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RESEARCH ARTICLE

Neural activation patterns associated with mouthbrooding, maternal care, infanticide and fry release in an African cichlid fish

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ABSTRACT

Parental care has evolved several times and is present across taxa. Parental care behaviors, such as food provisioning and protection, are critical for offspring success. However, infanticide can co-exist with parental care in the same species. The mechanisms underlying the switch from care to consumption and from offspring dependence to independence are relatively unknown, especially in fishes, the oldest and largest group of vertebrates. Mouthbrooding, an extreme example of parental care present in dozens of genera of fishes, provides an excellent opportunity to investigate the brain regions important for parental care. The maternal mouthbrooding African cichlid fish *Astatotilapia burtoni* broods developing young inside the mouth for approximately 14 days, then provides post-release maternal care by protecting fry inside the mouth when threatened. Following the post-release maternal care phase, females can exhibit infanticide and consume their own offspring. We used immunohistochemistry for the neural activation marker pS6 to identify differences in neural activation among mouthbrooding, maternal-care-providing and infanticide-exhibiting females, and between pre- and post-release fry. We identified five brain regions (Dc-5, ATn, nPPa, Vd-c and Dl-g) that are differentially activated among mouthbrooding, maternal care and infanticide females as well as six regions (Dm, Vv, Vd, Vs-m, TPp, PGZ and INL of retina) differentially activated between pre- and post-release fry. This study identifies both shared and distinct circuitry that may support transitions between parental care states and from care to infanticide, as well as regions in developed fry that support the transition from pre- to post-release.

KEY WORDS: *Astatotilapia burtoni*, Behavior, Brain, Parental care, pS6, Teleost

INTRODUCTION

Parental care is present across taxa, has evolved independently several times, and is critical for increasing offspring survival and species persistence (Gilbert and Manica, 2015; Reynolds et al., 2002). Despite the importance of parental care, infanticide (killing of conspecific offspring prior to independence from adults or the end of the larval stage), including consumption of one own's offspring, has also evolved in diverse taxa (Klug and Bonsall, 2007; Parmigiani and vom Saal, 2016). Although infanticide and parental care seem in conflict, these behaviors can co-exist in a single species. For example, sand gobies, mice and burying beetles all provide some form of parental care but also engage in infanticide (Elwood, 1985; Klug et al., 2006; Oldekop et al., 2007). And although consumption of offspring is typically maladaptive, infanticide can be beneficial when offspring are developing slowly, offspring have low reproductive value, paternity is uncertain or food availability is low (Gray et al., 2007; Manica, 2002, 2004). In these cases, infanticide can help parents recover some of the costs invested in offspring (Manica, 2002). How does the brain promote caring or infanticidal behaviors within the same species, and is the neural circuitry underlying these decisions evolutionarily conserved?

The neural circuitry involved in maternal care and infanticide is relatively well studied in mammalian models. For example, optogenetic activation of galanin-expressing neurons in the mouse medial preoptic area (MPOA) that project to the periaqueductal gray (PAG) suppresses pup attacks, and optogenetic activation of galanin MPOA projections to the ventral tegmental area (VTA) increases motivation to interact with pups (Kohl et al., 2018). Estrogen receptor 1 (ER1)-containing cells in the mouse MPOA are activated during pup retrieval, and pup retrieval elicits larger calcium responses in MPOA ER-1-containing cells from lactating mice compared with virgin mice (Fang et al., 2018). The mouse medial amygdala (MeA) is active during parental care behaviors (Kohl et al., 2018), and in rats, excitotoxic lesions of the MeA stimulate maternal care behaviors (Sheehan et al., 2001). Several neurotransmitters and neuropeptides are implicated in parental care and infanticidal behaviors in mammalian models, including vasopressin (Bendesky et al., 2017), dopamine (Lonstein, 2002), oxytocin (Rich et al., 2014) and urocortin-3 (Autry et al., 2021). Although recent work has begun to identify the neural mechanisms of parental care in fishes (Butler et al., 2020; Kent and Bell, 2018; Maruska et al., 2020b; Wei et al., 2021), little is known about the neural circuitry underlying the switch between care and infanticidal behaviors in the same species and whether these circuits are evolutionarily conserved.

Mouthbrooding is an extreme form of parental care that occurs in at least 53 genera of teleost fishes, as well as one frog species (Jiménez de la Espada, 1872; Oppenheimer, 1970). Mouthbrooding individuals protect developing young in their mouths for periods of days to months and typically cease feeding during this period (Oppenheimer, 1970; Smith and Wootton, 1994). Despite the cessation of feeding, whole-clutch infanticide does not typically occur in mouthbrooding parents (Mrowka, 1987). The maternal mouthbrooding African cichlid fish *Astatotilapia burtoni* is an excellent model to investigate how the brain controls the conflicting decisions of infanticide and maternal care (Maruska and Fernald, 2018). Females brood developing young in their mouths for approximately 2 weeks, then provide post-release maternal care for several days by allowing free-swimming fry to re-enter their mouths

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for protection (Renn et al., 2009). Both male and female adult *A. burtoni* will cannibalize fry in communities, but during the mouthbrooding and maternal care phases, mothers typically do not exhibit infanticide (Renn et al., 2009). However, following the post-release maternal care phase, mothers will consume their own young (Renn et al., 2009). How the brain triggers this switch between care and consumption of offspring is unknown.

During the mouthbrooding period, *A. burtoni* fry develop in their mother's buccal cavity for approximately 14 days before being released. The sensory world and social environment differ dramatically between pre-release fry within the mother's buccal cavity and post-release fry. Prior to release, fry remain in the buccal cavity in close contact with fry of the same cohort and derive nutrition from their yolk sacs (Flegler-Balon, 1989). Following release, fry must forage, shoal with conspecifics, interact with unrelated fish and evade predation, which includes recognizing their mother to seek refuge in her mouth if needed. The cognitive demands after fry release are therefore high, but which brain regions are important for supporting this drastic lifestyle change are unknown for offspring from any mouthbrooding species. Understanding which brain nuclei are involved in this transition will provide insights into the neural mechanisms underlying offspring survival in mouthbrooding species and the evolution of these neural circuits more broadly.

The goal of this study was to test whether neural activation patterns differ (1) among mouthbrooding females, females providing maternal care and females exhibiting infanticide, and (2) between fry before and after release from the mother's mouth. We compared neural activation in nine brain regions in mothers exhibiting different behaviors and nine brain regions and the inner nuclear layer (INL) of the retina between pre- and post-release fry. We identified distinct activation patterns associated with mouthbrooding, maternal care and infanticide, and with pre- and post-release fry. Because fishes are the largest and oldest group of vertebrates, our results have important implications for the evolution of parental care circuits and offspring development across vertebrates.

MATERIALS AND METHODS

Experimental animals

Astatotilapia burtoni (Günther 1894) derived from a wild-caught stock originally collected by Dr Russell Fernald in the 1970s from Lake Tanganyika, Africa, were laboratory raised under conditions similar to those of their natural environment (~28°C, pH 8.0, 12 h:12 h light:dark cycle). Fish were fed cichlid flakes (AquaDine, Healdsburg, CA, USA) daily and brine shrimp (Sally's Frozen Brine Shrimp, San Francisco, CA, USA) twice weekly. Prior to experiments, fish were housed in mixed-sex groups in aquaria with gravel-covered bottoms and halved terracotta pots to serve as shelters. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA, and were in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011.

Experimental procedures

To identify the neural correlates of maternal care and infanticide, mouthbrooding adult females were collected from community aquaria in the early brooding stages (1–2 days post-spawning) and transferred to experimental 38 liter tanks. Only one female was placed in each tank. Females were randomly assigned to either pre-release mouthbrooding (Br), post-release maternal care (MC) or infanticide (Inf) experimental groups (Fig. 1). Pre-release mouthbrooding females used here were identical to those used in a previous study from the laboratory and were collected in the late brood stage (~12 days post spawning) (Maruska et al., 2020b). Almost all (7/9) maternal care females were collected concurrently with the brooding females from 2016 to 2018. The remaining maternal care and all the infanticide females were collected from 2019 to 2021; however, laboratory conditions and husbandry practices remained identical throughout all collections. Maternal care females were collected 1–3 days after fry release, when >80% of their free-swimming brood remained. Infanticide females were collected 3–5 days post fry release, when <60% of their free-swimming brood remained, indicating they had consumed ≥40% of their offspring. Females were monitored daily for fry release, and fry were counted daily to monitor infanticide. None of the females in any group were fed during the experimental period prior to collection, with the exception of infanticide females that consumed their offspring after release. However, females may have consumed algae growing on the gravel in experimental tanks. We chose not to feed females in order to reliably induce infanticide (previous observations revealed that fed females exhibit less infanticide) and to collect a sufficient number of females that had consumed ≥40% of their fry. During dissection, fry in the stomach and intestines were counted to confirm infanticide occurred.

