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Vasopressin mediates nonapeptide and glucocorticoid signaling and social dynamics in juvenile dominance hierarchies of a highly social cichlid fish

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ABSTRACT

Early-life social experience can strongly affect adult behavior, yet the behavioral mechanisms underlying developmental trajectories are poorly understood. Here, we use the highly social cichlid, Burton's Mouthbrooder (*Astatotilapia burtoni*) to investigate juvenile social status and behavior, as well as the underlying neuroendocrine mechanisms. We placed juveniles in pairs or triads and found that they readily establish social status hierarchies, with some group structural variation depending on group size, as well as the relative body size of the group members. Next, we used intracerebroventricular injections to test the hypothesis that arginine vasopressin (AVP) regulates juvenile social behavior and status, similar to adult *A. burtoni*. While we found no direct behavioral effects of experimentally increasing (via vasotocin) or decreasing (via antagonist Manning Compound) AVP signaling, social interactions directed at the treated individual were significantly altered. This group-level effect of central AVP manipulation was also reflected in a significant shift in whole brain expression of genes involved in nonapeptide signaling (AVP, oxytocin, and oxytocin receptor) and the neuroendocrine stress axis (corticotropin-releasing factor (CRF), glucocorticoid receptors (GR) 1a and 1b). Further, social status was associated with the expression of genes involved in glucocorticoid signaling (GR1a, GR1b, GR2, mineralocorticoid receptor), social interactions with the dominant fish, and nonapeptide signaling activity (AVP, AVP receptor V1aR2, OTR). Together, our results considerably expand our understanding of the context-specific emergence of social dominance hierarchies in juveniles and demonstrate a role for nonapeptide and stress axis signaling in the regulation of social status and social group dynamics.

1. Introduction

Early-life environments can have strong and lasting impacts on organismal phenotypes (Bateson, 2001; Bateson et al., 2004; Weaver, 2009). For social species, social stimuli are among the most influential in the early environment (Branchi et al., 2013a; Champagne and Curley, 2005; Taborsky et al., 2012; Taborsky, 2016), and juveniles can spend a substantial portion of their time interacting socially, including with parents (maternal, paternal, or biparental interactions, Champagne and Curley, 2005; McClelland et al., 2011), siblings (Branchi et al., 2013a; Buist et al., 2013; D'Andrea et al., 2007), parents with (and without) helpers (Arnold and Taborsky, 2010; Taborsky et al., 2012), as well as other members of the social group (Branchi et al., 2006, 2013b; D'Andrea et al., 2007; Smith et al., 2010; White et al., 2002). Observing social interactions among other group members can also influence juvenile experience and behavior, such as juvenile bonobos observing social conflict and consolation (Clay and de Waal, 2013). Together, these attributes of the early social environment influence the quantity and quality of social experiences and sensory cues perceived, which in turn shape behavioral phenotype via persistent changes in the underlying neural mechanisms (e.g., Antunes et al., 2021; Branchi et al., 2013a; Champagne and Curley, 2005; McClelland et al., 2011). Studies of early-life social experiences are critical to understanding how gene-by-environment interactions drive developmental plasticity and variation

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in adult phenotypes (Taborsky, 2017). Individual variation in social behavior, in particular, is relevant to fitness and health outcomes (Bennett et al., 2006; Meyer-Lindenberg and Tost, 2012; Silk, 2007; Solomon-Lane et al., 2015; Wilson, 1980), suggesting that behavioral development is a potent target for natural selection.

The behavioral mechanisms underlying variation in behavioral development are the specific attributes of social interactions experienced in early-life that cause long-term changes in behavior. Across species, these processes remain understudied (Kasumovic, 2013; Taborsky, 2016). In general, social interactions are determined by the social organization of a species, the local structure and dynamics of a social group, as well as ecological factors (Chase, 2022; Creel et al., 2013; Drews, 1993; Emlen and Oring, 1977; Krause et al., 2010; Saltz et al., 2016; Shizuka and Johnson, 2020). Even within a shared environment, social experiences vary across individuals, starting in early life. For example, mouse pups in a mixed age, communal nest receive varying levels of maternal care and sibling interaction, which affects their social behavior (Branchi et al., 2013a), and the social network position of young male long-tailed manakins predicts future reproductive success (McDonald, 2007). Across species, social dominance hierarchies are a common form of social organization, and they exert powerful effects on social experience. Social status is defined by asymmetrical agonistic interactions, directed from dominant individuals to subordinates, such that subordinates consistently yield to dominants (Chase and Seitz, 2011; Drews, 1993; Wilson, 1980). Social dominance is most often studied in adults, in part, because one of the most important benefits of dominance is access to reproductive opportunities (Ellis, 1995). However, juveniles of many species also form status relationships, including juvenile crayfish, *Procambarus clarkii* (Girard) (Sato and Nagayama, 2012), bluebanded gobies, *Lythrypnus dalli* (Solomon-Lane et al., 2016), blue-footed boobies, *Sula nebouxii* (Drummond and Canales, 1998), primate species with inherited maternal rank (Engh et al., 2009), such as yellow baboons, *Papio c. cynocephalus* (Pereira, 2010), and humans (Thomsen, 2020), to name just a few. For juveniles and adults, status influences the nature, frequency, and outcome of social interactions within a group. For juveniles, these experiences can lead to developmental plasticity and altered adult phenotype (Taborsky, 2016).

The highly conserved nonapeptide, arginine vasopressin (AVP), constitutes a prime candidate mechanism by which social experience and behavior may shape behavioral development (Baran, 2017). Vasopressin has been well-studied, and it is an important source of variation in adult social behavior within and across species (Caldwell, 2017; Godwin and Thompson, 2012; Goodson and Bass, 2001; Goodson and Thompson, 2010; Kelly and Goodson, 2014). For example, AVP often varies across statuses and/or is associated with aggression, a key agonistic behavior in the establishment and maintenance of status (e.g., fish: Godwin and Thompson, 2012; Loveland and Fernald, 2017; Oldfield and Hofmann, 2011; Reddon et al., 2015; reptiles and amphibians: Wilczynski et al., 2017; birds: Goodson et al., 2012; mammals: Grieb et al., 2021; Lee et al., 2019b). Importantly, the direction of effect is species- and context-dependent (Goodson and Thompson, 2010; Kelly and Goodson, 2014).

In understanding the role of the early social environment in behavioral development, AVP has the potential to mediate both types of behavioral mechanisms: social sensory cues (e.g., AVP mediation of olfaction) and variation in social interactions (Baran, 2017; Taborsky, 2016). For example, early-life maternal separation in rat pups alters developmental changes in AVP and oxytocin receptors (Lukas et al., 2010), social play experience in juvenile rats activates AVP and oxytocin neurons in sex-specific ways (Reppucci et al., 2018), and early-life manipulation of AVP signaling affects zebra finch (*Taeniopygia guttata*) social behavior, including attachment, affiliation (Baran et al., 2016a), and adult pair maintenance behavior (Baran et al., 2016b). Early-life effects on nonapeptide signaling and behavior can persist into adulthood (Bales and Perkeybile, 2012; Branchi et al., 2013a; Veenema, 2012). Furthermore, AVP is a potent stimulator of the neuroendocrine stress axis (hypothalamic-pituitary-adrenal/interrenal in fish; HPA/I) (Aguilera and Rabadan-Diehl, 2000; Gesto et al., 2014), which is a primary mechanism by which environmental conditions and experience are translated into physiological responses (Crespi and Denver, 2005). Specifically, AVP can signal for the release of corticotropin-releasing factor (CRF) from the hypothalamus, causing the anterior pituitary to release adrenocorticotropic hormone, which signals for the release of glucocorticoids (cortisol in fish) from the adrenal / interrenal glands to affect physiology and behavior (Denver, 2009; Lowry and Moore, 2006; Wendelaar Bonga, 1997). Many early-life social effects exert long-term influence on adult phenotype via persistent changes in HPA/I axis function (Champagne and Curley, 2005; Crespi and Denver, 2005; Ensminger et al., 2018; Jonsson and Jonsson, 2014), such as altered neural glucocorticoid receptor (GR) expression (e.g., fish Antunes et al., 2021; Nyman et al., 2017; birds Banerjee et al., 2012; mammals: Champagne and Curley, 2005).

We investigated juvenile social experience and status, and potential regulation by AVP, in Burton's Mouthbrooder (*Astatotilapia burtoni*), a highly social African cichlid fish that has become a model system in social neuroscience (Fernald and Maruska, 2012; Hofmann, 2003). This species is well-suited to understanding early-life social experiences and the behavioral mechanisms of behavioral development. First, laboratory populations make it feasible to manipulate and observe all life history phases, including early life. Second, adults (Fernald and Maruska, 2012; Hofmann, 2003; Weitekamp et al., 2017) and juveniles (Fernald and Hirata, 1979; Solomon-Lane and Hofmann, 2019) express a suite of highly evolutionarily conserved social behaviors, including aggression, affiliation, courtship, and cooperation. Adult *A. burtoni* form mixed-sex, hierarchical communities with males of dominant or subordinate status, along with females (Fernald and Hirata, 1977; Fernald and Maruska, 2012). Social hierarchies also form in experimental, all-female groups (Renn et al., 2012). In the laboratory, juveniles readily form social status relationships in short (30 min) behavior assays (Solomon-Lane and Hofmann, 2019), as well as in stable social groups (Fernald and Hirata, 1979; Fraley and Fernald, 1982), which suggests status may be a useful framework for understanding how social experiences accrue during development. Third, early-life social experiences can trigger developmental plasticity (Alvarado et al., 2015; Fraley and Fernald, 1982; Solomon-Lane and Hofmann, 2019). Experimental manipulations of the early social environment—such as rearing juveniles in social groups, pairs, physical isolation, and total isolation—can alter growth rate, emergence of male nuptial coloration, reproductive maturation (Fraley and Fernald, 1982), social behavior (Fraley and Fernald, 1982; Solomon-Lane and Hofmann, 2019), and neuroendocrine gene expression (Solomon-Lane and Hofmann, 2019). Importantly, these studies did not observe behavior in the rearing environments; therefore, the specific experiences driving these effects are not yet known (Taborsky, 2016).

