre") and after ("post") a sham perturbation ($n = 2$ control groups) or intruder challenge ($n = 6$ social groups). Dominant males in social groups that experienced an intruder (A) exhibited significantly more aggressive attacks but reduced border conflicts to defend their territories against other males post-challenge. There were no differences in attacks directed at females or lateral displays directed at other males at either time point. Resident dominant males from control groups or groups that experienced an intruder exhibited no differences in (B) leading or courtship displays directed at females in a reproductive context, or (C) circulating levels of sex (testosterone) or stress (cortisol) hormones at either time point. Box plots extend from the 25th to the 75th percentile, the whiskers extend from the smallest to the largest values 1.5× the interquartile range. and the median is represented as a line. Individual data points are plotted as filled circles and colored based on the group challenge experienced by each dominant male, including no intruder (grey) or an intruder present (mauve and red). Asterisks represent significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resident dominant males in our experiment ($n = 23$), three males initially lost dominance status in response to the social perturbation (due to either an intruder male or the control condition of removing and then replacing all group members) and another two dominant males lost dominance status the following day (Supplementary Table 1). In total, five resident dominant males lost their territory and associated dominance status by the end of the experiment (2 from control communities, 3 from communities that experienced an intruder) and joined the shoal of subordinates and females. Next, we found that dominant males who maintained their status despite an intruder challenge ($n = 12$) carried out significantly more attacks ($R^2_{adj} = 0.160$, $F(1,18) = 4.615$, $p_{adj} = 0.046$), but engaged in fewer border conflicts ($R^2_{adj} = 0.211$, $F(1,18) = 6.072$, $p_{adj} = 0.024$), compared to dominant males in control communities ($n = 8$) (Fig. 2A). There were no significant differences in hormones (testosterone or cortisol per body mass), composite scores of different types of behavior (aggressive, reproductive, or social), or space use based on the presence or absence of an intruder (Fig. 2B,C; Supplementary Table 2). Interestingly, aggression and space use were not correlated in resident dominant males before the social challenge (Supplementary Fig. 1; rho = −0.102, $p = 0.642$, $N = 23$), but showed significant correlation post-challenge in dominant males that experienced an intruder (Supplementary Fig. 1; rho = 0.697, $p = 0.012$, $N = 12$).

We then asked whether we could detect distinct patterns in neural activity across brain regions in the SDMN in response to intrusion. We counted pS6-positive cells (as a proxy of neural activity) in the Dlg, Dlv, Dm1, Dm3, Vd, Vv, Vs, and POA of resident dominant males who experienced an intruder ($n = 12$) and those who did not ($n = 8$). We found a significant difference only in Dm1 ($R^2_{adj} = 0.302$, $F(1,11) = 6.183$, $p_{adj} = 0.030$), where males who experienced an intruder within the social group showed reduced activity (Supplementary Fig. 2, Supplementary Table 4).

Because the multivariate nature of our dataset can obscure meaningful group differences when each measure is examined separately, we