A total of 32 females were collected: 11 mouthbrooding females, 9 post-release maternal care females and 12 infanticide females. All females were collected in the morning, between 09:00 and 11:00 h. Standard length (SL) and body mass (M_b) were recorded, and condition factor [K; K=100(M_b/SL^3)] was calculated. Females were anesthetized in ice-cold cichlid system water and killed via rapid cervical transections. Ovaries were removed and weighed to calculate gonadosomatic index (GSI) as a measure of reproductive investment. Brains were exposed, then heads were fixed in 4% paraformaldehyde in 1× phosphate buffered saline (PBS) at 4°C for 24 h. Heads were then transferred to 1× PBS for~24 h at 4°C. Brains were removed from heads, cryoprotected in 30% sucrose made in 1× PBS for 24 h at 4°C, embedded in optimal cutting temperature (OCT) medium (TissueTek, Sakura, Torrance, CA, USA) and sectioned in the transverse plane at 20 µm using a cryostat (Cryostar NX50), then collected on alternate sets of charged slides (Superfrost plus, VWR, Radnor, PA, USA) and stored at −80°C until staining.

To identify differences in neural activation of fry before (pre-release) and after (post-release) release from the mother's mouth, 13 pre-release fry were collected 1–2 days before fry release (~12 days after brooding onset) and 15 post-release fry were collected on the day of fry release (~12–14 days after brooding onset) (Fig. 1A). We collected up to two fry from each female and neural activation was quantified in each fry collected. Total length was recorded, and fry were anesthetized in ice-cold cichlid system water and killed via rapid cervical transection. Whole heads were fixed, rinsed, cryoprotected and sectioned as stated above for females.

pS6 Immunohistochemistry

To identify activated neurons, immunohistochemistry staining for the phosphorylated ribosome marker pS6 was performed, as used previously (Butler et al., 2018; Maruska et al., 2020b). S6 phosphorylation is associated with increased translation, and pS6 is present in neurons that were activated within ~1 h prior to euthanization (Knight et al., 2012; Ruvinsky and Meyuhas, 2006), but is also useful for detecting differences among more stable steady states of neural activity (Maruska et al., 2020b). Slides were thawed and sectioned tissue was surrounded with a hydrophobic barrier (Immedge pen, Vector Laboratories, Newark, CA, USA).

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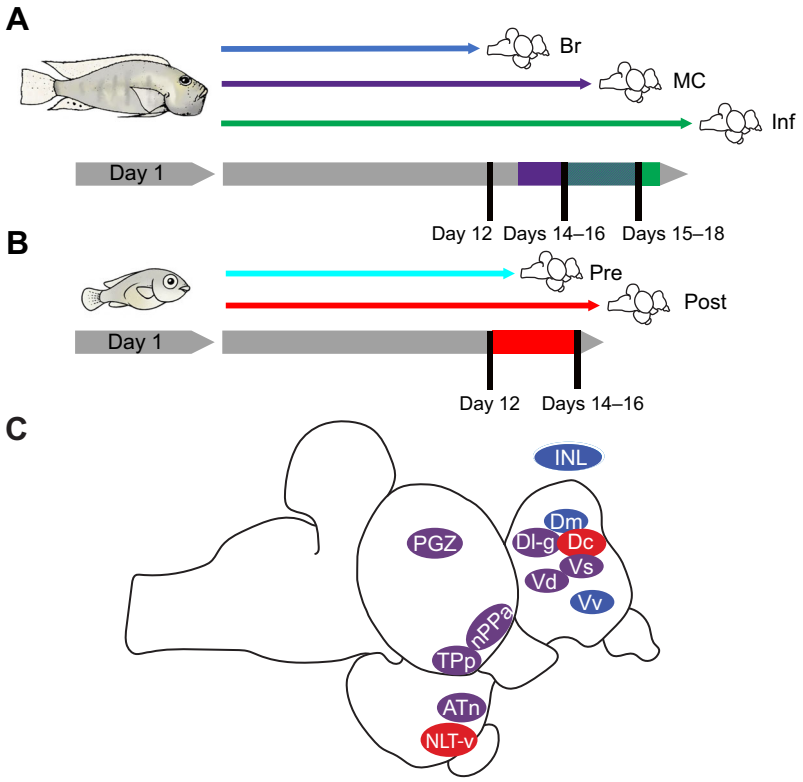

Fig. 1. Experimental protocol for *Astatotilapia burtoni* collection and approximate locations of brain regions analyzed for pS6-stained cells as a proxy for neural activation. (A) Mouthbrooding (Br) females were identified 1–3 days post-spawning in community tanks and transferred to experimental tanks. Brooding females (blue) were collected after 12 days. Maternal care (MC)-providing females (purple) were collected 14–16 days post-spawning, when >80% of their brood remained and the female exhibited maternal care behaviors. Infanticide (Inf) females (green) were collected 15–18 days post-spawning when <60% of the brood remained, indicating the female had consumed >40% of her brood. Hatched bar represents the overlap in collection time of maternal-care-providing females and infanticide females. (B) Pre-release fry (cyan) were collected 12 days post-spawning and 1–2 days before the mouthbrooder released her brood. Post-release fry (red) were collected 12–14 days post-spawning on the day of fry release. (C) Sagittal view of the cichlid brain with locations of analyzed brain regions. Regions in blue were quantified only in pre- and post-release fry. Regions in red were quantified only in mouthbrooding, maternal care and infanticide females. Regions in purple were quantified in both fry and adult females. ATn, anterior tuberal nucleus; Dc-4, central part of the dorsal telencephalon, subdivision 4; Dc-5, central part of the dorsal telencephalon, subdivision 5; Dl-g, lateral part of the dorsal telencephalon, granular zone; Dm, medial part of the dorsal telencephalon; PGZ, periventricular gray zone of the tectum; NLTv, lateral tuberal nucleus, ventral subdivision; nPPa, parvocellular preoptic nucleus, anterior part; TPp, periventricular nucleus of the posterior tuberculum; Vd, dorsal part of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; Vv, ventral part of the ventral telencephalon.

Slides were then washed with 1× PBS (3×10 min), non-specific binding was blocked (0.2% bovine serum albumin, 0.3% Triton-X and 5.0% normal goat serum made in 1× PBS; 2 h), and slides were incubated with primary pS6 antibody (1:1500; Cell Signaling Technologies pS6 ribosomal protein S235/236 antibody no. 4858) overnight (∼18 h) at 4°C. Slides were rinsed with 1× PBS (3×10 min), incubated with biotinylated goat anti-rabbit IgG secondary antibody (Vector Labs BA-1000; 1:277) at room temperature for 2 h, and reacted with 3,3′-diaminobenzidine (Vector Labs DAB Substrate Kit) substrate for 30 min. Slides were dehydrated through an alcohol series (50%, 70% and 95% for 1 min each; 100% 2×2 min each), cleared with xylene (2×3 min) and coverslipped using cytoseal-60. Pre-absorption of the primary pS6 antibody with pS6 blocking peptide did not result in staining (Butler et al., 2018).

Imaging and analysis

Slides were visualized on a Nikon Eclipse Ni microscope and images were taken using a digital color camera (Nikon DS-Fi2) controlled with Nikon NIS elements software. Quantification of pS6-stained cells was performed by individuals blind to the experimental condition. Borders were drawn around regions of interest (ROI) and gridlines were applied. Boxes were randomly selected (3–5 depending on ROI size) and the number of pS6-stained cells within selected boxes was counted. pS6-stained cell density was calculated as the number of pS6-stained cells divided by the area of the boxes that were quantified (Tables S1, S2). For a given region, 3–4 consecutive sections were quantified at the same location within the nucleus ROI across animals. Data from each animal were averaged together to calculate a mean density of pS6-stained cells in each brain region.