Finally, although it has yet to be investigated in juvenile *A. burtoni*, AVP has important roles in the regulation of adult social behavior and status (for review, see Maruska et al., 2022, *this issue*). Vasopressin and its receptors have been mapped throughout the adult brain (Butler et al., 2021; Greenwood et al., 2008; Huffman et al., 2012; Loveland and Fernald, 2017), and there are key overlaps with the social decision-making network, a highly-conserved set of brain regions that, together, are involved in the regulation of social behavior across vertebrates (Huffman et al., 2012; O'Connell and Hofmann, 2011). Vasopressin neurons are found in three cell populations of the preoptic area of the hypothalamus, which is a part of the social decision-making network: small, rostroventral parvocellular cells; caudal magnocellular cells; and caudal gigantocellular cells, which are the largest. These cell populations can vary depending on a variety of factors (Butler et al., 2021; Godwin and Thompson, 2012; Huffman et al., 2012; Kelly and Goodson, 2014; Loveland and Fernald, 2017; Silva and Pandolfi, 2019), including social status (Greenwood et al., 2008) and behavior (Loveland and Fernald, 2017) in adult male *A. burtoni*, and reproductive state and

behavior in adult female *A. burtoni* (Butler et al., 2021). In general, subordinate male *A. burtoni* behavior is associated with AVP expression in the parvocellular cell population, whereas dominance behavior is associated with AVP expression the magnocellular and gigantocellular AVP populations (Greenwood et al., 2008; Loveland and Fernald, 2017). Finally, brain V1aR receptor expression is increased in males ascending from subordinate to dominant status, and blocking that receptor reduces aggression in these animals (Huffman et al., 2015). In general, both nonapeptides—AVP and oxytocin—are relevant to this research due to the promiscuity of receptors, which can bind both ligands (Kelly and Goodson, 2014).

Here, we conducted two experiments to test the overarching hypothesis that *A. burtoni* social organization, and its regulation, is maintained across life history stages. In the first experiment, we observed the social behavior of juveniles over one week in pairs and triads that varied in the relative body sizes of group members (Fig. 1). We hypothesized that, like adults, juveniles form social hierarchies in all social groups and that, similar to adults (Alcazar et al., 2014; Alward et al., 2021; Weitekamp and Hofmann, 2017), relatively larger individuals are more likely to attain higher social status. Forming these very small groups allowed us to thoroughly study group dynamics and individual experiences in the simplest possible contexts. In addition, group structure and dynamics may not scale predictably with increasing group size (Chase et al., 2003); therefore, including multiple group types provides insight into a range of possible juvenile experiences. In the second experiment, we manipulated AVP activity using intracerebroventricular (ICV) injection of arginine vasotocin or the V1aR antagonist Manning Compound (MC). We used the triads containing a size matched pair (Fig. 1) because this treatment showed the least asymmetric antagonism in Experiment 1, and we hypothesized that AVP regulates the emergence of social status relationships and affects patterns of neural gene expression. In both drug- and vehicle-injected fish, we analyzed whole brain expression of candidate genes related to nonapeptide and HPA/I axis signaling, including AVP, AVP receptor V1aR2, IT, the isotocin receptor (ITR) CRF, GR subtypes 1a (GR1a), 1b (GR1b), 2 (GR2), and the mineralocorticoid receptor (MR). Together, these experiments aimed to uncover the social organization and experiences of juvenile *A. burtoni* to better understand behavioral development and its potential regulation by AVP.

2. Methods

2.1. Animals

The juveniles used in this study were a laboratory population of *A. burtoni* that have descended (~65–70 generations) from a wild-caught stock (Fernald and Hirata, 1977). The adult parents of the juveniles were housed in naturalistic, mixed-sex social groups. Dominant males are territorial, reproductively active, and colorful, whereas subordinate males shoal with females, are reproductively suppressed, and drab in coloration. Male status is socially regulated, and individuals regularly transition between status phenotypes (Fernald and Maruska, 2012; Hofmann, 2003). Dominant males court gravid females, which in turn lay their eggs in the dominant male's territory. The female immediately scoops up her eggs into her mouth, where the male fertilizes them. The mother incubates the developing larvae in her buccal cavity for approximately two weeks. Under natural (and some laboratory) conditions, mothers continue to guard their offspring for up to 10 more days following the initial release of free-swimming fry from her mouth (Renn et al., 2009). In these experiments, we removed developing larvae from the mother's mouth 6–12 days post fertilization. Once they were sufficiently developed (approximately 12 days post-fertilization, when freely swimming with no remaining yolk), we transferred juveniles into communities in 35 L aquaria) with other juveniles of similar age. Juveniles remained in these multi-brood communities until they were placed in experimental social groups (described below). Although fish were not observed while in these communities, juveniles interact socially starting at a very early age. Behaviors, such as chasing, nipping, and aggressive displays, emerge in a predictable sequence as juveniles approach reproductive maturity, which can occur as early as 15 weeks, depending on the early-life social conditions (Fernald and Hirata, 1979; Fraley and Fernald, 1982). It is not known the extent to which juveniles form status relationships in the wild, and factors such as proximity to nearby adults may influence juvenile behavior.

All fish used in these experiments were juveniles between the ages of 10 and 18 weeks old. Within this age range, the suite of social behaviors that immature fish express should be similar (Fernald and Hirata, 1979). Fish were all silver (drab) in coloration, and none had or developed nuptial coloration, which would indicate reproductive maturity for males. No fish appeared gravid or were observed brooding eggs, indicating reproductive maturity for females. Because sex cannot be

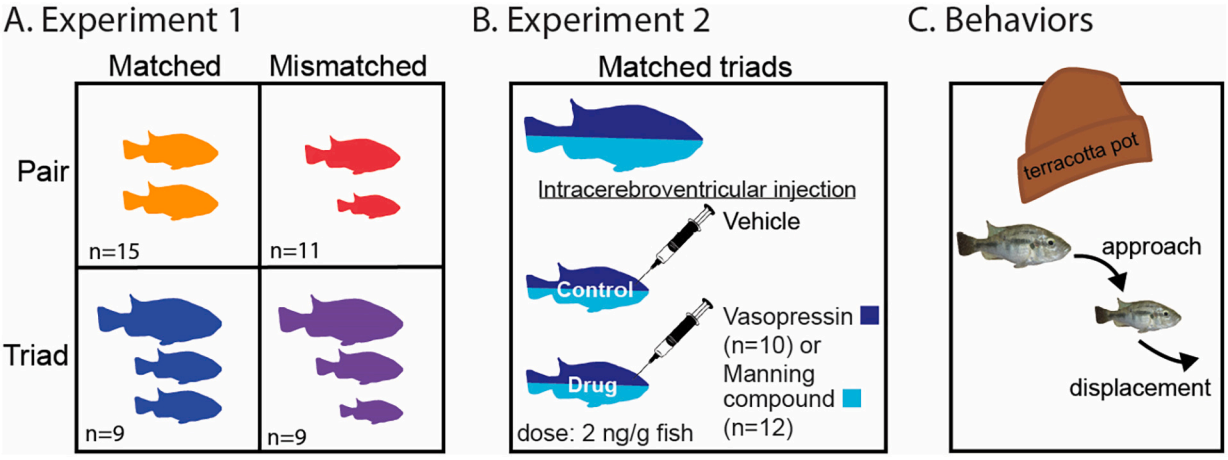

Fig. 1. Experimental designs. A) Experiment 1 included social groups of two (pairs) and three (triads) fish of specific relative body sizes. The mismatched pairs contained a relatively larger and smaller fish. The matched pairs contained two fish of the same size. The mismatched triads contained a relatively larger, medium, and relatively smaller fish. The matched triads contained a relatively larger fish, with two smaller fish that were size matched with each other. B) Experiment 2 used intracerebroventricular injection to manipulate vasopressin signaling in one of the size matched fish (vasopressin or antagonist Manning Compound). The other size matched fish received a vehicle injection. The large fish was not injected. C) Experimental aquaria included a terracotta pot as shelter / territory. We observed social interactions, including approaches, where one fish swims directly towards any part of another fish, within three body lengths. If that approached fish swims away in any direction, it is a displacement.

determined anatomically until maturation, the sex ratios of the pairs and triads are not known; however, the sex ratio of *A. burtoni* broods is approximately 1:1 (Heule et al., 2014). All research was done in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.2. Experimental housing conditions (Experiments 1 & 2)

Experimental fish were selected from community aquaria containing juveniles hatched in the same calendar month. We removed juveniles using hand nets and measured standard length (SL) using calipers (to the nearest 0.1 mm) and body mass (g) using a balance (to the nearest 0.0001 g). We then temporarily housed fish individually in 250 mL plastic beakers while social groups were formed (⟨1h). Fish assigned to the same social group (pairs or triads, see below) were introduced into the experimental aquarium at the same time. Experimental aquaria were small (3.27 L, 23 × 15 × 15 cm), acrylic tanks with one terracotta shard placed against the short end of the enclosure (to act as a territory / shelter). For Experiment 1, which lasted 7 days, air was supplied through a single air stone. The fish were fed once a day with Hikari plankton (Pentair Aquatic Eco-Systems, Cary, NC) and were maintained on a 12:12 light dark cycle. Fish in Experiment 2 were in the aquaria for 1 h, which obviated the need for aeration or feeding.

2.3. Experiment 1: juvenile social behavior and status

2.3.1. Social group formation

We formed social groups of two (pairs) and three (triads) fish, and then we observed social behavior over 7 days (Days 1, 3, and 7). We included two treatments of pairs and triads: groups with and without size matched fish (Fig. 1A). In the pairs, the two fish were either the same size (matched, $n = 15$) or one fish was larger than the other (mismatched, $n = 11$). In the triads, one treatment group contained a size matched pair, along with a larger juvenile (matched, $n = 9$). The other treatment group contained three juveniles that all differed in size (mismatched, n = 9). Individuals were size matched as closely as possible based on SL (< 0.5 mm difference). Fish were considered to be of different sizes if one was at least 3 mm SL longer than the other. Adult *A. burtoni* are highly sensitive to differences in body size (Alcazar et al., 2014; Alward et al., 2021; Weitekamp and Hofmann, 2017), and this size difference was sufficient in juveniles to bias status in a short-term assay (Solomon-Lane and Hofmann, 2019). Between fish that were size matched (all conditions), the average difference in SL was 0.06 ± 0.018 mm (range: 0–0.4 mm SL), and the average difference in mass was 0.018 ± 0.0039 g (range: 0.0007–0.084 g). In comparison, between fish that were size mismatched (all conditions), the average difference in SL with the next larger or smaller fish (i.e., large vs. medium and medium vs. small, but not large vs. small) was 4.32 ± 0.11 mm (range: 3.1–6.9 mm SL), and the average difference in mass was 0.18 ± 0.012 g (range: 0.052–0.4 g) (Supplemental Fig. 1).