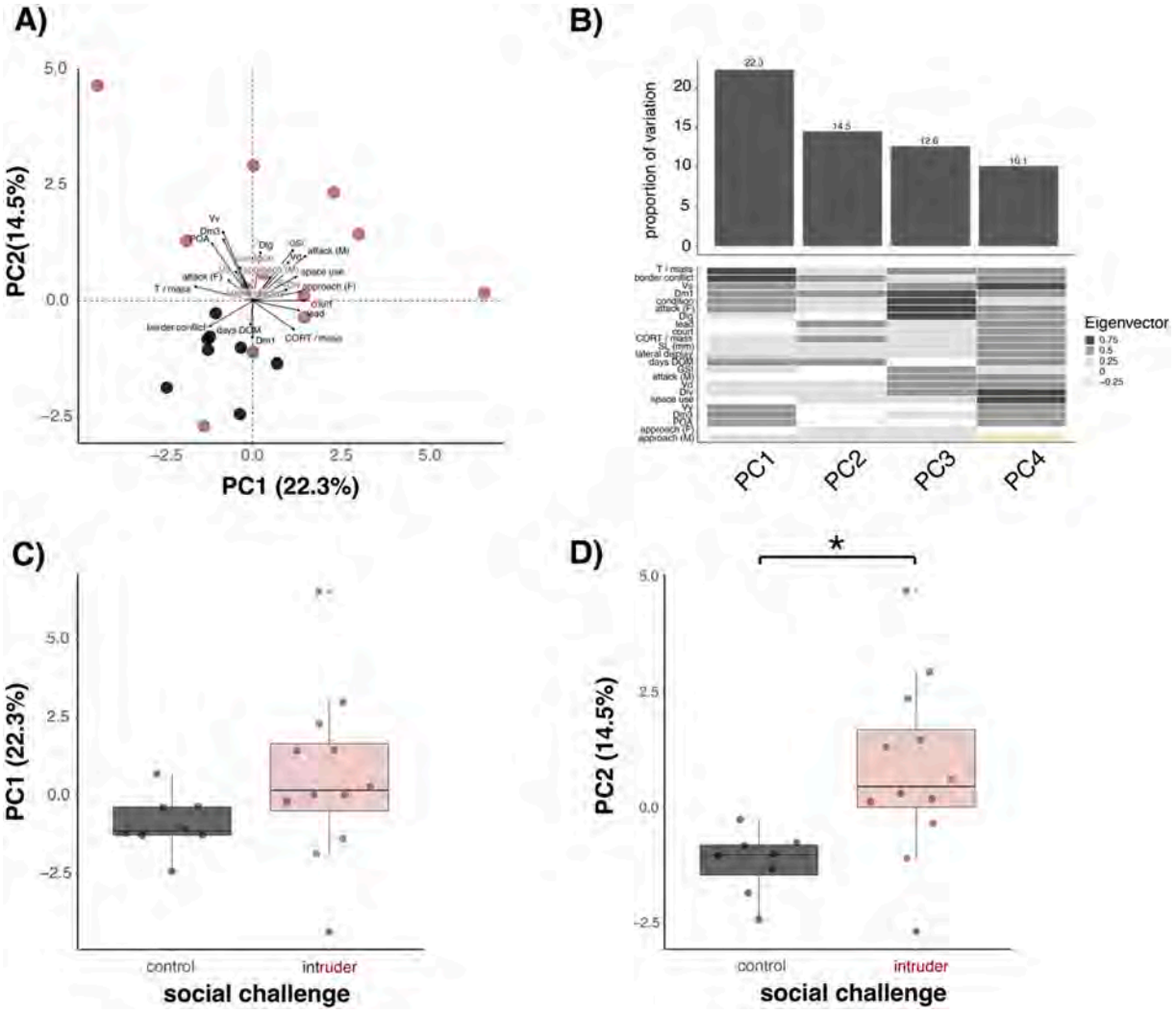

Fig. 3. Resident dominant males can be categorized based on the presence or absence of an intruder. (A) Principal Component Analysis (PCA) on morphology (condition, standard length, gonadosomatic index), days dominant, space use, aggressive displays (lateral display, attack, border conflict), reproductive displays (court, lead), social displays (approach), hormone levels (cortisol, testosterone), and neural activity across 8 brain regions (Dlg, Dlv, Dm1, Dm3, Vd, Vv, Vs, POA) in resident dominant males in social groups that did (pink) or did not (grey) experience an intruder. PC2 (explaining 14.5 %) separates dominant males with border conflicts, lead displays, and neural activity in Dm1 (all reduced in dominant males who experienced an intruder) loading most strongly. (B) PCA Eigenvector Plot represents the percentage of variation across resident dominant males explained by the first four PCs (above) along with a tile plot representing correlation of traits included in the PCA with each PC (below). Box plots compare multivariate variation across resident dominant males explained by PC1 (C) and PC2 (D), where individual data points represent resident dominant males in social groups that did (pink) or did not (grey) experience an intruder. See Fig. 2 for box plot descriptions. Asterisks indicate significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used Principal Component Analysis (PCA) to isolate the dimensions in variance space – including behavior (aggression, space use, reproductive and social displays), hormones (testosterone, cortisol), morphology (body length, condition), and neural activity patterns (expressed as cell counts) – in dominant males who did (n−12) or did not ($n = 8$) experience an intruder challenge (Fig. 3A). Testosterone, aggressive behavior, and (in the opposite direction) reproductive displays loaded most strongly on Principal Component 1 (PC1, which explains 22.3 % of the variance), which did not significantly separate the treatment groups ($F(1,18) = 2.247$, $p = 0.151$) (Fig. 3B,C). PC2 (14.5 %), however, significantly separated dominant males that experienced a social challenge from control males ($F(1,18) = 7.862$, $p = 0.0112$) (Fig. 3B,D). The factors that loaded most strongly on PC2 included border conflicts, lead displays, and neural activity in Dm1 (which are all reduced in dominant males who experienced an intruder challenge).