We quantified activation in nine brain nuclei implicated in maternal care and social behavior in mouthbrooding, post-release maternal care and infanticide females: the supracommissural nucleus of the ventral telencephalon (Vs), the dorsal part of the ventral telencephalon, caudal subdivision (Vd-c), the granular subdivision of the lateral part of the dorsal telencephalon (Dl-g), two subdivisions (4 and 5) of the central part of the dorsal telencephalon (Dc-4, Dc-5), the anterior tuberal nucleus (ATn), the periventricular nucleus of the posterior tuberculum (TPp), the ventral part of the lateral tuberal nucleus (NLTv), and the parvocellular preoptic nucleus, anterior part (nPPa) (Fig. 1B,C).

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In pre- and post-release fry, we quantified activation in the INL of the retina and in nine brain nuclei implicated in social behavior and sensory processing: the medial part of the supracommissural nucleus of the ventral telencephalon (Vs-m), the dorsal part of the ventral telencephalon (Vd), the ventral part of the ventral telencephalon (Vv), the medial part of the dorsal telencephalon (Dm), the granular subdivision of the lateral part of the dorsal telencephalon (Dl-g), the anterior tuberal nucleus (ATn), the periventricular nucleus of the posterior tuberculum (TPp), the periventricular gray zone of the tectum (PGZ), and the parvocellular preoptic nucleus, anterior part (nPPa) (Fig. 1B).

Statistical analysis

All analysis was performed in SigmaPlot 12.3, IBM SPSS Statistics 27 and R 4.1.0. The Iglewicz and Hoaglin robust test for multiple outliers was used to check data for outliers with a *z*-score of 3.5 (Iglewicz and Hoaglin, 1993). Normality was confirmed using Shapiro–Wilk tests. Standard length, body mass and condition factor were compared across mouthbrooding, maternal care and infanticide females using one-way ANOVAs followed by Tukey's *post hoc* tests. Total length was compared between pre- and post-release fry using a Student's *t*-test. Brain activation data for adults and fry were compared using linear mixed models (LMMs) in IBM SPSS Statistics 27 with brain region as a repeated factor. Animal ID was used as a random factor, and condition and brain region were fixed factors. Because fry total length differed between groups and female standard length differed among groups, we used fry total length and female standard length as covariates.

To identify brain regions that may co-vary in their activation patterns, we used Spearman correlation coefficients to produce heat maps, and significant clusters were identified with hierarchical clustering performed using the pvclust package in R (Suzuki and Shimodaira, 2006). To determine whether neural activation patterns across the brain could predict fish group membership, we used discriminant function analysis. Group means replaced missing values in cases where a given brain region could not be quantified in an individual. No more than 1/11 values per region were replaced in brooding females, 1/9 values per region in maternal care providing females or 4/12 values per region in infanticide females were replaced.

RESULTS

Neural activation during mouthbrooding, maternal care and infanticide

Body mass, GSI and condition factor did not differ among female groups (Table 1). However, maternal-care-providing females had longer standard lengths compared with infanticide females (Table 1; *post hoc*: Inf–MC, P=0.036; Inf–Br, P=0.245; MC–Br, P=0.552).

Table 1. Body mass, standard length, gonadosomatic index (GSI) and condition factor of mouthbrooding, maternal-care-providing and infanticide-exhibiting female *Astatotilapia burtoni*

	Body mass (g)	Standard length (mm)	GSI	Condition factor
Mouthbrooding	1.27±0.36	37.72±1.27	0.72±0.25	2.35±0.40
Maternal care	1.48±0.45	39.78±2.04	0.74±0.38	2.42±0.85
Infanticide	1.22±0.27	34.75±0.74	0.86±0.41	2.95±0.76
$F_{2,31}$	1.50	**3.57**	0.51	2.60
P	0.24	**0.04**	0.61	0.09

Values are reported as means±s.d. Bold denotes significance at P<0.05.

To account for this difference in standard length, we included standard length as a covariate in our linear mixed model.

We analyzed nine brain regions for differences in neural activation among mouthbrooding, maternal care and infanticide group females. There was an overall effect of treatment ($F_{2,332}$=14.820, P<0.001) and region ($F_{8,36}$=92.621, P<0.001), and an interaction between treatment and region ($F_{16,37}$=5.326, P<0.001). Five of the nine regions analyzed had significant differences among groups (Fig. 2). In the Vd-c (Fig. 2A), mouthbrooding females had greater activation compared with both maternal care and infanticide females. Mouthbrooding and maternal care females had greater activation in the Dl-g compared with infanticide females (Fig. 2B). Maternal-care-providing females had greater activation in Dc-5 compared with both mouthbrooding and infanticide females (Fig. 2C). All groups were different from each other in the nPPa, with the greatest activation in mouthbrooding females and the least in infanticide females (Fig. 2D). In the ATn, mouthbrooders had greater activation compared with infanticide females, but not maternal care females (Fig. 2E). No differences were observed among groups in the Vs, Dc-4, NLTv and TPp (Fig. S1; see Table 2 for *post hoc* values).

We performed correlation analysis to identify relationships among different brain regions based on activation. In mouthbrooding females, the Dl-g and Vs, Dl-g and ATn, ATn and Vs, ATn and TPp, ATn and NLT-v, NLT-v and Vs, and Vs and TPp were positively correlated. ATn and nPPa and Dl-g and Vd-c were negatively correlated (Fig. 3A,D; Table S3). Hierarchical clustering revealed one significant cluster, consisting of the Dl-g and Vs. In maternal care females, activation in the Vd-c and Dc-4, Dl-g and ATn, Dl-g and nPPa, Dl-g and TPp, TPp and nPPa, TPp and ATn, and ATn and nPPa were positively correlated (Fig. 3B,E; Table S3). Two significant clusters were identified: one containing the TPp, Dl-g, ATn, NLT-v and nPPa, and a second containing the Dc-5 and Vs. In infanticide females, Dc-5 and Dc-4, Dc-4 and nPPa, Dc-4 and Vd-c, Vd-c and Vs, and nPPa and ATn were positively correlated (Fig. 3C,F; Table S3). One significant cluster was identified containing Dc-5, ATn, Dc-4, nPPa and NLT-v.

To determine whether activity across all nine regions produced distinct brain activation patterns in brooding, maternal care and infanticide females, we performed discriminant function analysis. The discriminant function analysis produced one significant function, explaining 69.0% of the total variation (eigenvalue=5.225, χ^2=33.403, P=0.015). Function 1 separated mouthbrooding and maternal care females from infanticide females, suggesting that mouthbrooding and maternal care result in more similar neural activation patterns compared with infanticide. Function 1 was strongly positively loaded by neural activation in Dc-5 and ATn and strongly negatively loaded by activation in Dc-4 (Fig. 4). Function 2 was not significant (eigenvalue=2.347, χ^2=13.289, P=0.102). The discriminant function analysis correctly classified group membership for 90.9% of mouthbrooding females, 55.6% of maternal care females and 75% of infanticide females.

Neural activation in pre- and post-release fry

Post-release fry were slightly larger than pre-release fry (TL, pre: 8.43±0.32 mm; post: 9.45±0.21 mm; t=2.769, P=0.0122), which likely reflects the different developmental stages of the fry at collection (e.g. pre-release collected at ∼12 days and post-release collected at ∼15 days post fertilization). These size differences were accounted for by including total length as a covariate in the linear mixed model.