2.3.2. Tagging

To visually distinguish between size matched fish in the pairs and triads, both of individuals were tagged using fishing line threaded through the dorsal muscle and tied into a loop (see detailed description in Solomon-Lane and Hofmann, 2018). Briefly, we anesthetized juveniles in 0.0006 g tricaine methanesulfonate (MS-222, Sigma Aldrich) / mL aquarium water. The solution was buffered with sodium bicarbonate to pH 7–7.5. Fish were removed from the MS-222 as soon as they stopped responding to touch, which always occurred after ventilation stopped. We used fishing line (Berkley Nanofil Fishing Line, 0.006 in average diameter) attached to the needle of an insulin syringe (BD Ultra-Fine™ Short Needle, 8 mm, 31G) to pierce through the dorsal muscle of the fish. The line was then tied in a square knot in a loop large enough for the fish to fully raise its dorsal fin. One of the size matched fish in a social group received a white tag (the color of the fishing line). The other fish received a black tag, which was colored using a Sharpie permanent marker and rinsed in aquarium water (Solomon-Lane and Hofmann, 2018). The large fish in the matched triads was not tagged, and the fish in the mismatched triads and pairs were not tagged.

2.3.3. Behavioral observations

Digital video cameras (Alibi Security System) were used to record behavior from above, such that all areas of the enclosure were visible, with the exception of the space underneath the piece terracotta shard. Behavior was recorded for one hour on Day 1, Day 3, and Day 7, where Day 1 was the first full day social groups were together (the day after tagging and group formation). Ten minutes of behavior from each day was scored using Solomon Coder (www.solomoncoder.com) and analyzed (RMB). Because social group size and the relative body sizes among fish were readily apparent, the observer was aware of the treatment group when scoring behavior. We scored all the social interactions among all individuals in the group. An approach was defined as one fish swimming directly towards any part of the other fish's body, within 3 body lengths. If the approached fish responded by moving away, in any direction, the behavior was recorded as a displacement for the initiator and a submission for the responder (Fig. 1C). Other social behaviors expressed by adult *A. burtoni*, such as threat displays, fighting, or courting (Fernald, 1977), were not observed (Fraley and Fernald, 1982) or scored in this study.

From these behaviors, we calculated three additional social measures. First, using the *compete* package in R, we calculated directional consistency for displacements, a measure of whether interactions between dyads are reciprocal, from perfectly reciprocal (0) or unidirectional (1). We also used *compete* to run a randomization test for each social group to determine if directional consistency was significantly greater (more asymmetrical) than expected by chance (Curley, 2016; Leiva et al., 2008). Second, we calculated agonistic efficiency, which is the proportion of approaches that lead to displacement (Solomon-Lane et al., 2014). If no interactions occurred, the value was set to 0. Finally, we calculated an adjusted version of David's Score to quantify dominance index (Gammell et al., 2003). David's Score reflects the outcome of an individual's agonistic interactions, as well as the outcomes of agonistic interactions among other group members. Most often when David's Score is calculated, agonistic interactions between dyads are defined as a binary win vs. loss. We incorporated both approaches and displacements, such that the agonistic outcome of individual *i* in interaction with another individual *j* is the number of times that *i* displaces *j*, divided by the total number of interactions (approaches) between *i* and *j* (i.e., *i* displaces *j* / (*i* approaches *j* + *j* approaches *i*)). The rest of the calculations were done as described in (Gammell et al., 2003) and coded in the *compete* package in R, with the correction for when frequencies of interactions vary across dyads (see Supplemental Information for adjusted code, Curley, 2016). Overall, our adjustment makes David's Score more similar to agonistic efficiency, but weighted by the relative efficiencies of group members in their dyadic interactions.

2.4. Experiment 2: central pharmacological manipulation of AVP

2.4.1. Social group formation

We removed juveniles from community aquaria (see above) with a hand net and formed matched triads as in Experiment 1 (Fig. 1B, $n = 22$). The fish were size matched as closely as possible based on SL (< 0.2 mm difference) (Supplemental Fig. 1). The larger juvenile was at least 3 mm SL larger than the matched pair. The sizes of the large fish were measured, but these values were accidentally not recorded; therefore, these values are not included in the analyses.

2.4.2. Central manipulation of AVP

After the juveniles were measured (SL, mass) and assigned to a social group (see above), the size matched fish within the triad were anesthetized for tagging and pharmacological manipulation. The larger

juvenile remained in its plastic beaker until the other members of its social group recovered. The timing of following procedures—anesthesia, tagging, and intracerebroventricular (ICV) injection—was standardized such that every fish was returned to their beaker 3 min after being initially removed. Juveniles were anesthetized in 0.6 g MS-222 / L aquarium water (buffered with sodium bicarbonate to pH 7.0–7.5). Immediately after the fish stopped responding to touch, it was removed from the MS-222 and tagged with white or black fishing line (see above). While still anesthetized, we used ICV injection to deliver either arginine vasotocin (2 ng/g fish in 50.6 nL phosphate buffer, (Arg8)-vasotocin, CPC Scientific, San Jose, CA) or V1aR2 antagonist Manning Compound (2 ng/g fish in 50.6 nL phosphate buffer, (Kruszynski et al., 1980) into the third ventricle of the brain. The other member of the size matched pair received a vehicle injection of phosphate buffer (50.6 nL) (Fig. 1B). This protocol has been used successfully in other small fish species to centrally manipulate neuromodulators (Pradhan et al., 2014; Solomon-Lane and Grober, 2012). We selected these dosages for arginine vasotocin and Manning Compound based on previous ICV studies in juvenile rainbow trout (*Onchorhynchus mykiss*) (Backström and Winberg, 2009) and in bluebanded gobies (*Lythrypnus dalli*) (Solomon-Lane & Grober, unpublished results).

The ICV injections were performed under a dissecting microscope using the Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). The anesthetized fish was held in place under the microscope with the experimenter's fingers (RMB), and the solution was injected into the third ventricle using a pulled capillary tube needle. The needle was lowered via micromanipulator through the top of the head and skull at the intersection of the midline and middle of the eyes. A series of pilot injections of methylene blue were used to verify this anatomical location (as in Solomon-Lane and Grober, 2012). Once the needle was in place, the Nanoject was used to eject the solution, bathing the brain and third ventricle. Following each injection, the needle was kept in the skull for an additional 5 s to reduce leakage. To verify proper Nanoject function, we followed every injection with a test injection under the dissecting scope to confirm that the needle was not blocked. None of the injections in this experiment was blocked. Between injections, the needle was wiped with ethanol and allowed to dry. The needle was changed as soon as any resistance was detected (indicating dulling of the tip), as well as between drug and vehicle injections. Following injection, fish were placed in a plastic beaker of fresh aquarium water for recovery. A transfer pipette was used to gently push water across the gills until the juvenile was steadily and independently breathing. The fish remained in recovery until equilibrium and normal swimming behavior was regained.

2.4.3. Behavioral observations

Once both injected fish had recovered, all three members of the triad were gently introduced into the experimental aquarium at the same time. From the time of anesthetization, fish were placed in experimental tanks an average of 19.3 ± 1.6 min later. Behavior was recorded from above using digital video cameras for one hour. All social interactions (approaches and displacements) among all fish were scored for 50 min of behavior, specifically minutes 12–62 after being introduced into the experimental aquarium with the novel social group. Three observers (who were unaware of the drug treatment of the triad – AVP vs. Manning Compound – as well as which individual received the drug or vehicle-injection) consistently scored 10-min segments of video (e.g., 12–22 min, 22–32 min, etc.). Observers EM and KL scored two 10-min segments, and HG scored one 10-min segment. Minutes 2–12 were excluded from analysis because of very low rates of behavior following tagging, injection, and transfer to the novel environment. We present the data as a sum of behaviors over the observation.

2.4.4. Whole brain gene expression

For both size matched fish within the triad, we measured the whole brain expression of nine candidate genes relevant to the regulation social behavior and status, including in adult *A. burtoni*. We decided to analyze whole brain because we did not have *a priori* expectations about which brain regions would be the most important to examine. After recording one hour of social behavior, we removed the size matched fish from the experimental aquaria using hand nets and rapidly decapitated them. The brains were dissected immediately, flash frozen on dry ice, and stored at −80 °C until processing. Pituitaries were not included with the brains. Candidate gene expression was quantified using qPCR and previously validated primers (Chen and Fernald, 2008; Greenwood et al., 2003; O'Connell and Hofmann, 2012) (Table 1). We measured the expression of AVP, V1aR2 (the receptor subtype that is broadly expressed in the brain, Kline et al., 2011), IT, ITR, GR1a, GR1b, GR2, MR, CRFb (paralog sequence from Chen and Fernald, 2008; Grone and Maruska, 2015), and reference gene 18S. We selected 18S as a reference gene because it has shown very little expression variation across social phenotypes in transcriptome studies of *A. burtoni* (O'Connell and Hofmann, 2012; Renn et al., 2008; Solomon-Lane and Hofmann, 2019). RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega, Madison, WI), and the Promega GoScript Reverse Transcription System (Promega, Madison, WI) was used for reverse transcription. PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) was used for quantitative PCR. Samples were processed according to manufacturers' recommendations. Relative gene expression levels were quantified using ΔΔCT analysis.

2.5. Statistics

All statistical analyses were conducted using R Studio (R version 4.1.2) (RStudio Team, 2022). Results were considered significant at the $p < 0.05$ level, and averages ± standard error of the mean are included in the text. The box of the box and whisker plots show the median and the first and third quartiles. The whiskers extend to the largest and smallest observations within or equal to 1.5 times the interquartile range. In Experiment 1, for comparisons across individuals within treatment groups—including SL, mass, total rates of approaching and displacing for all fish across all days, and David's Scores on Day 7—we used repeated measures *t*-tests or one-way ANOVA if the data met the requirements for parametric statistics, or we used Friedman tests for non-parametric analyses. We used Wilcoxon tests with Bonferroni

Table 1
Primers used for quantitative real-time PCR analyses.