The variables that differed across resident dominant males, independent of treatment, included attacks and border conflicts directed at other males, as well as neural activity in area Dm1. For each of these response variables we used a general linear mixed effects model and AIC scores to understand which variables affected this difference while accounting for the effect of the social group that dominant males reside in. We used an iterative process and compared AIC scores to select a model that best represented the variables that influenced these traits across dominant males (Supplementary Table 5). Because neural activity was

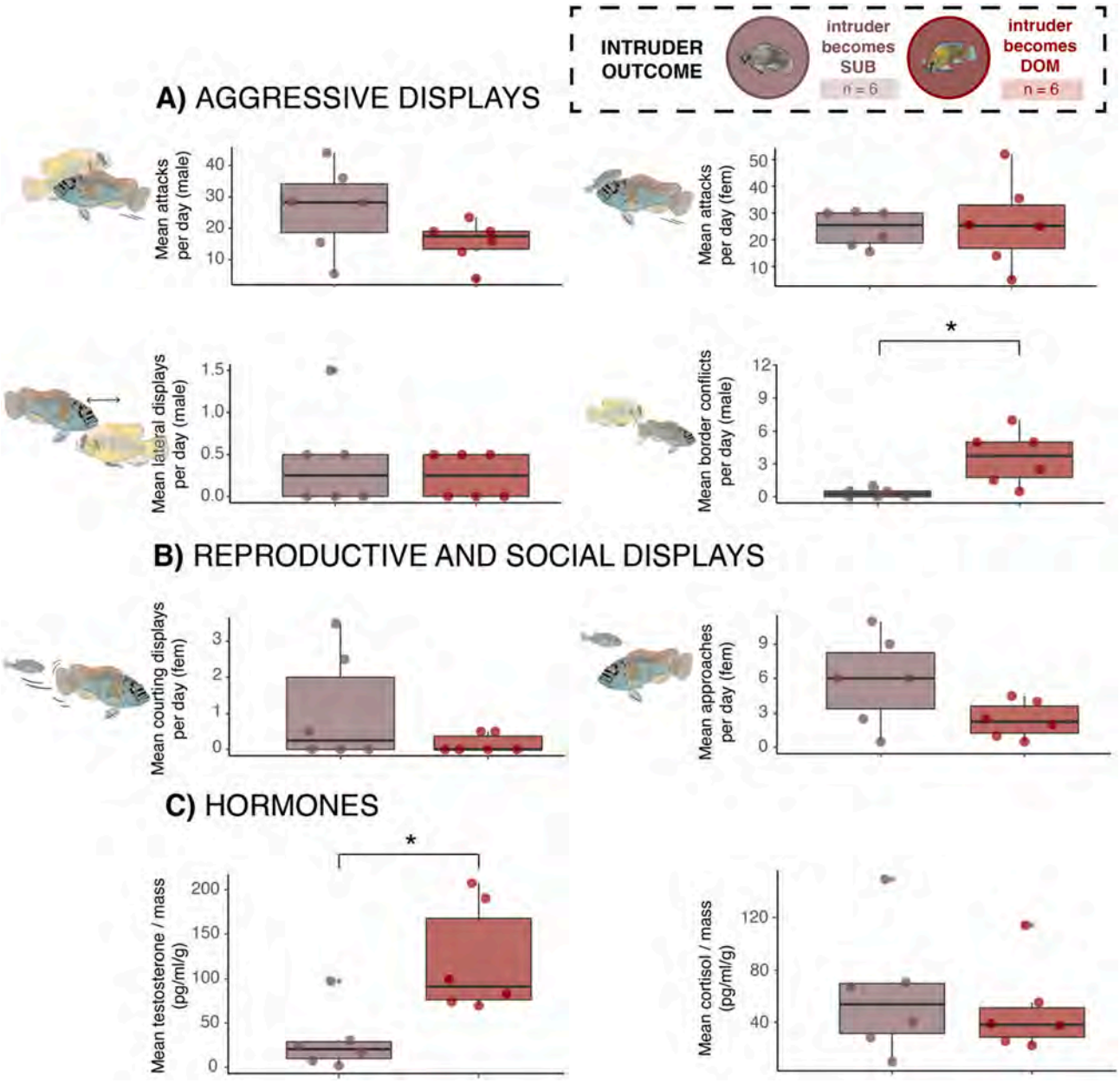

Fig. 4. Patterns of behavior and hormones in resident dominant males in social groups that experienced an intruder who ultimately became subordinate ($n = 4$ social groups) or dominant ($n = 2$ groups). Across resident males in social groups that experienced an intruder who became subordinate or dominant, there were no differences in (A) aggressive attacks directed at males or females, lateral displays directed at other males, or border conflicts to defend their territory. There were also no differences in (B) leading or courtship displays directed at females in a reproductive context, or (C) circulating levels of stress (cortisol) hormones though resident dominant males in groups that experienced an intruder who became dominant exhibited significantly higher levels of testosterone. See Fig. 2 for box plot descriptions. Individual data points are colored based on groups where an intruder became subordinate (mauve) or dominant (red). Asterisks indicate significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