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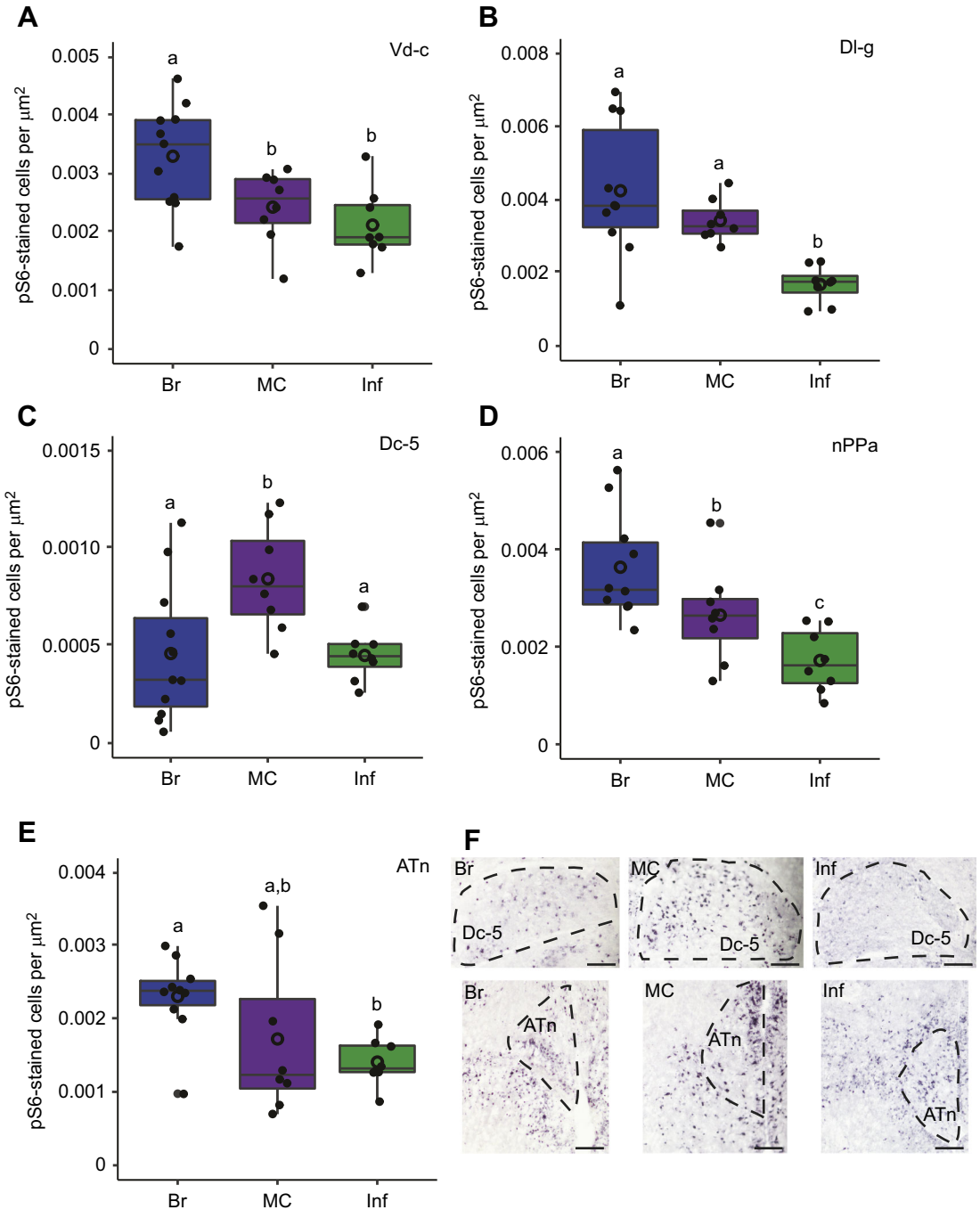

Fig. 2. Differences in neural activation patterns shown by pS6-stained cell densities among mouthbrooding (Br), maternal care (MC) and infanticide (Inf) female *A. burtoni*. Activation in the Vd-c (A) was greater in mouthbrooding females compared with maternal care and infanticide females. In the Dl-g (B), mouthbrooding and maternal care females had greater activation than infanticide females. Maternal care-providing females had greater activation in the Dc-5 (C) compared to both other groups. In the nPPa (D), mouthbrooding females had the greatest activation, followed by maternal-care-providing females and then infanticide females. Mouthbrooding females had greater activation compared with infanticide females in the ATn (E), but activation in maternal care females was similar to that of both brooding and infanticide females. (F) Representative photomicrographs of pS6 staining in the Dc-5 and ATn of mouthbrooding, maternal-care-providing and infanticide exhibiting females. Distracting artifacts were removed from photomicrographs using Photoshop as needed. Sample sizes: *N*=11 mouthbrooding (Vd-c, Dc-5, ATn); *N*=10 mouthbrooding (nPPa, Dl-g); *N*=8 maternal care; *N*=8 infanticide. Different letters indicate statistical differences at $P<0.05$. Scale bars: 100 µm in all photomicrographs. Boxes extend to the furthest data points of the first and third quartiles. Medians are represented by solid lines. Means are indicated by open circles. Filled circles represent individual data points. Whiskers extend to the furthest data point within 1.5× the interquartile range. Outliers beyond the 1.5× interquartile range are not statistical outliers. See Fig. 1 for abbreviations.

We analyzed activation in nine brain regions and the INL of the retina in pre- and post-release fry. There was an overall effect of region ($F_{9,23}$=46.676, P<0.0001) and treatment ($F_{1,40}$=39.630, P<0.0001), and an interaction between region and treatment ($F_{9,23}$=23.136, P=0.006). Five brain regions, the Dm, Vs-m, Vd, PGZ and TPp, and the INL of the retina had differential activation between groups, with greater activation in post-release fry in each of these regions (Fig. 5). The Vv, ATn, Dl-g and nPPa did not show

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Table 2. *Post hoc* region×treatment interaction statistics for mouthbrooding (Br), maternal care (MC) and infanticide (Inf) female neural activation in *A. burtoni*

Region	*F*	Br versus MC *P*	Br versus Inf *P*	MC versus Inf *P*
Vd-c	8.938	**0.006**	**<0.001**	0.364
Vs-m	1.172	0.428	0.128	0.498
Dl-g	9.353	0.180	**<0.001**	**0.011**
Dc-4	0.944	0.184	0.774	0.318
Dc-5	4.568	**0.010**	0.827	**0.008**
nPPa	10.742	**0.017**	**<0.001**	**0.034**
NLTv	0.299	0.943	0.478	0.544
ATn	4.596	0.059	**0.006**	0.348
TPp	1.387	0.997	0.141	0.170

Bold indicates significant differences at $P<0.05$.

significant differences in neural activation between pre- and post-release fry (Fig. S2; see Table 3 for *post hoc* values). The greater activation in these brain regions and the retina in post-release fry suggests increased cognitive demands associated with social interactions, foraging and sensory stimulation following release from the mother's mouth.

To examine relationships in activation among different brain regions, we performed correlation analyses. Spearman correlations revealed neural activation in the Dm and PGZ, PGZ and Dl-g, nPPa and Vs, and Vv and nPPa were positively correlated, whereas neural activation in the Vd and Dm was negatively correlated in pre-release fry (Fig. 6A,C; Table S4). Hierarchical clustering revealed a significant cluster containing the Vd and the INL. In post-release fry, neural activation in the Dm and Vv was positively correlated, whereas activation in the Vd and TPp was negatively correlated (Fig. 6B,D; Table S4). Two clusters were identified: one consisting of the Dm and Vv and a second consisting of the Vd, nPPa and PGZ. To determine whether distinct neural activation patterns were present in pre- and post-release fry, we also performed discriminant function analysis. The analysis revealed one function, which was not significant (eigenvalue=30.271, χ^2=12.049, P=0.099). Overall, these data reveal differences in neural activation among mouthbrooding, maternal care and infanticide females, and between pre- and post-release fry in several distinct nuclei (Fig. 7).