Gene	Primer sequence (forward)	Primer sequence (reverse)	Amplicon size (bp)
GR2	TGC CTC TGT CAC TGC CAC CGT AG	AGT CGT CTG CGT CTG AAG TAA CTG	109
GR1a	TCA TAA GAT CTG TTT GGT GTG CTC	GTA GTT GTG CTG GCC TTC AAC	1058
GR1b	TGT TGG CTT CTC CGG TTC ATC AC	GTT GTG CTG GCC ATC TGT GTT T	223
MR	CGT TAA TGG AGT CGT GGA AAT C	GAG GAC GGT TGT CTC AGT GG	130
CRF	CGA ACT CTT TCC CAT CAA CGT CCA	AGC GCC CTG ATG TTC CCA ACT TTA	121
AVP	AGG CAG GAG GGA GAT CCT GT	CAG GCA GTC AGA GTC CAC CAT	98
V1aR2	GAA AGA AGA CTC AGA CAG TAG CC	ACC ATC ACT ACA CAC ATC TCG	209
IT	GGA AAC AGC TCA CTG TGT GGA	AGC ACA GCG TCC TCC TTC AG	91
ITR	GGC TTA CAT GCT CTG CTG GA	AGC AGC ATG GAG ATA ATG AAG G	105
18S	CCC TTC AAA CCC TCT TAC CC	CCA CCG CTA AGA GTC GTA TT	7460

GR1a, glucocorticoid receptor 1a; GR1b, glucocorticoid receptor 1b; GR2, glucocorticoid receptor 2; MR, mineralocorticoid receptor; CRF, corticotropin-releasing factor; AVP, vasopressin; V1aR2, vasopressin receptor; IT, isotocin; ITR, isotocin receptor.

correction for post hoc analysis of significant Friedman results. We used two-way mixed factorial ANOVAs to test the effects of group size (pair vs. triad) and presence of a matched pair (matched vs. mismatched), or an interaction effect, on total directional consistency (displacements) and agonistic efficiency. We used Chi-Square tests to determine whether large fish were significantly more likely to have the highest David's Score in mismatched pairs, mismatched triads, and matched triads. In Experiment 2, we compared AVP triads to Manning triads—including SL and mass; how well size-matched fish were; large, drug-injected, and vehicle-injected fish approaches and displacements; and the proportion of approaches and displacements received by the large fish (large fish "attention")—using *t*-tests if the data met the requirements or non-parametric Wilcoxon tests, if not. We compared approaches and displacements across AVP and Manning large vs. drug-injected vs. vehicle-injected using Friedman tests. To correct for the multiple comparisons of approaches and displacements, we used a Bonferroni correction, and adjusted *p*-values are reported in the text. Post hoc analysis of significant Friedman results was done with Wilcoxon tests with Bonferroni correction. We used Principal Components Analysis (PCA) to identify how behavior, status, and gene expression clustered. Finally, we used two-way mixed factorial ANOVAs to test the effects drug treatment (AVP vs. Manning), experimental role of the fish (large, drug-injected, vehicle-injected), or an interaction effect, on David's Scores, whole-brain expression of candidate genes, and Principal Components (PC) 1–3. Genes that were not normally distributed were first log-transformed (V1aR2, ITR, GR1b). Cohen's d is reported to estimate effect size (small effect: $0.2 < d < 0.5$; moderate: $0.5 < d < 0.8$; large: $0.8 < d$) for pairwise comparisons of parametric statistics. Eta squared is reported to estimate effect size for three-way comparisons of parametric statistics (small effect: $0 < \eta^2 < 0.01$; moderate: $0.01 < \eta^2 < 0.06$; large: $0.06 < \eta^2$). Kendall's W is reported to estimate effect size (small effect: $0.1 < W < 0.3$; moderate: $0.3 < W < 0.5$; large: $0.5 < W$) for nonparametric statistics.

3. Results

3.1. Variation in body size in Experiments 1 and 2

In Experiment 1, juveniles ranged in size from 13.8 to 28.3 mm SL (average 20.8 ± 0.29 mm) and 0.11–0.64 g (average 0.25 ± 0.012 g). We successfully manipulated the size classes, such that matched groups did not differ, and mismatched groups did differ, significantly in size. In the matched pairs, there was no significant difference in SL ($t_{12} = -0.59$, $p = 0.56$) or mass ($t_{12} = -0.036$, $p = 0.97$) between black and white tagged fish. In matched triads, SL ($\chi^2(2) = 17$, $p = 0.0002$, $W = 0.95$) and mass ($\chi^2(2) = 13.6$, $p = 0.0011$, $W = 0.75$) differed significantly among the larger, black tagged, and white tagged fish. Post hoc analysis showed the large fish was significantly longer and heavier than the black tagged (SL: $p = 0.027$; mass: $p = 0.012$) and white tagged fish (SL: $p = 0.027$; mass: $p = 0.012$), but there were no significant differences between the black and white tagged fish (SL: $p = 1.0$; mass: $p = 1.0$). In mismatched pairs, the larger fish was significantly longer ($t_7 = 35.75$, $p = 3.48e\text{-}9$, $d = 2.24$) and heavier ($t_7 = 8.41$, $p = 6.64e\text{-}5$, $d = 1.10$) than the smaller fish. Finally, in mismatched triads, SL ($\chi^2(2) = 18$, $p = 0.00012$, $W = 1.0$) and mass ($\chi^2(2) = 12$, $p = 0.0025$, $W = 1.0$) differed significant among larger, medium, and small fish. Post hoc analysis showed that larger fish were significantly longer than medium fish ($p = 0.012$) and smaller fish ($p = 0.027$), and medium fish were significantly longer than small fish ($p = 0.027$). We found the same patterns for body mass, although they did not reach significance ($p = 0.094$ for all pairwise comparisons).

In Experiment 2, juveniles ranged in size from 18.4 to 23.9 mm SL (average 21.7 ± 0.2 mm) and 0.12–0.33 g (average 0.22 ± 0.0074 g). There was no significant effect of drug treatment (AVP vs. Manning) ($F_{1,18} = 0.63$, $p = 0.44$) or experimental role (drug- vs. vehicle-injected) on SL ($F_{1,18} = 0.007$, $p = 0.94$), although there was a trend for an interaction effect ($F_{1,18} = 3.45$, $p = 0.08$). For mass, there was no significant effect of drug treatment ($F_{1,18} = 0.26$, $p = 0.62$), but there was a significant effect of drug- vs. vehicle-injected ($F_{1,18} = 7.10$, $p = 0.016$, $d = 0.40$). There was no significant interaction effect ($F_{1,18} = 0.19$, $p = 0.67$). Post hoc analysis showed the mass of vehicle-injected was significantly larger than drug-injected fish ($p = 0.043$). There were no differences in how well size matched fish were for SL ($W = 58.5$, $p = 0.48$) or mass ($W = 31.5$, $p = 0.18$) between AVP and Manning triads.

3.2. Experiment 1: juvenile social behavior and status

3.2.1. Juveniles form social dominance relationships

To test our first hypothesis that juveniles form social status, we analyzed the effects of group size (pair vs. triad) and having a matched pair (matched vs. mismatched) on directional consistency, agonistic efficiency (Table 2), and David's Score (Table 3). We found that matched groups had significantly lower directional consistency (Fig. 2A) and agonistic efficiency (Fig. 2B) than mismatched groups. There was no effect of group size, nor were there significant interactions between matched status and group size. These treatment differences suggest that juvenile social interactions are patterned and hierarchical. Therefore, we focused our analyses of David's Scores on observation Day 7 (Table 3; Fig. 2C), after which time social status had an opportunity to stabilize. In pairs, fish were categorized as having higher or lower David's Scores. In triads, categories were highest, middle, or lowest David's Scores. In matched pairs and mismatched pairs, the David's Scores of the higher ranked fish were significantly higher than the lower ranked fish. In matched triads, there were significant differences in David's Scores across status ranks; however, post hoc analysis showed no significant pairwise differences among ranks, although there was a trend between the highest and lowest David's Score ranks. In mismatched triads, there were significant differences in David's Scores across status classes, and post hoc analysis showed significant differences among all statuses.

3.3. Behavioral basis of social status

To determine whether social status can be defined by characteristic rates of behavior on Day 7, we compared approaches and displacements across status classes (Table 3), as determined by David's Scores. Significantly higher approach rates were indicative of high status in matched and mismatched pairs, as well as mismatched triads. However, post hoc analysis of the mismatched triads showed no significant pairwise differences. In matched triads, rates of approaching did not vary significantly by status. Dominant fish in pairs also displaced significantly more frequently. For both mismatched and matched triads, there were significant differences in displacements across ranks, but post hoc analysis showed no significant pairwise differences. See Supplemental Fig. 2 for displacements and agonistic efficiency from Day 1 and Day 3 (statistics in Supplemental Table 1).

3.4. Relatively larger fish are more likely to be socially dominant in some contexts

To test our hypothesis that size benefits higher social status, we tested whether large fish in the mismatched pairs, mismatched triads, and matched triads were significantly more likely to have the highest David's Score in the social group. In mismatched pairs, 100 % (11 of 11) of the larger fish are also more dominant, which was significantly greater than chance ($\chi^2 = 11$, $df = 1$, $p = 0.00091$). In mismatched triads, there was a trend for large fish to be more likely to be dominant ($\chi^2 = 4.67$, $df = 2$, $p = 0.097$), with 66.6 % (6 of 9) of the large fish as the most dominant. Two medium and one small fish had the highest David's Score. In matched triads, large size did not benefit dominance ($\chi^2 = 2.67$, $df = 2$, $p = 0.26$), with just 55.6 % (5 of 9) of the large fish as the most dominant.

Table 2
The effects of group size and/or presence of a matched pair on social measures in Experiment 1.

Behavior	Effect	DFn	DFd	F statistic	p-value	Effect size	Direction of effect
Directional Consistency	Group size	1	40	0.71	0.40		
	Matched	**1**	**40**	**11.09**	**0.0019**	**−1.026**	**Mismatched > matched**
	Interaction	1	40	1.52	0.22		
Agonistic efficiency	Group size	1	40	0.89	0.35		
	Matched	**1**	**40**	**6.59**	**0.014**	**−0.75**	**Mismatched > matched**
	Interaction	1	40	1.40	0.24		

Results of two-way mixed factorial ANOVAs. Group size refers to pairs vs. triads. Matched refers to the presence of a size matched pair in the social group. DFn (degrees of freedom in the numerator; levels of factor - 1). DFd (degrees of freedom in the denominator; sample size - levels of factor). Effect size is Cohen's d. Significant results in bold.