only measured after the completion of the experiment, only the analyses of the post-challenge data include pS6-positive cell counts in Dm1 as a response variable. The addition of individual as a random effect (to account for repeated measures over time) and the social group as a random effect (to account for the influence of the social group) had no significant effect on attacks directed at other males or Dm1 activity. In other words, in the presence of an intruder, resident dominant males carried out significantly higher attacks toward other males and showed significantly decreased levels of Dm1 activity, compared to controls, even after accounting for individual differences and social group as random effects. While there was no significant effect of the intruder on border conflicts after controlling for repeated measures over time and the effect of the social group, there was a significant interaction effect between treatment (intruder or none) and time (pre- or post-challenge) on the number of border conflicts exhibited by dominant males (Supplementary Table 5).

3.3. Dominant males exhibited distinct responses based on the outcome of an intruder challenge

Despite being socially dominant in their community of origin, only two of the six intruder males (33 %) successfully established a territory in our study. These divergent outcomes allowed us to test the hypothesis that the response of resident dominant males to a social challenge differs depending on whether the intruder successfully establishes dominance status or not. We first examined patterns of behavior and circulating hormone levels across all resident dominant males who experienced an intruder challenge. We found that resident dominant males exhibited significantly higher numbers of border conflicts ($R^2_{adj} = 0.451$, $F(1,10) = 10.050$, $p_{adj.} = 0.010$) in communities where the intruder became