DISCUSSION

We compared neural activation patterns among mouthbrooding, maternal-care-providing and infanticide-exhibiting female *A. burtoni* to test whether neural activation differs among mouthbrooding mothers, caring mothers and mothers that have consumed their own offspring. We identified differential neural activation patterns associated with each of these states and found nuclei that co-vary and may represent functional networks. Discriminant function analysis revealed a significant function that

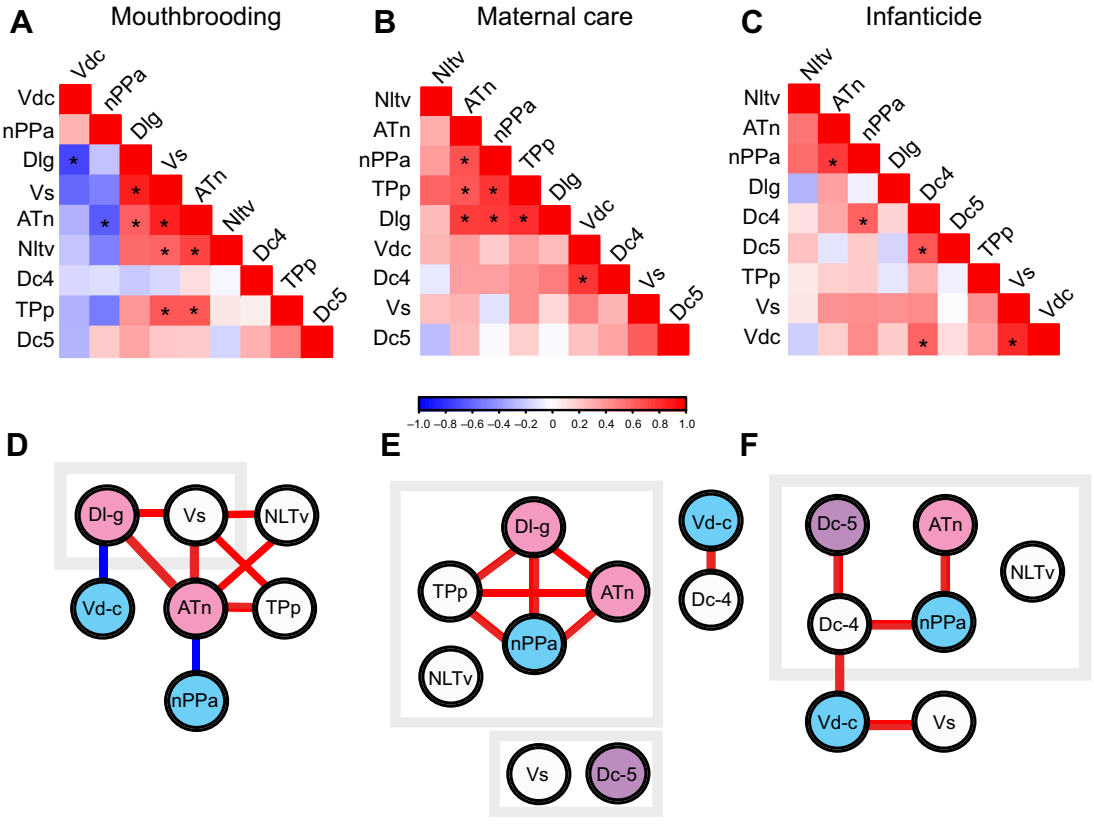

Fig. 3. Spearman correlation coefficients and hierarchical clustering reveal distinct co-variation networks among mouthbrooding, maternal-care-providing and infanticide-exhibiting female *A. burtoni*. Co-activation heatmaps of Spearman correlation coefficients (color scale) across nine brain regions shows differences in activation patterns among mouthbrooding (A), maternal care (B) and infanticide (C) females. Asterisks indicate significant correlations at $P<0.05$. Diagrams of significantly correlated regions based on Spearman correlations and hierarchical clustering in mouthbrooding (D), maternal care (E) and infanticide (F) females. Node color represents regions with greatest activation in mouthbrooding (blue), maternal care (purple), or mouthbrooding and maternal care (pink) females. White nodes represent regions with no significant differences among groups. Red lines indicate positive correlations whereas blue lines indicate negative correlations. Gray boxes indicate significant clusters. See Fig. 1 for abbreviations.

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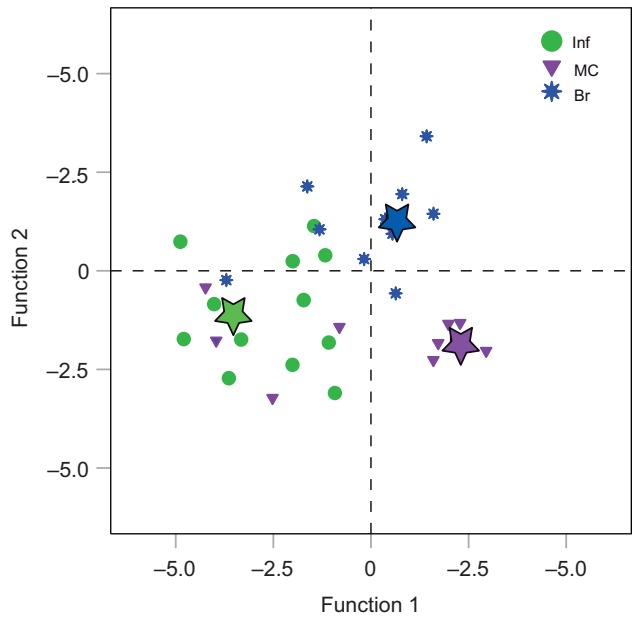

Fig. 4. Discriminant function analysis separates infanticide females from maternal care and mouthbrooding females in *A. burtoni*. Discriminant function analysis correctly classified 90.9% of mouthbrooding females, 55.6% of maternal care females and 75% of infanticide females. Function 1 explains 69% of the variation (eigenvalue=5.225, χ^2=33.403, *P*=0.015). Function 2 explains 31% of the variation (eigenvalue=2.347, χ^2=13.289, *P*=0.102). Circles and triangles represent individual fish and stars indicate group centers. Sample sizes: *N*=12 infanticide; *N*=9 maternal care; *N*=11 mouthbrooding.

separated mouthbrooding and maternal care females from infanticide females based on neural activation alone, suggesting different circuitry involved in offspring-promoting compared with offspring-hindering behaviors. Further, we identified differential activation patterns and distinct networks of co-variation between pre- and post-release fry. Together, our results provide insights into the nuclei implicated in the behavioral switch from care to cannibalism and the nuclei that support the increased cognitive demands associated with a fry's release from its mother's mouth.

Neural activation during mouthbrooding, maternal care and infanticide

Mouthbrooding females had greater activation in the Dl-g and ATn compared with infanticide females but not maternal care females. Previous work from our laboratory to examine neural activation specifically related to mouthbrooding-associated energetic state identified differences in neural activation among mouthbrooding females, fed females and starved females (Maruska et al., 2020b). In the Dl-g, mouthbrooding females in that study had lower activation compared with starved females but greater activation compared with fed females (Maruska et al., 2020b), suggesting some stimuli from fry might alter activation in this brain region. This fry stimulus is likely still present during the maternal care phase, perhaps explaining the similarity between Dl-g activation in brooding and caring mothers. Evidence in rodents suggests that the putative homologue to the ATn, the ventromedial hypothalamus, plays roles in feeding and blood glucose regulation (King, 2006). For example, in mice, brain-derived neurotrophic factor in the ventromedial hypothalamus is important for satiety and energy balance (Xu et al., 2003), suggesting that the increased activation in the ATn during mouthbrooding compared with infanticide may in part facilitate the cessation of feeding while fry are present in the mother's mouth. Norepinephrine and GABA concentrations increase in the ventromedial hypothalamus in response to hypoglycemia (Beverly et al., 2001), suggesting that activation in the ATn may increase in response to starvation during mouthbrooding. Maternal-care-providing females showed a trend toward a lower condition factor compared with infanticide-exhibiting females, indicating that maternal-care-providing females still have reduced body conditions and potentially explaining the similarity in ATn activation between mouthbrooding and maternal care females. In contrast with these findings, mouthbrooding females do not have different neural activation in the ATn compared with starved or fed females (Maruska et al., 2020b), perhaps suggesting different responses in the ATn to different food sources, such as cichlid flakes compared with the high protein intake associated with infanticide, or alternatively, activation of different neuron phenotypes in each condition. In maternal-care-providing females, activation in the ATn and Dl-g is positively correlated, and the two regions are grouped in a significant cluster, suggesting they may act in the same circuit to promote maternal behaviors. The ATn and Dl-g may serve to integrate fry stimuli and energetic state during both maternal care and mouthbrooding.

