Distribution and Female Reproductive State Differences in Orexigenic and Anorexigenic Neurons in the Brain of the Mouthbrooding African Cichlid Fish, Astatotilapia burtoni

Danielle Tiffany Porter

Louisiana State University and Agricultural and Mechanical College

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DISTRIBUTION AND FEMALE REPRODUCTIVE STATE DIFFERENCES IN OREXIGENIC AND ANOREXIGENIC NEURONS IN THE BRAIN OF THE MOUTHBROODING AFRICAN CICHLID FISH, ASTATOTILAPIA BURTONI

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science in The Department of Biological Sciences

by
Danielle Tiffany Porter
B.S., University of Alabama, 2012
August 2015
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Abbreviations

A  anterior thalamic nucleus
AC  anterior commissure
aGn  anterior glomerular nucleus
AP  accessory pretectal nucleus
ATn  anterior tuberal nucleus
CC  cerebellar crest
CCe  corpus cerebelli
CCeG  granular layer of corpus cerebelli
CCeM  molecular layer of corpus cerebelli
CCeP  purkinje layer of corpus cerebelli
CeAlc  central nucleus of the amygdala
CG  central gray
CM  corpus mammillare
CP  central posterior thalamic nucleus
CTn  central thalamic nucleus
CZ central zone of tectum
Dc-1  central part of the dorsal telencephalon, subdivision 1
Dc-2  central part of the dorsal telencephalon, subdivision 2
Dc-3  central part of the dorsal telencephalon, subdivision 3
Dc-4  central part of the dorsal telencephalon, subdivision 4
Dc-5  central part of the dorsal telencephalon, subdivision 5
Dd  dorsal part of the dorsal telencephalon
Dd-d  dorsal part of the dorsal telencephalon, dorsal subdivision
Dl-d  dorsal part of lateral zone of the dorsal telencephalon
Dl-g  granular zone of lateral zone of the dorsal telencephalon
Dl-v1  ventral part of the lateral zone of the dorsal telencephalon, subdivision 1
Dl-v2  ventral part of the lateral zone of the dorsal telencephalon, subdivision 2
Dm  medial part of the dorsal telencephalon
Dm-1  medial part of the dorsal telencephalon, subdivision 1
Dm-2c  medial part of the dorsal telencephalon, caudal subdivision 2
Dm-2r  medial part of the dorsal telencephalon, rostral subdivision 2
Dm-3  medial part of the dorsal telencephalon, subdivision 3
DON  descending octaval nucleus
Dp  posterior part of the dorsal telencephalon
DP  dorsal posterior thalamic nucleus
dtt  descending trigeminal tract
DWZ  deep white zone of tectum
Dx  unassigned subdivision of dorsal telencephalon
E  entopeduncular nucleus
ECL  external cell layer of olfactory bulb
EG  granular eminence
FR  fasciculus retroflexus
GL  glomerular layer of olfactory bulb
Gn  glomerular nucleus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>gTN</td>
<td>ganglion of the terminal nerve</td>
</tr>
<tr>
<td>hc</td>
<td>horizontal comissure</td>
</tr>
<tr>
<td>ICL</td>
<td>internal cellular layer of olfactory bulb</td>
</tr>
<tr>
<td>IIIn</td>
<td>oculomotor nucleus</td>
</tr>
<tr>
<td>In</td>
<td>intermediate thalamic nucleus</td>
</tr>
<tr>
<td>IP</td>
<td>interpeduncular nucleus</td>
</tr>
<tr>
<td>LFB</td>
<td>lateral forebrain bundle</td>
</tr>
<tr>
<td>LL</td>
<td>lateral lemniscus</td>
</tr>
<tr>
<td>LT</td>
<td>lateral tegmental nucleus</td>
</tr>
<tr>
<td>LZ</td>
<td>limited zone of the diencephalon</td>
</tr>
<tr>
<td>MFN</td>
<td>medial funicular nucleus</td>
</tr>
<tr>
<td>MLF</td>
<td>medial longitudinal fasciculus</td>
</tr>
<tr>
<td>MON</td>
<td>medial octaval nucleus</td>
</tr>
<tr>
<td>NC</td>
<td>nucleus corticalis</td>
</tr>
<tr>
<td>NCIL</td>
<td>central nucleus of the inferior lobe</td>
</tr>
<tr>
<td>NDILc</td>
<td>caudal part of the diffuse nucleus of the inferior lobe</td>
</tr>
<tr>
<td>NDILl</td>
<td>lateral part of the diffuse nucleus of the inferior lobe</td>
</tr>
<tr>
<td>NDILm</td>
<td>medial part of the diffuse nucleus of the inferior lobe</td>
</tr>
<tr>
<td>nGMp</td>