Table 3
Differences in social measures among individuals within a treatment group of Experiment 1.

Behavior / Treatment group	DF	χ^2	p-value	Effect size	Post hoc / direction of effect
David's Scores					
Matched pairs	**1**	**9.0**	**0.0027**	**0.60**	**High rank > low rank**
Mismatched pairs	**1**	**10.0**	**0.0016**	**0.91**	**High rank > low rank**
Matched triads	2	13.0	0.0015	0.72	High > middle: p = 0.11 High > low: p = 0.068 Middle > low: p = 0.11
Mismatched triads	**2**	**16.0**	**0.00034**	**0.89**	**High > middle: p = 0.043** **High > low: p = 0.043** **Middle > low: p = 0.043**
Approaches					
Matched pairs	**1**	**10.0**	**0.0016**	**0.67**	**High rank > low rank**
Mismatched pairs	**1**	**10.0**	**0.0016**	**0.91**	**High rank > low rank**
Matched triads	2	3.39	0.14		
Mismatched triads	2	6.67	**0.036**	**0.37**	High > middle: p = 0.44 High > low: p = 0.11 Middle > low: p = 0.09
Displacements					
Matched pairs	**1**	**9.0**	**0.0027**	**0.60**	**High rank > low rank**
Mismatched pairs	**1**	**10.0**	**0.0016**	**0.91**	**High rank > low rank**
Matched triads	**2**	**9.33**	**0.0094**	**0.52**	High > middle: p = 0.35 High > low: p = 0.10 Middle > low: p = 0.098
Mismatched triads	**2**	**9.93**	**0.007**	**0.55**	High > middle: p = 0.088 High > low: p = 0.068 Middle > low: p = 0.10

Results of Friedman Tests. Effect size is Kendall's W. Significant results in bold.

3.5. Experiment 2: pharmacological manipulation

3.5.1. Social status and behavior

Because matched triads showed the least asymmetric antagonism across all our measures of social organization in Experiment 1 (directional consistency, agonistic efficiency, David's Scores, displacements), we reasoned that this group composition would be best suited to test our hypothesis that AVP regulates social status and behavior in juvenile *A. burtoni*. We first analyzed the effects of drug treatment and the experimental role of the fish (large vs. drug-injected vs. vehicle-injected) on David's Scores (Table 4). We found that fish did form social status relationships, but there was no effect of pharmacological manipulation. Specifically, large fish had significantly higher David's Scores compared to drug- and vehicle-injected fish (Fig. 3A). We next tested for effects on social behavior (Table 4). In both the AVP and Manning matched triads, there were differences in approaches and displacements across large, drug-injected, and vehicle-injected fish, with the large fish approaching and displacing significantly more than the smaller, size matched fish (Table 5). There was no significant effect of drug treatment (AVP vs. Manning) on rates of dyadic interaction for the large, drug-injected, or vehicle-injected fish ($p > 0.15$, see Supplemental Fig. 3).

Given that the vast majority of approaches (83 %) and displacements (89 %) were performed by the large fish, we next asked whether large fish interacted differentially with the drug-injected compared to the vehicle-injected fish in AVP and Manning triads (Table 4). To our surprise, we found a significant interaction effect between drug treatment and experimental role for the proportion of approaches the large fish directed towards drug-injected vs. vehicle-injected fish. In AVP triads, the vehicle-injected fish received a significantly higher proportion of large fish approaches than AVP-injected fish. In Manning triads, there was a trend for Manning-injected fish to receive a higher proportion of large fish approaches than vehicle-injected fish (Fig. 3B). For the proportion of large fish displacements, there was a trend for an interaction effect in the same pattern as for approaches. There were no significant main effects for either approaches or displacements.

3.5.2. Whole brain gene expression

Although there were no significant behavioral or social status differences between drug-injected and vehicle-injected fish, we were intrigued by the finding that manipulating AVP signaling could shift the attention of the dominant (large) fish in the triad towards the individual presumed to have relatively lower AVP activity (i.e., the vehicle-injected fish in AVP triads and the drug-injected fish in Manning triads, respectively). This result hinted at a (possibly compensatory) physiological or molecular response that was not evident at the behavioral level. We therefore asked whether drug treatment and/or experimental role (drug-injected vs. vehicle-injected fish) affected whole brain expression of nine candidate genes involved in nonapeptide or glucocorticoid signaling. We found that the expression patterns of two of these genes were affected: V1aR2 and CRF. (Table 4). V1aR2 expression was significantly higher in fish in the AVP triads (drug- and vehicle-injected together) compared to Manning triads, but there were no differences between drug- and vehicle-injected fish. For CRF expression, there was a significant interaction effect between the drug treatment of the triad and whether the fish was injected with vehicle or drug. Pairwise post hoc analysis showed Manning-injected fish had significantly higher CRF expression than vehicle-injected fish in Manning triads. The expression

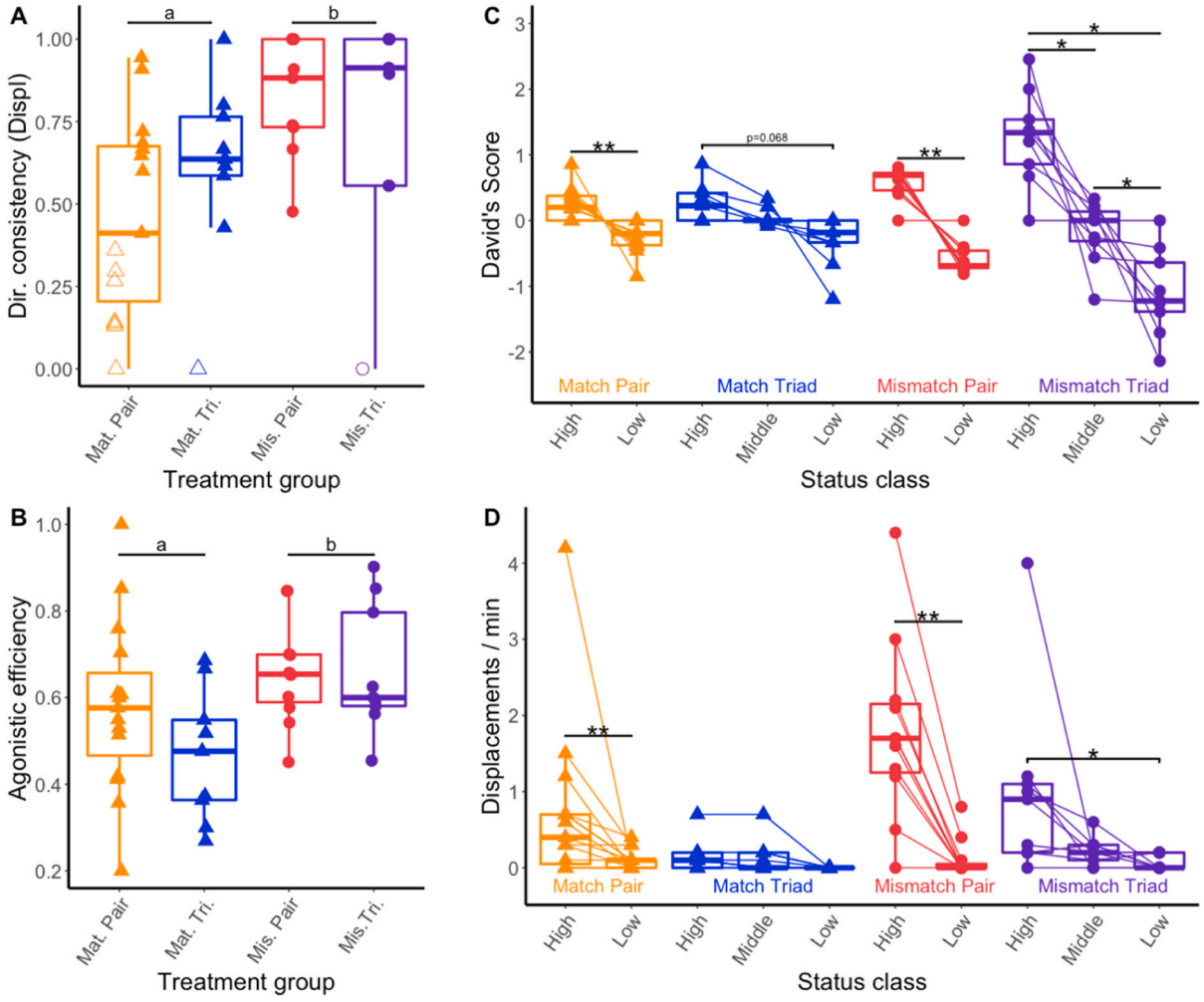

Fig. 2. Experiment 1 social measures. A) Directional consistency index for total displacements (sum of Days 1, 3, and 7). Filled shapes indicate that group's directional consistency was significantly more directional than chance. Open shapes indicate no significant difference from 0 (perfectly reciprocal). B) Total agonistic efficiency (sum of displacements / sum of approaches for all fish from Days 1, 3, and 7). C) Adjusted David's Score on Day 7. D) Displacements per minute on Day 7. For C and D, the fish with the highest David's Score was categorized as high status, the middle-ranked fish was middle status, and the fish with the lowest David's Score was categorized as low status. Different letters indicate significant differences between status classes ($p < 0.05$). *p < 0.05, **$p < 0.01$.

data for all genes are shown in Supplemental Fig. 4, with the statistical results in Supplemental Table 2.