In addition to its function in energetics, the ATn may support fry defense during mouthbrooding and maternal care. Maternal-care-providing and brooding females must display defensive behaviors to avoid predator and conspecific attacks on fry. Brooding *A. burtoni* females initiated aggression toward gravid females when a gravid female was placed in the brooding female's experimental tank, and ATn activation was greater during aggressive contexts compared with reproductive and control conditions (Field and Maruska, 2017). In mice, the ventromedial hypothalamus is critical for female aggression (Hashikawa et al., 2017), and in rats, lesions to the ventromedial hypothalamus reduce maternal aggression (Hansen, 1989). The potential involvement of the ATn in energetic regulation and maternal aggression is further supported by our discriminant function analysis, which revealed that activation in the ATn strongly and positively loads the function that separates mouthbrooding and maternal care females from infanticide females. The ATn may play a role in several maternal care and mouthbrooding behaviors, including fry defense, aggression and integration of energetic state.

Maternal-care-providing females had greater activation in Dc-5 compared with both mouthbrooding and infanticide females. Our previous work showed that mouthbrooders had greater activation in Dc-5 compared with both fed and starved females, suggesting that Dc-5 plays a role in maternal behaviors (Maruska et al., 2020b). In bluegill sunfish, stimulation of Dc results in nestbuilding, an important parental care behavior (Demski and Knigge, 1971). Discriminant function analysis revealed that activation in Dc-5 strongly loads the function that separates mouthbrooding and maternal-care-providing females from infanticide females, further supporting the importance of Dc-5 in maternal behaviors. Some evidence suggests that the teleost Dc is homologous to the mammalian isocortex (Mueller et al., 2011; Northcutt, 2011). The isocortex is involved in functions such as spatial reasoning, sensory processing and motor control, all of which are important for fry location and retrieval during the *A. burtoni* maternal care phase. Activation in Dc-5 is clustered with the Vs in maternal-care-providing females. The Vs receives sensory inputs, and in weakly electric fish, *Gymnotus carapo*, fibers from the Vs project to the central dorsal telencephalon (Corrêa et al., 1998), indicating that perhaps these nuclei act in a common circuit to recognize and

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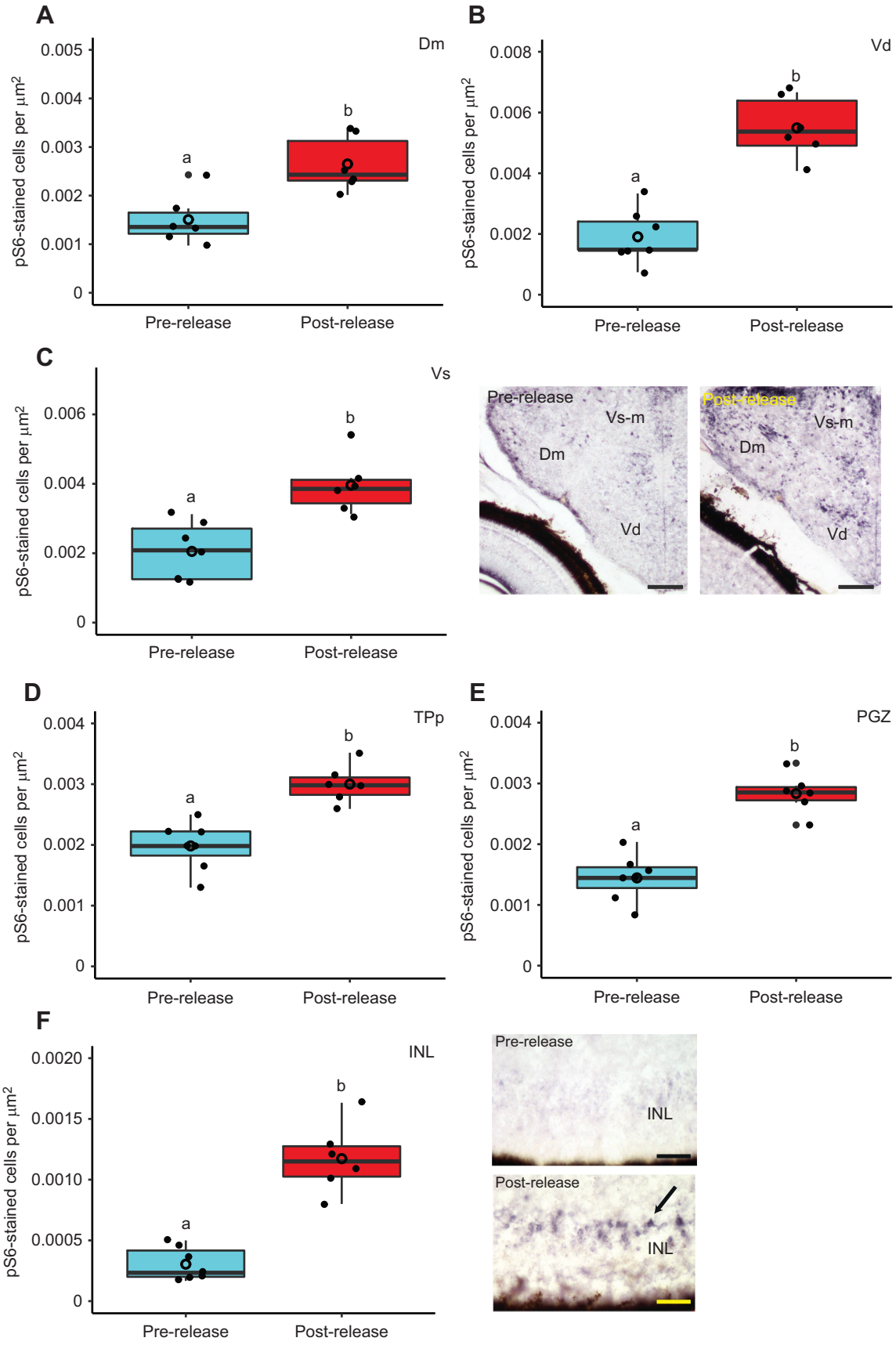

Fig. 5. See next page for legend.

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Fig. 5. Differences in neural activation patterns shown by pS6-stained cell densities between pre- and post-release *A. burtoni* fry. Activation was greater in post-release fry in the (A) Dm, (B) Vd, (C) Vs, (D) TPp, (E) PGZ of the tectum and (F) inner nuclear layer (INL) of the retina. Photomicrographs depict differences in pS6-stained cell density between pre- and post-release fry in the Dm, Vs-m and Vd, and in the INL of the retina. Sample sizes: *N*=7 pre-release (Vd, Vs-m, INL); *N*=6 pre-release (Dm, TPp); *N*=5 pre-release (PGZ); *N*=6 post-release (Dm, Vd, Vs-m, INL); *N*=5 post-release (TPp, PGZ). Different letters indicate statistical differences at $P<0.05$. Scale bars: 100 µm (Dm, Vs-m, Vd), 25 µm (INL). Distracting artifacts were removed from photomicrographs using Photoshop as needed. See Fig. 2 for boxplot description. See Fig. 1 for abbreviations.

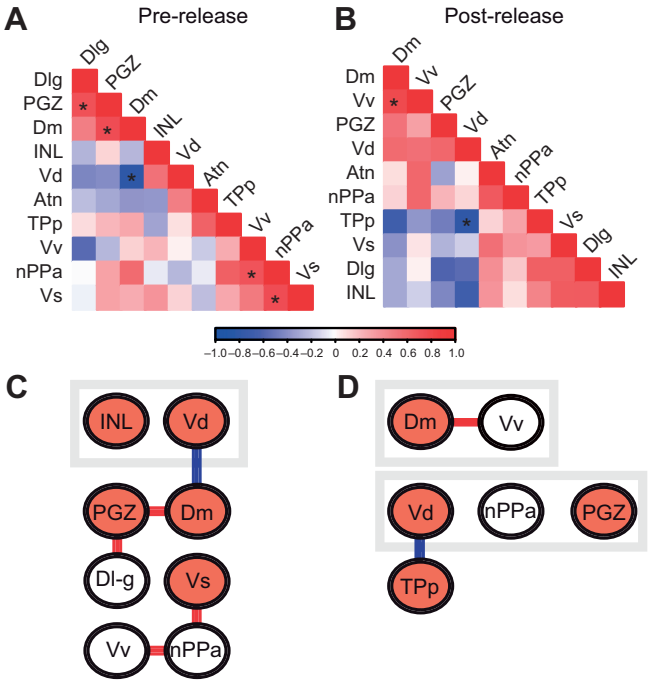

Fig. 6. Spearman correlation coefficients and hierarchical clustering reveal distinct co-variation networks between pre- and post-release *A. burtoni* fry. Heatmap of Spearman correlation coefficients across nine brain regions and the inner nuclear layer of the retina in (A) pre- and (B) post-release fry. Asterisks indicate significant correlations at $P<0.05$. Diagrams of significantly correlated regions based on Spearman correlations and hierarchical clustering in (C) pre-release and (D) post-release fry. Node color represents regions with greatest activation in post-release fry (red) or no difference (white). Red lines indicate positive correlations while blue lines indicate negative correlations. Gray boxes indicate significant clusters. See Fig. 1 for abbreviations.

retrieve fry. Thus, Dc-5 appears to be especially important for maternal care behaviors after fry release, possibly related to sensory, decision-making and motor demands associated with fry protection and retrieval.