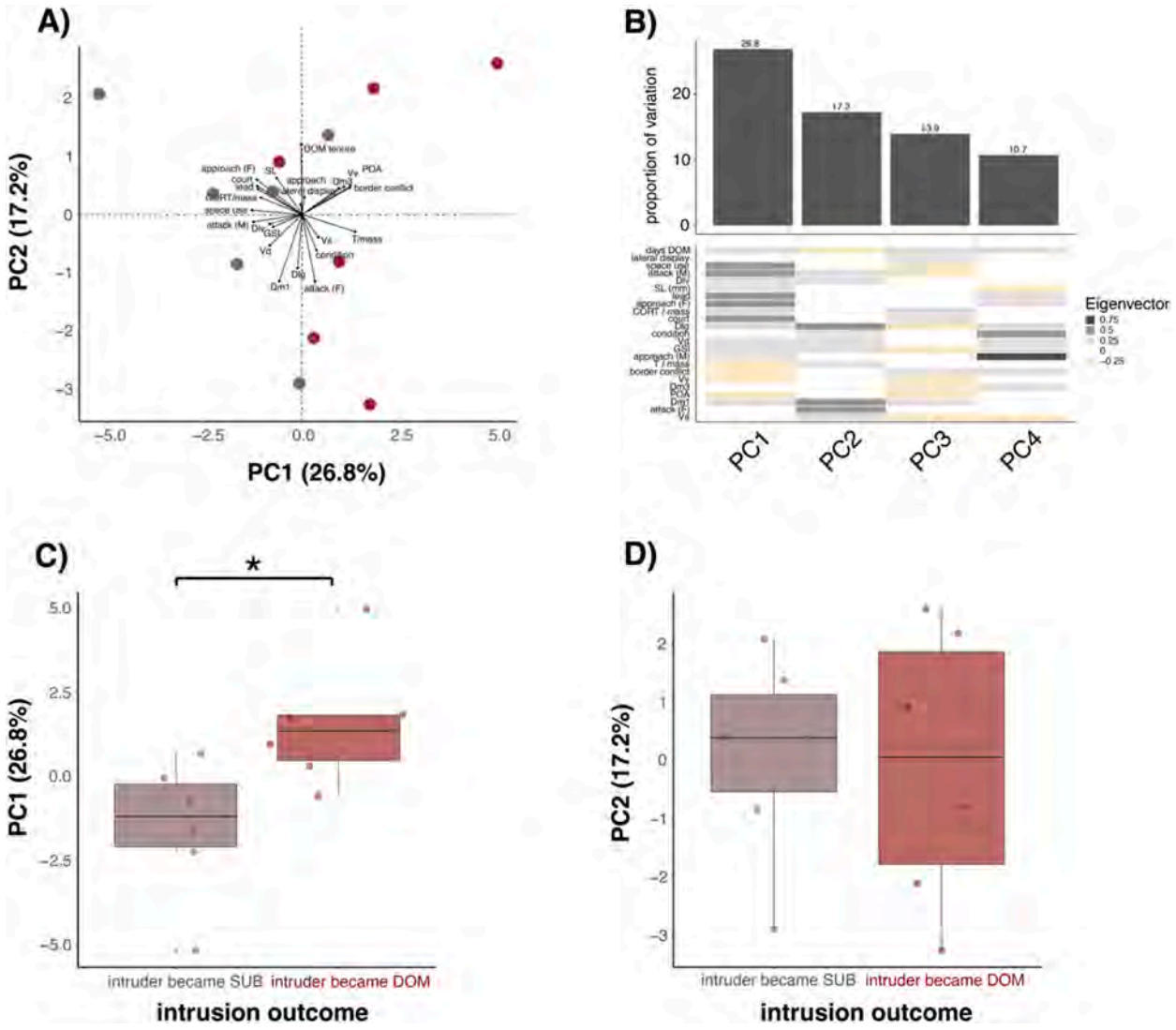

Fig. 5. Resident dominant males can be categorized based on the outcome of a social intruder challenge. (A) Principal Component Analysis (PCA) on morphology (condition, standard length, gonadosomatic index), days dominant, space use, aggressive displays (lateral display, attack, border conflict), reproductive displays (court, lead), social displays (approach), hormone levels (cortisol, testosterone), and neural activity across 8 brain regions (Dlg, Dlv, Dm1, Dm3, Vd, Vv, Vs, POA) in resident dominant males in social groups that experienced an intruder that became subordinate (mauve) or dominant (red). PC1 (explaining 26.8 %) separates resident dominant males with border conflicts and androgen levels (both higher in dominant males who experienced an intruder that became dominant), as well as space use, aggressive attacks, reproductive courting displays, and social approaches toward females (all higher in dominant males who experienced an intruder that became subordinate) loading most strongly. (B) PCA Eigenvector Plot represents the percentage of variation across resident dominant males explained by the first four PCs (above) along with a tile plot representing correlation of traits included in the PCA with each PC (below). Resident dominant males differ based on the outcome of the social intruder challenge when comparing variation explained by PC1 (C) but not PC2 (D). See Fig. 2 for box plot descriptions. Asterisks indicate significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dominant ($n = 6$) rather than subordinate (n = 6) (Fig. 4A). There was no difference in any other behavioral displays, composite behavior scores of different types of behavior (aggressive, reproductive, or social), space use, or hormone levels (testosterone or cortisol per body mass) (Fig. 4B, C; Supplementary Table 2). It should also be noted that none of the pre-challenge measures across dominant males predicted the eventual social status of the intruder (Supplementary Figs. 3–5). The relative size of resident dominant males to the intruder male did not differ between communities where the intruder secured dominance status (84–97 %) and those where the intruder failed to do so (91–102 %) (Kruskal-Wallis test: $\chi^2 = 1.103$, df = 1, $p = 0.294$; Supplementary Fig. 5B).

We then asked if we could detect distinct patterns in neural activity across brain regions in the SDMN based on the outcome of the intrusion. When we compared dominant males who experienced an intruder that became subordinate ($n = 6$) with those who experienced an intruder that became dominant (n = 6), there were no significant differences in cell counts, as a proxy for neural activity, in any of the eight brain regions investigated (Supplementary Fig. 6, Supplementary Table 6).