3.6. *Integrative analysis of behavior, status, and whole brain gene expression*

Given the multivariate and integrative nature of our dataset, we used PCA to identify the factors that contribute to juvenile phenotype and explain specific components of individual variability, depending on drug treatment and experimental role. Drug- and vehicle-injected fish were the focal fish in the PCA, and we included focal fish displacements of the other size matched fish, displacements of the large fish, submissions to the size matched and large fish, David's Scores, the proportion of large fish approaches received, and neural gene expression for all genes (AVP, V1aR2, IT, ITR, CRF, GR1a, GR1b, GR2, and MR). Gene expression was not measured for the large fish; therefore, they were not included as focal fish in the analysis. Their behavior is, however, reflected in social interactions with the focal fish. Here, we focus on the first three principal components (PCs), which together explained 50.5 % of the variation in the data (Fig. 4A, Supplemental Fig. 5A,B). PC1 (20.5 %) reflected social status in conjunction with glucocorticoid signaling. Specifically, David's Scores, GR1a expression, and GR1b expression loaded strongly on PC1, as did (in the opposite direction) expression levels of GR2 and MR expression and submissions to the large fish (Fig. 4A). PC1 did not differ among the treatment groups (Table 4, Supplemental Fig. 5C). PC2 (17.2 %) reflected the activity of nonapeptide and glucocorticoid systems as mRNA levels of AVP, GR1a, GR1b, IT, ITR, and CRF loaded most strongly on this dimension. PC2 was significantly higher in fish in the AVP triads (drug- and vehicle-injected) compared to fish in Manning triads (Table 4, Fig. 4B). Finally, PC3 (12.8 %) reflected the interplay between social interactions with the large fish and nonapeptide signaling. Displacement of and attention by the large fish loaded most strongly on this dimension, along with mRNA levels of AVP, V1aR2, and ITR (Fig. 4C). For PC3 (12.8 %), there were no differences among the treatment groups (Table 4).

4. Discussion

We investigated the behavioral and neuroendocrine basis of social behavior and status in juvenile *A. burtoni*. By quantifying the experiences of juveniles in different social environments, we aimed to identify potential behavioral mechanisms of behavioral development (Taborsky, 2016). We present strong evidence that juveniles form status relationships, consistent with the few past studies (Fernald and Hirata, 1979; Fraley and Fernald, 1982; Solomon-Lane and Hofmann, 2019). Overall, size matched fish decreased agonistic asymmetry in the pairs and triads.

Table 4
The effects of group size and/or presence of a matched pair on social measures in Experiment 2.

Behavior	Effect	DFn	DFd	F statistic	p-value	Effect size	Direction of effect
David's Scores	[Group] Drug treatment	1	20	3.78e-16	1.0		
	[Individual] Exp. role	**1.23**	**24.58**	**38.06**	**0.0001**	**0.65**	**Large > drug: $p < 0.0001$** **Large > vehicle: $p < 0.0001$** Vehicle vs. drug: $p = 1.0$
	Interaction	1.23	24.58	1.23	0.29		
Large fish attention: approaches	[Group] Drug treatment	1	20	−3.71e-14	1.0		
	[Individual] Exp. role	1	20	1.40	0.25		
	Interaction	**1**	**20**	**7.17**	**0.014**	0.26	**AVP vehicle > AVP drug: $p = 0.003$** MC drug > MC vehicle: $p = 0.10$
Large fish attention: displacements	[Group] Drug treatment	1	20	−3.78e-16	1.0		
	[Individual] Exp. role	1	20	0.17	0.69		
	Interaction	1	20	3.06	0.096		
V1aR2	[Group] Drug treatment	**1**	**16**	**9.40**	**0.007**	**0.14**	**AVP > MC**
	[Individual] Exp. role	1	16	0.12	0.74		
	Interaction	1	16	1.40	0.25		
CRF	[Group] Drug treatment	1	16	0.089	0.77		
	[Individual] Exp. role	1	16	0.00028	0.99		
	Interaction	**1**	**16**	**8.26**	**0.011**	**0.10**	**MC drug > MC vehicle: $p = 0.0015$** AVP drug vs. AVP vehicle: $p = 0.53$
PC1	[Group] Drug treatment	1	15	0.41	0.53		
	[Individual] Exp. role	1	15	0.003	0.96		
	Interaction	1	15	0.53	0.48		
PC2	[Group] Drug treatment	**1**	**15**	**5.15**	**0.038**	**0.13**	**AVP > MC**
	[Individual] Exp. role	1	15	0.091	0.77		
	Interaction	1	15	0.46	0.51		
PC3	[Group] Drug treatment	1	15	0.052	0.82		
	[Individual] Exp. role	1	15	0.096	0.76		
	Interaction	1	15	2.53	0.13		

Results of two-way mixed factorial ANOVAs. "[Group] Drug treatment" refers to whether the drug-injected fish in the triad received vasopressin (AVP) or Manning compound (MC). "[Individual] Exp. Role" refers to the experimental role of the size matched fish, either drug-injected (both AVP and MC) fish or vehicle-injected fish (from both AVP and MC triads). Vasopressin receptor (V1aR2). Corticotropin-releasing factor (CRF). Principal component (PC). DFn (degrees of freedom in the numerator; levels of factor - 1). DFd (degrees of freedom in the denominator; sample size - levels of factor). Effect size is Eta squared. Significant results in bold.

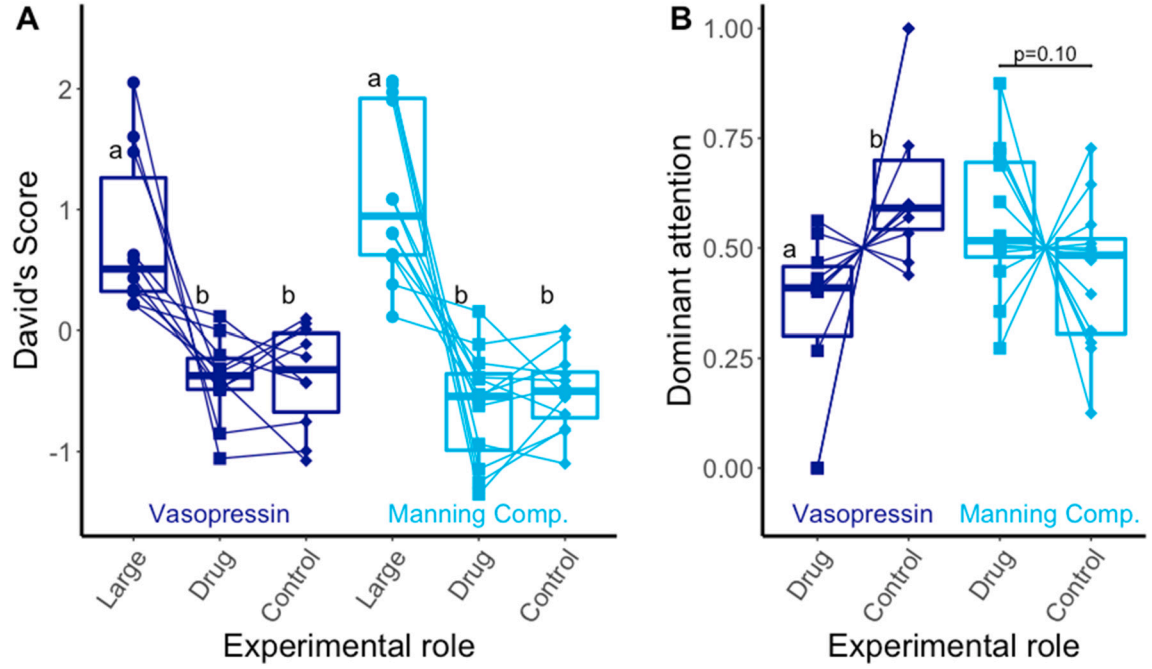

Fig. 3. Experiment 2 social measures. A) Adjusted David's Score in vasopressin (AVP) and Manning Compound matched triads. B) The proportion of large fish approaches that were directed towards the drug-injected fish in AVP and Manning Compound triads. Different letters indicate significant differences.

Focusing on David's Score, a dominance index, we found significant status differences on Day 7 between members of the mismatched and matched pairs, as well as the mismatched triads. In matched triads, however, David's Score did not differ across status classes. This suggests that juveniles either cannot resolve social status relationships in this context, or resolution may not be essential (Fig. 2C). To test whether AVP signaling regulates social behavior and/or status, we manipulated central AVP activity in matched triads—the social group with the lowest asymmetrical antagonism. We identified no direct effects on social behavior or status of the treated animals (Supplementary Fig. 3), but

Table 5
Differences in social measures among individuals within a treatment group in Experiment 2.

Treatment group and behavior	DF	χ^2	p-value	Effect size	Post hoc / direction of effect
Approaches					
AVP	2	16.3	**0.0015**	**0.81**	**Large > drug: p = 0.018** **Large > vehicle: p = 0.006** Drug vs. vehicle: p = 1.0
Manning	2	16.2	**0.0015**	**0.67**	**Large > drug: p = 0.003** **Large > vehicle: p = 0.001** Drug vs. vehicle: p = 1.0
Displacements					
AVP	2	16.7	**0.0012**	**0.84**	**Large > drug: p = 0.017** **Large > vehicle: p = 0.006** Drug vs. vehicle: p = 1.0
Manning	1	18.8	**0.00042**	**0.78**	**Large > drug: p = 0.001** **Large > vehicle: p = 0.001** Drug vs. vehicle: p = 1.0

Results of Friedman Tests. Effect size is Kendall's W. Significant results in bold.

there was a subtle yet robust effect on the large (untreated) fish. The large fish directed a larger proportion of approaches to the vehicle-injected fish in AVP triads and drug-injected fish in Manning Compound triads (Fig. 3C), suggesting that the large fish paid more attention to the fish with presumably lower AVP signaling. Pharmacological manipulation also significantly shifted whole brain neuroendocrine gene expression profiles in fish (drug- and vehicle-injected) in AVP vs. Manning Compound triads, including CRF, AVP, IT, ITR, and GR1a, and GR1b (Fig. 4A, B). Finally, we identified integrated brain and behavior profiles associated with social status (Fig. 4A) and attention (proportion of approaches) received from the large, dominant fish (PC3, Fig. 4A, C). Together, these experiments provide a key step towards understanding juvenile social behavior, social group structure, and underlying regulation by nonapeptide and HPA/I axis signaling.