We found greater neural activation in the Vd-c and nPPa of mouthbrooding females compared with maternal care and infanticide females. In our previous study, we also observed greater activation in the Vd-c of mouthbrooders compared with starved females, which in turn had greater activation than fed females (Maruska et al., 2020b). These patterns suggest that the Vd-c may be important for integration of the energetic state and associated brooding behaviors of mouthbrooders while the developing fry are still in the mouth. In the nPPa, we observed that maternal-care-providing females had lower activation in the nPPa compared with mouthbrooding females, but greater activation compared with infanticide females. Mouthbrooding females also have similar neural activation to starved females in the nPPa, suggesting the greater activation in the nPPa of mouthbrooding females may be due to the starved energetic state of mouthbrooders compared with maternal care and infanticide females that are eating (Maruska et al., 2020b). The greater activation in the nPPa of both mouthbrooders and maternal care females compared with infanticide females could in part be due to activation of specific neuronal phenotypes important for maternal behaviors. For example, mouthbrooders and maternal-care-providing females have greater activation of galanin-expressing cells in the nPPa compared with infanticide females (Butler et al., 2020), supporting the idea that specific nPPa neuron populations help mediate maternal versus infanticide behaviors. In mice, estrogen receptor expressing cells in the MPOA are implicated in maternal care behaviors (Fang et al., 2018). Estrogen receptors α and β are expressed in the nPPa of *A. burtoni* (Maruska et al., 2020a), and activation of cells expressing these receptors could in part also underly differences in activation among brooding, maternal care and infanticide females. Activation in both the Vd-c and nPPa are correlated with activation in other nuclei in brooders, further supporting the potential roles of these regions in a mouthbrooding-promoting circuit. These results highlight the potential importance of the Vd-c and nPPa in integrating energy status and mouthbrooding behavior.

Table 3. *Post hoc* region×treatment interaction statistics for neural activation in pre- and post-release *A. burtoni* fry

Region	*F*	*P*
Dm	**12.449**	**<0.001**
Vs-m	**17.931**	**<0.001**
Vd	**46.170**	**<0.001**
Vv	1.628	0.228
Retina	**52.510**	**<0.001**
Tectum	**27.185**	**<0.001**
ATn	0.286	0.608
Dl-g	4.507	0.060
TPp	**14.529**	**<0.001**
nPPa	4.609	0.057

Bold indicates significant differences at $P<0.05$.

No differences were found in neural activation of the Vs, Dc-4, NLTv or TPp among mouthbrooding, maternal care and infanticide females. Although these regions are implicated in integration of feeding, energetic and maternal behaviors in *A. burtoni* females (Maruska et al., 2020b), they may play less of a role in the circuitry regulating the decision to perform maternal care behaviors versus infanticide. The lack of differences among groups in these regions may also simply reflect the role of these regions in multiple social contexts, or that different populations of neurons are activated in each context that cannot be discerned based on pS6 staining alone. For example, the TPp and its putative homolog the ventral tegmental area (VTA) are implicated in maternal care, feeding and aggression in various contexts (Fang et al., 2018; Mahadevia et al., 2021; Yokobori et al., 2012), and the Vs is involved in sensory processing (Butler and Maruska, 2016; Field et al., 2018). Our hierarchical clustering and correlation analysis revealed that although the TPp, Vs, NLTv and Dc-4 are not differentially activated among groups, these regions are still grouped with and correlated with regions that do display differential activation across groups. For example, in teleosts, several of these regions project to the dorsal or ventral telencephalon, which include several regions implicated in brooding and maternal care (Corrêa et al., 1998; Rink and

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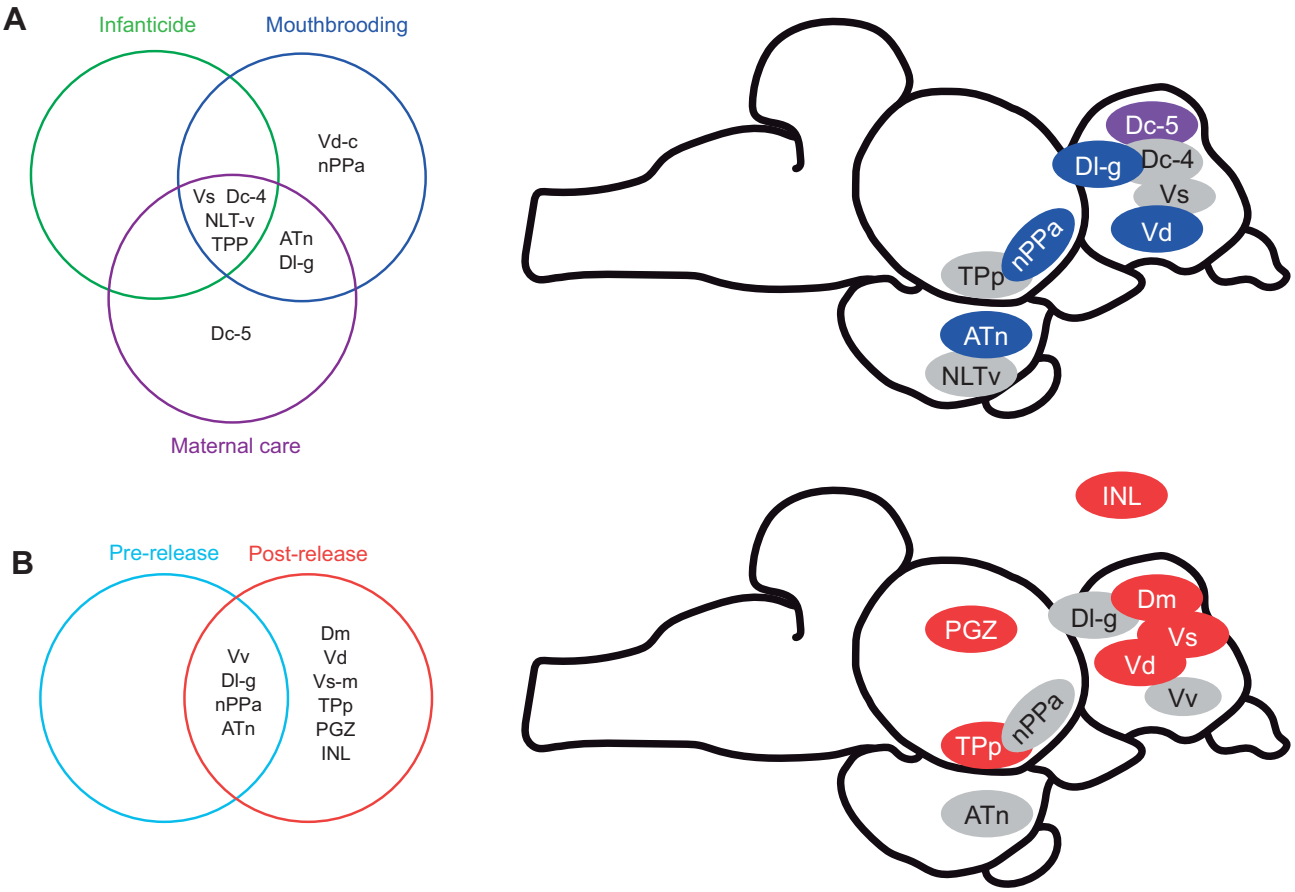

Fig. 7. Neural activation differences in the *A. burtoni* brain associated with mouthbrooding, maternal care, infanticide and fry release. Venn diagrams and schematic lateral view of the cichlid brain depicting neural activation differences (A) among mouthbrooding, maternal care and infanticide females and (B) between pre- and post-release fry. Regions are sorted by the condition in which neural activation was greatest, and regions in overlapping circles indicate similar activation. Lateral view of the cichlid brain depicts which regions have greatest activation (A) during maternal care (purple) or brooding (blue) and (B) in post-release in fry (red). Gray indicates no differences in neural activation among groups.