Because the multivariate nature of our dataset can obscure meaningful group differences when each measure is examined separately, we used PCA to examine the effect of intrusion outcome on patterns of behavior (aggression, space use, reproductive and social displays), hormones (testosterone, cortisol), morphology (body length, condition), and neural activity (expressed as cell counts) across brain regions from all resident dominant males who experienced an intruder (Fig. 5A). PC1 (explaining 26.8 % of the variance) significantly separated dominant males depending on whether they experienced an intruder that became subordinate or dominant ($F(1,10) = 7.118$, $p = 0.024$) (Fig. 5B,C). Distinct types of aggressive displays loaded most strongly on PC1 to separate dominant males that experienced an intruder that became either dominant or subordinate. Territorial defense (border conflicts) and androgen levels were higher in dominant males who experienced an intruder who became dominant, while overt aggressive (e.g., attacks), space use, reproductive displays (e.g., courts) and social displays (e.g., approaches) toward females were higher in dominant males who experienced an intruder who became subordinate. Dominant males did not separate depending on intrusion outcome along PC2 (17.2 %; $F(1,10) = 0.118$, $p = 0.897$) (Fig. 5D) or any other PC axis.

The variables that differed significantly across resident dominant males based on the outcome of the social intrusion were border conflicts directed at other males and levels of testosterone. We used separate general linear mixed effects models and AIC scores to understand which variables affected these differences while accounting for the effect of the social group that dominant males reside in. We used an iterative process and compared AIC scores to select a model that best represented the variables that influenced border conflicts and levels of testosterone across dominant males in groups with an intruder (Supplementary Table 7). The addition of individual as a random effect (to account for repeated measures over time) and the social group as a random effect (to account for the influence of the social group) had no significant effect on border conflicts. In contrast, while we found a significant effect of intruder outcome on testosterone levels using a model that ignored the social group, our best fit model with social group as a random effect had a higher AIC and reported no significant difference in testosterone levels based on the intruder outcome. In other words, resident dominant males carried out significantly higher border conflicts in groups where the intruder also became dominant, compared to groups were the intruder failed to establish dominance status, even after accounting for individual differences and social group as random effects (Supplementary Table 7). Interestingly, resident dominant males are characterized by higher levels of testosterone in groups where the intruder also became dominant, but these differences appear to be driven by the surrounding social group.