4.1. Experiment 1: behavioral basis of juvenile social status

Our quantitative analysis of social behavior and group structure clearly demonstrates that juvenile *A. burtoni* form social status relationships, like juveniles of many other social species (e.g., Drummond and Canales, 1998; Engh et al., 2009; Sato and Nagayama, 2012; Solomon-Lane and Grober, 2016). Adult male status classes are one of the most visible and well-studied attributes of *A. burtoni* (Fernald, 1977; Fernald and Hirata, 1977; Maruska and Fernald, 2018), and adult females can also form hierarchies in the absence of males (Renn et al., 2012). It has long been known that during behavioral development (Fernald and Hirata, 1979; Fraley and Fernald, 1982), social behaviors typical of adult dominance emerge sequentially in juvenile *A. burtoni*, such as chasing (as early as 11 days old), nipping, frontal displays, etc. Preliminary territories emerged as early as 26 days old, with permanent territories as early as 57 days (Fraley and Fernald, 1982), indicating the presence of stable dominants and subordinates. By observing fish in their groups with this level of detail, we identified important variation across treatment groups, including that status and the importance of body size can be more ambiguous than we expected. Juveniles provide an exciting opportunity to uncover the proximate mechanisms regulating aggression, submission, status, and group structure across developmental stages and without the influence of mature gonads or reproductive behaviors. For example, future work can investigate whether juveniles show similar neuroendocrine changes to adults during status ascent (e.g., Maruska et al., 2013a) and/or descent (e.g., Maruska et al., 2013b).

We quantified multiple measures of agonistic asymmetry, the foundation of social status (Drews, 1993), to gain a comprehensive understanding of juvenile social structure and avoid assumptions based on knowledge of adults. Matched and mismatched pairs and triads varied across social measures in distinct ways, which supports our overarching finding that juvenile social structure is hierarchical and nuanced. As expected, mismatched pairs and triads had the clearest hierarchical

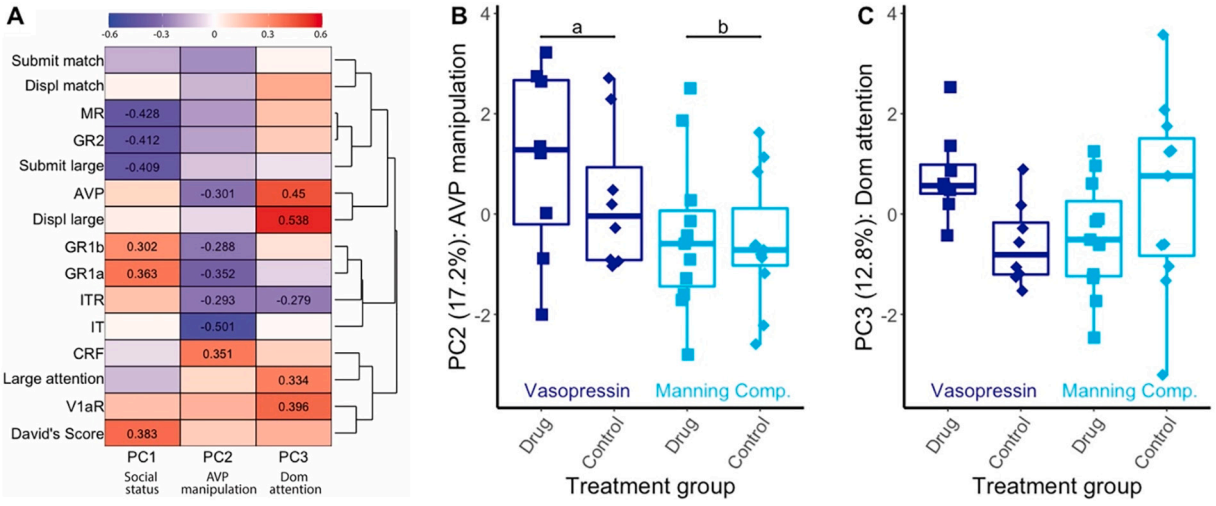

Fig. 4. Principal components analysis (PCA) of behavior—including displacements (displ), submissions (submit), adjusted David's Score, and the proportion of large fish approaches received (large attention)—and whole-brain gene expression for vasopressin (AVP), vasopressin receptor V1aR2, isotocin (IT), isotocin receptor (ITR), corticotropin-releasing factor (CRF), glucocorticoid receptors (GR) 1a, 1b, and 2, and mineralocorticoid receptor (MR). A) Heatmap of eigenvalues showing the PCA variables that load on PC1 (20.5 %), PC2 (17.2 %), and PC3 (12.8 %). Numerical values are shown for variables stronger than ±0.25. Rows are hierarchically clustered. B) Differences in PC2 and C) PC3 across AVP drug-, AVP vehicle-, Manning drug-, and Manning vehicle-injected fish. Percentages refer to the amount of variance explained by that component. Different letters indicate significant differences among groups ($p < 0.05$).

organization: directional consistency and agonistic efficiency were higher, and David's Scores showed distinct status classes. Dominant fish frequently initiated social interactions with the subordinate(s) (approaches), which consistently resulted in the subordinate(s) swimming away (being displaced / submitting). In contrast, subordinates approached dominants significantly less often, and those approaches were less likely to lead to displacement. These social dynamics share key attributes with adult *A. burtoni* (Fernald, 1977; Fernald and Hirata, 1977). Adults of many other species actively maintain dominance through agonistic interactions initiated from the dominant to the subordinate (Creel et al., 2013; Drews, 1993), such as mice (Chase et al., 2022; Williamson et al., 2016), hens (Chase et al., 2022), and naked-mole rats (Clarke and Faulkes, 2001). In natural *A. burtoni* social groups, juveniles shoal with subordinate males and females. In the presence of adults, juveniles should always be subordinate, but it is not yet known whether juveniles have a social organization among themselves, within the group with adults or separately. Although juvenile (and adult, Hofmann et al., 1999) *A. burtoni* have socially-regulated growth that should lead to local variation in body size (Fraley and Fernald, 1982), groups in the wild are unlikely to have size classes as distinct as the mismatched experimental groups.

In the presence of size matched fish, social groups do not fully align along measures of agonistic asymmetry. This variation in social structure across treatments provides an opportunity to disentangle structural effects from those of status and behavior. For example, dominant olive baboons (*Papio anubis*) have higher cortisol levels during periods of social instability but relatively lower cortisol during periods of stability (Sapolsky, 1992), and mice (*Mus musculus*) from highly despotic hierarchies show status differences in testosterone, whereas there were no status differences in hierarchies with low despotism (Williamson et al., 2017). We found that matched pairs and triads had significantly lower directional consistency and agonistic efficiency than the mismatched groups (Fig. 2A, B). Although directional consistency did not differ significantly between matched pairs and triads, all but one of the matched triads (89 %) were significantly more directional than expected by chance (Fig. 2A), in contrast to just over half (53 %) of matched pairs. Despite this reciprocal pattern of social interaction in matched pairs, agonism was sufficiency asymmetrical for dominant fish to have significantly higher David's Scores and rates of displacement than subordinates. In matched triads, there were trends for David's Scores to differ across statuses, but rates of displacement did not differ (Fig. 2).

That measures of agonistic asymmetry align differently in different social contexts is not wholly unexpected or uncommon. For example, frequent, reciprocated displacements could be play (i.e., chasing), especially if the animals are young (Graham and Burghardt, 2010), or prolonged status conflict. Additional context is needed to differentiate. We do not yet know whether juveniles are unable to form more agonistically asymmetrical social groups in these contexts or whether they do not need or benefit from doing so. We do not expect inability is the cause based on studies of size matched adult cichlids (Alcazar et al., 2014; Alward et al., 2021; Reddon et al., 2011), as well as numerous other species (e.g., Franck and Ribowski, 1989; Jonart et al., 2007; Sato and Nagayama, 2012; Solomon-Lane and Grober, 2016). Measuring glucocorticoid levels could elucidate whether certain social group structures (e.g., well-defined status classes), or positions within the group (e.g., dominant vs. subordinate), are more or less stressful. Across hierarchical vertebrates, relative glucocorticoid levels are influenced by social stability and how status is established and maintained (Creel et al., 2013). For adult *A. burtoni*, cortisol levels depend on individual attributes (e.g., tenure in rank: Huffman et al., 2015; Parikh et al., 2006); or color morph: Dijkstra et al., 2017), as well as the social group stability Fox et al., 1997; Friesen et al., *in review*; Maguire et al., 2021) (for review, see Maruska et al., 2022, *this issue*).

We were surprised to find that body size did not benefit dominance across all treatment groups. Relatively large size can be an advantage for high social status across species, in part due to increased competitive abilities (Emlen, 2008). We found that all (100 %) larger fish were dominant in mismatched pairs, but only 66.6 % and 55.6 % of large fish were dominant in mismatched triads and matched triads, respectively. This was contrary to our prediction that the large juvenile would clearly establish dominance over the smaller, size matched fish, while the size matched fish might require more time (which we did not investigate here). Adult *A. burtoni* males are highly sensitive to differences in body size (as small as 5 % difference in body mass) (Alcazar et al., 2014; Alward et al., 2021; Weitekamp and Hofmann, 2017), and a relative size advantage benefits juvenile dominance in short term, paired assays (Solomon-Lane and Hofmann, 2019). Interestingly, these results are consistent with research in Lake Malawi cichlids (*Pseudotropheus tropheops* and *Metriaclima zebra*) showing that the social structure of pairs do not scale predictably to larger groups, even of three or four animals (Chase et al., 2003). For example, a systematic study of juvenile crayfish triads—a size matched pair with a larger, smaller, or equal sized third—revealed that only when the size matched individuals were defeated together by a larger individual was their initial, established status destabilized (Herberholz et al., 2016). A detailed temporal analysis of status establishment (e.g., in mice, hens, and cichlids (Chase et al., 2022; Williamson et al., 2016) could show that matched triads are less hierarchical because it is difficult for the large fish to establish dominance simultaneously over two, equally competitive fish. Assortative patterns of social interaction influenced by size or size differences, such as for guppies (*Poecilia reticulata*) and sticklebacks (*Gasterosteus aculeatus*) (Croft et al., 2005), may have a strong effect on whether and how status is established and maintained. The kinds of social experiences that juvenile *A. burtoni* accrue, including through bystander effects (Desjardins et al., 2010, 2012; Grosenick et al., 2007; Roleira et al., 2017; Weitekamp et al., 2017), social learning (Rodriguez-Santiago et al., 2020; Rodriguez-Santiago et al., 2022), and overall group stability (Maguire et al., 2021) can shape phenotypic development (Taborsky, 2016).