Wullimann, 2004). This suggests that these regions may still be involved in the neural circuitry underlying mouthbrooding, maternal care and infanticide despite their similar activation across treatments.

Previous work in rodents suggests infanticide may rely on a dedicated neural circuit, separate from parental care. Ablation of MPOA galanin cells important for parental care in mice did not result in infanticidal behaviors in male or female parental mice, suggesting that infanticide and parental care behaviors rely on distinct neural circuits (Wu et al., 2014). Further, in male rodents, neural activation underlying infanticide is similar to that underlying aggression. For example, the VMH, a region important for aggression in rodents, is also implicated in infanticidal behaviors in male mice (Renier et al., 2016). Here, we show that infanticide-exhibiting females have lower activation in the ATn, the teleost homolog to the VMH, compared with mouthbrooding females. This suggests that infanticide exhibited by *A. burtoni* females is not rooted in aggression, but could be due to other factors such as hunger. As females recover from mouthbrooding-induced starvation, infanticide may become adaptive to help regain body condition and invest in gonadal growth to prepare the female to brood once again. From hierarchical clustering analysis and Spearman correlations, we show similarities in the brain nuclei implicated in mouthbrooding and maternal care, suggesting some overlap in the circuitry underlying these parental behaviors. This, along with our discriminant function analysis results, suggests that the circuitry underlying infanticide is distinct from mouthbrooding and maternal care, and may be more related to other states such as hunger. This is consistent with that seen in mammals, where infanticide by male parents involves infant-directed aggression, whereas in females, infanticide is typically driven by resource limitation and offspring are consumed as a source of nutrition (Kohl et al., 2017). More work is needed to identify the functional connections and circuitry that underlie brooding, care and infanticide, the neurotransmitters and hormones involved in regulating these circuits, as well as the stimuli that initiate the transitions from mouthbrooding to maternal care and from care to infanticide.

Pre- and post-release fry neural activation

We identify here, for the first time, which brain regions in the offspring's brain are involved in the dramatic transition between residence inside the mother's mouth to a free-swimming fry. In rodents, the immediate early gene *cfos* increases in several nuclei including the suprachiasmatic, supraoptic and periventricular nuclei of the hypothalamus following birth (Hoffiz et al., 2021). However, to our knowledge, ours is the first description of neural activation associated with transition from inside to outside a parent in a non-mammalian vertebrate. Post-release fry had greater neural activation compared with pre-release fry in five brain regions and the INL of

the retina. Two of these regions, the Dm and Vd, are implicated in choice behavior in zebrafish (Lau et al., 2011). Post-release fry have greater opportunities for choice in behaviors such as foraging, predator avoidance and interactions with conspecifics, which may be reflected by greater neural activation in the Dm and Vd. Hierarchical clustering grouped the Vd and Dm, further supporting their roles in a shared circuit. The Vs-m, Vd and PGZ as well as the INL of the retina are involved in sensory processing, and greater activation of these regions in post-release fry may reflect the more complex sensory environment after release. Both the Vd and Vs receive input from the olfactory bulbs in fish (Forlano and Bass, 2011) and are implicated in processing chemosensory signals in a social context in *A. burtoni* (Field et al., 2018). Further, the Vs is involved in mechanosensory processing during male–male territorial interactions in *A. burtoni* (Butler and Maruska, 2016), and is known to receive auditory and visual inputs in other fish species (Goodson and Bass, 2002; Ito et al., 1986). The PGZ of the tectum plays a role in visual processing, and in goldfish, the optic tectum responds to visual stimuli in a color-dependent manner (Gibbs and Northmore, 1998). Neurons in the tectum respond to motion and aid in prey detection and capture in larval zebrafish (Förster et al., 2020). In addition to vision, the tectum receives input from somatosensory processing regions (Xue et al., 2006), and evidence suggests the tectum is critical for the integration of multi-modal sensory stimuli and the subsequent motor output (Salas et al., 2006). Greater activation in post-release fry in these regions reflects the more complex social and sensory environments post-release compared with pre-release inside the mother's buccal cavity.

Greater activation in the TPp of post-release fry compared with pre-release fry may reflect the increase in goal-oriented behaviors, such as foraging. The TPp is a nucleus of the mesolimbic reward system, contains dopaminergic cells and is homologous, in part, to the mammalian VTA (O'Connell et al., 2011; O'Connell and Hofmann, 2011). Pre-release fry do not forage and rely on their yolk sac for nutrition, whereas post-release fry have fully reabsorbed yolk sacs and forage for their food. Further, the yolk sac provides pre-release fry with a constant source of nutrition whereas post-release fry likely experience periods of hunger, promoting goal-directed behaviors such as foraging. Hierarchical clustering grouped the TPp with regions important for sensory processing (INL, Dl-g and Vs) and social behaviors (nPPa) in post-release fry, suggesting that the TPp may work with these regions to respond to sensory stimuli in the environment and perform reward-motivated behaviors. The increase in reward-oriented behaviors such as foraging and group shoaling may in part account for the greater activation in the TPp of post-release fry, as feeding and social interactions are critical for a fry's success.

No differences in neural activation were observed between pre- and post-release fry in the Dl-g, nPPa, Vv or ATn. However, each of these regions except the ATn were placed in clusters by hierarchical clustering analysis and correlations were identified that included each of these regions. This suggests that although these nuclei play roles in diverse processes, they may function in the circuits underlying important behaviors for both pre- and post-release fry. Although the sensory environment and cognitive demands associated with fry release are dramatically different from those associated with remaining in the mother's mouth, pre-release fry must still process relevant stimuli and maintain physiological homeostasis. For example, tilapia fry have fully formed and functioning retinas prior to release from the brooder's mouth (Grün, 1975), suggesting visual processing may occur before the initial release. Thus, fry may learn to recognize their siblings within their mother's mouth. Even without the challenges of foraging and evading predation, fry inside the brooder's mouth must maintain basal processes such as circadian rhythms and nutrient metabolism. Although the fry's movements within the buccal cavity are not yet characterized, late brooding fry are capable of movement and may move within their mother's mouth to avoid hypoxic conditions. Characterizing the movements and behaviors of the fry during the brooding period will provide better insights into the cognitive demands associated with the late mouthbrooding period and the transition to free-swimming fry.

Conclusions

Neural activation patterns differ among mouthbrooding, maternal-care-providing and infanticide-exhibiting females as well as between pre- and post-release fry. Analyses of activation patterns in nine regions revealed five regions with differential activation among mouthbrooding, maternal care and infanticide females, as well as distinct co-variation patterns in each group. Discriminant function analysis separated infanticide females from mouthbrooding and maternal care females based on activation patterns alone, highlighting different circuitry for maternal tasks to promote offspring survival compared with those involved in offspring consumption. Several brain regions were also identified as important in two or all three female groups, suggesting both shared and distinct circuitry involved in these parental decision states. However, females exhibiting infanticide showed the most different activation patterns, indicating that consumption of one's own offspring is perceived very differently from care behaviors in the brain. In pre- and post-release fry, six of the 10 regions analyzed showed greater activation after release and distinct co-variation networks associated with each group, suggesting activation is driven by different sensory environments, cognitive demands and behaviors. Together, these results highlight the brain regions that may support important transitions in the reproductive cycle and during development, and provide important comparative data to examine the evolution of neural circuits involved in infanticide and parental behaviors.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: E.J.R., J.M.B., K.P.M.; Methodology: E.J.R., J.M.B., K.P.M.; Formal analysis: E.J.R., J.M.B., K.P.M.; Investigation: E.J.R., J.M.B., K.P.M.; Resources: K.P.M.; Data curation: E.J.R., J.M.B., K.P.M.; Writing - original draft: E.J.R.; Writing - review & editing: E.J.R., J.M.B., K.P.M.; Visualization: E.J.R., J.M.B., K.P.M.; Supervision: K.P.M.; Project administration: K.P.M.; Funding acquisition: K.P.M.

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Data availability

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

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