3.4. Subordinate males, but not females, exhibited distinct neural responses based on the outcome of an intruder challenge

While we did not measure any overt behavioral responses of the surrounding social group (i.e., subordinate males and females) to an intruder challenge, subordinate males and females are likely to closely observe the interactions of intruder and resident dominant males (Desjardins et al., 2012; Rodriguez-Santiago et al., 2020), which can subsequently affect neural activity patterns (Desjardins et al., 2010). We asked whether we could detect distinct patterns in neural activity across 8 brain regions in the SDMN (Dlg, Dlv, Dm1, Dm3, Vd, Vv, Vs, POA) in either subordinate males (control: $n = 9$; intruder: $n = 30$) or females (control: $n = 17$; intruder: $n = 48$). Note that this and subsequent analyses included the five males that descended from dominant to subordinate status after the social challenge. We found no significant differences in neural activity patterns based on the presence or absence of an intruder in the social group (Supplementary Fig. 2; Supplementary Table 4).

We then restricted our analyses to groups that had experienced an intruder and asked if we could detect distinct patterns in neural activity across 8 brain regions in either subordinate males (subordinate intruder: $n = 22$; dominant intruder: $n = 8$) or females (subordinate intruder: $n = 32$; dominant intruder: $n = 16$) based on the outcome of the intrusion. While there were no differences in neural activity in females based on the intrusion outcome, we found distinct patterns of neural activity in subordinate males (Supplementary Fig. 6; Supplementary Table 6). Specifically, subordinate males exhibited significantly higher levels of neural activity in the Dlg ($R^2_{adj} = 0.271$, $F(1,16) = 7.305$, $p_{adj} = 0.016$), Dlv ($R^2_{adj} = 0.406$, $F(1,16) = 12.620$, $p_{adj} = 0.003$), and Vv ($R^2_{adj} = 0.271$, $F(1,16) = 7.314$, $p_{adj} = 0.016$) in groups where the intruder was subordinate at the end of the experiment (Supplementary Fig. 6; Supplementary Table 6).

4. Discussion

Our study demonstrated that social context mediates the response of *A. burtoni* males to an intruder in the social group. Broadly, resident dominant males respond to an intruder male with increased aggressive displays directed at other males and decreased territorial defense through ritualized border conflicts. Dominant males also displayed a positive association between space use and aggression after a group-level intrusion. We found that the territorial phenotype of resident dominant males differed in social groups where the intruder failed to secure dominance status compared to groups where the intruder became dominant. Resident dominant males in groups where an intruder became dominant were characterized by increased defensive displays (border conflicts) and elevated testosterone levels (though differences in androgens appeared to be driven by the surrounding social group). In contrast, resident dominant males in groups where an intruder became subordinate tended to increase space use and exhibit more overt aggressive (attacks) and reproductive (court) displays, as well as social (approach) displays directed at females. While our study identified that resident dominant males can be separated based on their territorial phenotype and the outcome of an intruder challenge, it is important to note that further investigation is needed to assign a causal direction to this relationship between dominant male behavior and the intrusion outcome.

4.1. Resident dominant male aggression toward an intruder was not explained by androgen levels or relative size

Androgens and body size can influence an individual's behavioral and hormonal response to aggression (Alward et al., 2021; Ball and Balthazart, 2019; Goymann et al., 2019; Moore et al., 2020). Variation in hormone levels can be up to two orders of magnitude among individuals (Kempenaers et al., 2008, see also Williams, 2008), and differences in