4.2. Experiment 2: distinct behavioral and neuroendocrine signatures for social behavior vs. status

We used matched triads to test the hypothesis that AVP signaling regulates juvenile social behavior and status because these groups showed the lowest asymmetrical antagonism in Experiment 1. We found no significant effects of AVP or Manning compound on the social (approaches or displacements) behavior or status of injected fish, analyzed as total behaviors or as dyadic interactions (Supplemental Fig. 3). In addition, the size matched drug- and vehicle-injected fish in AVP and Manning triads were both subordinate to the large fish, and David's Scores did not differ significantly between them (Fig. 3A). Vasopressin has been investigated in a wide variety of species and contexts (Caldwell, 2017; Dumais and Veenema, 2016; Goodson and Thompson, 2010; Kelly and Goodson, 2014), and our results contrast with many that demonstrate a direct regulation of social behavior. For example, central administration of a V1aR antagonist in juvenile rats caused significant and opposite changes in play frequency in males and females (Veenema et al., 2013). Because regulation by AVP is highly species- and context-specific (Goodson and Thompson, 2010; Kelly and Goodson, 2014), we focus our discussion on exogenous manipulation of AVP in fishes (Godwin and Thompson, 2012; Maruska et al., 2022; Silva and Pandolfi, 2019), in particular comparisons with adult *A. burtoni*.

In adult *A. burtoni*, systemic administration of AVP in adult dominant males led to decreased aggression and increased circulating cortisol levels. Although a sickness response appears to have contributed to this decrease in behavior and loss of dominance (Huffman et al., 2015), other studies in this and other teleost fish species have observed increased social withdrawal following AVP administration, and the opposite from Manning Compound, including with consequences for social status (Backström and Winberg, 2009; Oldfield and Hofmann, 2011; Thompson and Walton, 2004). Systemic injection of Manning Compound in

adult male *A. burtoni* appeared to mediate a decrease in aggression along with an increase in courtship in ascending adult male *A. burtoni* (Huffman et al., 2015), a pattern that is also seen using ICV injection in white perch (*Morone americana*) (Salek et al., 2002) and using systemic injection in blueheaded wrasse (*Thalassoma bifasciatum*) (Semsar et al., 2001). Importantly, due to the promiscuity of AVP and oxytocin receptors, these effects may be mediated by AVP or OT receptors (Kelly and Goodson, 2014). Using ICV (or peripheral) injection, it is not feasible to activate or block specific neuron populations (e.g., gigantocellular neurons in the regulation of dominance, (Loveland and Fernald, 2017), which is ultimately necessary for a detailed understanding of the neural substrates that regulate behavior and status (Kelly and Goodson, 2014). Projections from AVP neurons in the preoptic area go to posterior pituitary for release of AVP in circulation, as well as to hindbrain, ventral telencephalon, and ventral thalamus (Dewan et al., 2011; Saito et al., 2004). Volumetric release of AVP can also reach other areas of the brain (Kelly and Goodson, 2014). Future studies must employ experimental approaches that allow for these more specific analyses.

Although we did not find effects on social behavior or status, we did identify multiple treatment effects at the level of the social group. First, the large, dominant juveniles, which initiated the majority of social interactions, preferentially directed approaches to the size matched fish that presumably had lower AVP signaling: the vehicle-injected fish in AVP triads and the Manning-injected fish in Manning triads (Fig. 3B). From our behavioral data (Supplementary Fig. 3), we do not yet know what signal(s) might communicate this information. A study in green anole lizards (*Anolis carolinensis*) found an effect of AVP manipulation on the social behavior of an unmanipulated partner that did not appear to be mediated by behavior. Campos et al. suggest AVP-induced neuroendocrine (e.g., cortisol and testosterone) and/or chemosensory signals may be responsible (Campos et al., 2020), which could be salient for *A. burtoni*, as well (e.g., Nikonov et al., 2017). Similarly, a study in rhesus macaques (*Macaca mulatta*) demonstrated that manipulation of nonapeptide signaling with intranasal AVP or oxytocin in females altered subordinate behavior in their untreated partners. This effect may be mediated by very subtle behaviors in the treated animals (Jiang and Platt, 2018), which is also a possibility for *A. burtoni*. More detailed analyses of the temporal dynamics of social interaction and status establishment (Chase et al., 2022; Lee et al., 2019a) may also provide insight into the underlying mechanism.

We hypothesize that the fish receiving more attention from the large fish would eventually become dominant over the other size matched fish. "Double-dominant" triads, in which an individual is dominant over two subordinates that have not formed a status relationship, are a common feature of dominance networks across species, and they typically become transitive triads (Shizuka and McDonald, 2015). In our PCA, we found that large, dominant fish attention loaded strongly on PC3 (12.8 %), in the same direction as displacements of the large fish and neural AVP and V1aR2 expression; ITR expression loaded strongly in the opposite direction (Fig. 4A, Supplementary Fig. 5B). That attention from the large fish loads in the same direction as displacements of the large fish, a relatively rare behavior, suggests that this subordinate fish is more competitive than the subordinate receiving less attention. A previous study in adult male *A. burtoni* also showed an increase in whole brain AVP and V1aR2 mRNA during social ascent (Huffman et al., 2015), providing additional support that the neural gene expression profile may be that of a fish that is poised to ascend in social status as soon as an opportunity arises. "Intermediate" status adult male *A. burtoni* also receive disproportionate attention from dominant males (Desjardins et al., 2012). Similarly, agonistic interactions in hierarches of hens (*Gallus gallus domesticus*) (Forkman and Haskell, 2004) and monk parakeets (*Myiopsitta monachus*) (Hobson and DeDeo, 2015) are most frequent among individuals closest in rank. Finally, data from Experiment 1 provides further support: the matched triad fish that received more attention from the large fish on Day 1 had significantly higher David's Scores and agonistic efficiency on Day 7 (data not shown).

The second social group level effect we identified was in patterns of whole brain gene expression. PC2 differed significantly between AVP and Manning triads, including both the drug- and vehicle-injected fish (Fig. 4B). Both nonapeptides, IT and AVP, loaded strongly on this axis, along with ITR, GR1a, and GR1b. CRF loaded strongly in the opposite direction, possibly as a consequence of the negative feedback loop between this releasing peptide and the glucocorticoid receptors (Denver, 2009). Although it did not load as strongly on PC2, the third social group-level effect we found involved V1aR2 expression, which was significantly higher in AVP (drug- and vehicle-injected) than Manning fish (Supplemental Fig. 4). Vasopressin has an important role initiating the HPA/I axis, which is a critical part of the stress response (Aguilera and Rabadan-Diehl, 2000). These results clearly show that the neural effects are at the level of the social group because they include both the drug-injected and vehicle-injected fish. Interestingly, these neural differences did not lead to differences in social behavior, status (Fig. 3, Supplemental Fig. 3), or social structure (i.e., directional consistency, agonistic efficiency, data not shown). It is possible that the differences in gene expression reflect compensatory mechanisms that result in similarities in behavior (De Vries, 2004). In addition, or alternatively, the context of establishing status in a matched triad as a smaller fish could also constrain behavioral expression.

Finally, one of the most exciting results from Experiment 2 is the identification of PC1 as an integrative metric that represents social status: David's Score, GR1a, and GR1b expression loaded strongly on PC1 in a concordant manner, while submissions to the dominant, large fish, as well as MR and GR2 mRNA levels loaded strongly in the opposite direction (Fig. 4A). Behaviorally, it is logical that submissions to the dominant fish are inversely related to David's Score, and we suggest that the opposing pattern of glucocorticoid receptor expression also fits mechanistically. Like many teleosts, *A. burtoni* has four glucocorticoid receptors: MR, GR1a, GR1b, and GR2, which differ substantially in their affinity for cortisol. For example, MR is 100-fold more sensitive to cortisol than GR2 in *A. burtoni* and is likely to be bound with cortisol at basal levels (in fish and tetrapods). In contrast, GR1a and GR1b are much less sensitive (Arterbery et al., 2011; Bury, 2017; Greenwood et al., 2003). Thus, MR and GR2 are key to baseline glucocorticoid signaling, rather than the stress response, and in our data, their patterns of expression covary (Fig. 4A). Interestingly, we have previously shown that GR1a responds to early-life social experience in juvenile *A. burtoni* (Solomon-Lane and Hofmann, 2019). We suggested based on this result that GR1a is involved in HPA/I axis negative feedback (Bernier et al., 2009; Bury, 2017; Denver, 2009; Kiilerich et al., 2018; Wendelaar Bonga, 1997), which is a common way for stress response to vary across individuals and a highly conserved mechanism by which early-life experiences exert long-term effects on adult phenotype (Champagne and Curley, 2005; Francis et al., 1999). Our data suggest that high social status may be characterized by higher GR1a and GR1b expression, which could indicate more responsive negative feedback, which can ameliorate the negative effects of chronic glucocorticoid exposure. The HPA/I axis, and negative feedback mechanisms, in particular, are complex, and it will be necessary to test these hypotheses directly. Given the social variation we saw Experiment 1 across matched and mismatched pairs and triads, it will also be important to test whether this social status phenotype is generalizable across social contexts and developmental stages.

5. Conclusion

Overall, with these experiments, we have begun to define the factors that shape juvenile *A. burtoni* social behavior, status, and group structure. This is an essential step towards understanding the types of social experiences juveniles accrue during development and the ways those experiences can be impactful, including for developmental plasticity. Early-life social experiences can have a powerful and lasting impact on adult phenotype (e.g., Buist et al., 2013; Creel et al., 2013; Jonsson and

Jonsson, 2014; Kasumovic and Brooks, 2011; Taborsky, 2016; White, 2010), including for juvenile *A. burtoni* (Solomon-Lane and Hofmann, 2019). Quantifying social experience is key to uncovering the behavioral mechanisms of behavioral development, as well as furthering our understanding of how the long-term effects of early-life experiences are mediated by neuroendocrine mechanisms (Champagne and Curley, 2005; Taborsky, 2016). Although we carried out this work in simplistic social contexts of pairs and triads, which are unlikely to accurately reflect social experience in larger and more complex groups found in nature (Chase et al., 2003), our results make clear that emergent social attributes like status and social group structure must be incorporated into models of behavioral development. Furthermore, we generated testable hypotheses about the neuroendocrine regulation of juvenile social experience by nonapeptides and HPA/I axis, which may be key mechanisms in the behavioral development of juvenile *A. burtoni* and other social species (Baran, 2017).

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Appendix A. Supplementary data

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