baseline and/or maximum levels of androgens can impact hormonal responsiveness and subsequent aggressive behavior. In our study, we found large variation in testosterone levels across resident dominant males, but it was not associated with patterns in aggressive behavior or testosterone levels post-challenge. Differences in standard length also play a key role in determining social status and contest outcomes in *A. burtoni* (Hofmann et al., 1999; Alcazar et al., 2014; Grosenick et al., 2007), and the relative standard length of intruder males can predict the behavioral response of resident males (Alward et al., 2021; Weitekamp and Hofmann, 2017). Previous work has determined that male *A. burtoni* can perceive a 5 % difference in standard length between an opponent and themselves (Alcazar et al., 2014). When resident dominant males are exposed to an intruder that is size-matched (<5 % smaller or larger than intruder) or larger (>5 % smaller than intruder) in relative standard length, they are quicker to perform physical (chases) and non-physical (lateral displays) aggressive behaviors and exhibit an increased number of non-physical displays compared to resident dominant males exposed to a smaller intruder (Alward et al., 2021). In our study, relative standard length of intruder males compared with resident males did not predict whether the intruder male would become subordinate or dominant. This difference may be attributed to the naturalistic group-level intrusion used in our study compared to more commonly examined dyadic or triadic-level intrusions.

While we characterized attributes of the dominant male territorial phenotype that differed across intruder contexts, many group-level factors could influence the response to a territorial intrusion. For instance, group-level aggression can impact the response to an intruder in a social group – in the daffodil princess cichlid, *Neolamprologus pulcher,* aggressive acts at the level of the individual can initiate additional aggressive interactions at the group-level (Anderson et al., 2020). We tested for differences in group summed aggression as a proxy for group-level aggression and found no significant differences across groups.

4.2. SDMN neural activity patterns vary by social status and intrusion outcome

To investigate neural activity associated with territorial behavior in response to different intruder contexts, we examined neural activity across 8 brain regions implicated in social behavior. In dominant males, we found that neural activity in the Dm1 (teleost homolog of *basolateral amygdala*) was significantly lower in males who experienced an intruder compared to those who did not. Interestingly, Weitekamp and Hofmann (2017) previously characterized unique neural profiles of both resident and neighboring dominant *A. burtoni* males exposed to the same intruder. These authors found that in neighboring, but not resident, dominant males the likelihood of engaging with an intruder was associated with reduced neural activity in the Dm1. In another fish species, the mudskipper, the medial telencephalon was also suggested to be involved in audience reaction (Wai et al., 2006). Taken together, this suggests that the reduced neural activity in the Dm1 of dominant males exposed to an intruder within the social group in our study may reflect audience effects of a group-level intrusion.

In subordinate males, we found that neural activity in the Dlv, Dlg (putative homologs of the *hippocampus*), and Vv (putative partial homolog of the *lateral septum*) was increased in males from social groups where an intruder male was subordinate, rather than dominant, at the end of the experiment. Increased neural activity in the Dlv and Dlg of subordinate males experiencing an intruding male who lost dominance status is consistent with the proposed role of the hippocampus in spatial memory (see reviews by Hojo and Kawato, 2018; Murakami et al., 2018; Ophir, 2017). The increased activity in the Vv in subordinate males may play a role in the processing of aggressive behavior they observe and the salience of a territorial challenge when an intruder male fails to secure dominance status (Oldfield et al., 2015). Across the ventral telencephalon in *A. burtoni,* neurons respond to odor in a status-specific manner that facilitates differential sensitivity when males are reproductively active and/or defending a territory (Nikonov and Maruska, 2019). Taken together, these results suggest that neural activity in the Dlv, Dlg, and Vv of subordinate males may play a role in the processing of aggression and the salience of a territorial challenge when an intruder male loses dominant status.

5. Conclusions

Our study combined multivariate linear regression analysis and principal component analysis to characterize an integrated territorial response including behavioral (aggressive, reproductive, and social behavior), hormonal, and neural activity patterns to quantify how dominant, resident males maintain and defend their territories over time in response to a social group intrusion. We found that resident dominant males broadly responded to a group-level intrusion with increased aggressive displays and decreased territorial defense but that this response differed based on the outcome of intrusion (although the causal direction of this relationship is unclear). Further, we found that surrounding group members (subordinate males) also display unique profiles of neural activity that differ in social groups where an intruder male becomes subordinate or dominant. Taken together, this research demonstrates that social status mediates an integrative territorial response to an intruder in a dynamic social group.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105241.

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