<td>magnocellular preoptic nucleus, gigantocellular division</td>
</tr>
<tr>
<td>nHd</td>
<td>dorsal habenular nucleus</td>
</tr>
<tr>
<td>nHv</td>
<td>ventral habenular nucleus</td>
</tr>
<tr>
<td>NI</td>
<td>nucleus isthmi</td>
</tr>
<tr>
<td>NLL</td>
<td>nucleus of the lateral lemniscus</td>
</tr>
<tr>
<td>NLT</td>
<td>lateral tuberal nucleus</td>
</tr>
<tr>
<td>NLTd</td>
<td>lateral tuberal nucleus, dorsal part</td>
</tr>
<tr>
<td>NLTi</td>
<td>lateral tuberal nucleus, intermediate part</td>
</tr>
<tr>
<td>NLTl</td>
<td>lateral tuberal nucleus, lateral part</td>
</tr>
<tr>
<td>NLTm</td>
<td>lateral tuberal nucleus, medial part</td>
</tr>
<tr>
<td>NLTv</td>
<td>lateral tuberal nucleus, ventral part</td>
</tr>
<tr>
<td>NLVc</td>
<td>central part of nucleus of the lateral valvulae</td>
</tr>
<tr>
<td>nPPa</td>
<td>parvocellular preoptic nucleus, anterior part</td>
</tr>
<tr>
<td>NMIL</td>
<td>medial nucleus of the interior lobe</td>
</tr>
<tr>
<td>nMLF</td>
<td>nucleus of medial longitudinal fasciculus</td>
</tr>
<tr>
<td>nMMp</td>
<td>magnocellular preoptic nucleus, magnocellular division</td>
</tr>
<tr>
<td>nPMp</td>
<td>magnocellular preoptic nucleus, parvocellular division</td>
</tr>
<tr>
<td>nPPp</td>
<td>parvocellular preoptic nucleus, posterior part</td>
</tr>
<tr>
<td>NPC</td>
<td>central pretectal nucleus</td>
</tr>
<tr>
<td>NPT</td>
<td>posterior tuberal nucleus</td>
</tr>
<tr>
<td>NRL</td>
<td>nucleus of the lateral recess</td>
</tr>
<tr>
<td>NRP</td>
<td>nucleus of the posterior recess</td>
</tr>
<tr>
<td>NT</td>
<td>nucleus taenia</td>
</tr>
<tr>
<td>nTE</td>
<td>nucleus of the thalamic eminentia</td>
</tr>
<tr>
<td>nTPI</td>
<td>nucleus of the pretecto isthmic tract</td>
</tr>
<tr>
<td>OB</td>
<td>olfactory bulbs</td>
</tr>
<tr>
<td>OC</td>
<td>optic chiasm</td>
</tr>
<tr>
<td>OEN</td>
<td>octavolateralis efferent nucleus</td>
</tr>
</tbody>
</table>
ON  optic nerve
PAG  periaqueductal gray
PBN  parabrachial nucleus
PC   posterior commissure
Pgd  dorsal pregglomerular nucleus
PGZ  periventricular gray zone of tectum
Pit  pituitary
POA  preoptic area
PPd  dorsal periventricular pretectal nucleus
PPv  ventral part of the periventricular pretectal nucleus
PSi  superficial pretectal nucleus, intermediate division
PSm  superficial pretectal nucleus, medial division
PSP  parvocellular superficial pretectal nucleus
PTT  paratal tegmental nucleus
Ri   inferior reticular nucleus
Rm   medial reticular nucleus
Rs   superior reticular formation
SGn  secondary gustatory nucleus
sgt  secondary gustatory tract
SOF  secondary olfactory layer of olfactory bulb
SR   superior raphe nucleus
STN  sensory trigeminal nucleus
SVn  secondary visceral nucleus
SWGZ superficial gray and white zone of tectum
T    tectum
TBT  tectobulbar tract
TGN  tertiary gustatory nucleus
TL   torus longitudinalis
TLa  torus lateralis
TMCa anterior mesencephalocerebellar tract
TMCp posterior mesencephalocerebellar tract
TPp  periventricular nucleus of posterior thalamus
TS   torus semicircularis
TSc  central nucleus of torus semicircularis
TSvl ventrolateral nucleus of torus semicircularis
VAO  ventral accessory optic nucleus
VCeG granular layer of valvula cerebelli
VCEM molecular layer of valvula cerebelli
VCeP purkinje layer of valvula cerebelli
Vc   central part of the ventral telencephalon
Vde  descending tract of the trigeminal nerve
Vd-c dorsal part of the ventral telencephalon, caudal subdivision
Vd-r dorsal part of the ventral telencephalon, rostral subdivision
Vi   intermediate nucleus of the ventral telencephalon
VL   vagal lobe
VIIIm facial motor nucleus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIIa</td>
<td>facial sensory nucleus</td>
</tr>
<tr>
<td>VI</td>
<td>lateral part of the ventral telencephalon</td>
</tr>
<tr>
<td>VLN</td>
<td>ventrolateral thalamic nucleus</td>
</tr>
<tr>
<td>VMn</td>
<td>ventromedial thalamic nucleus</td>
</tr>
<tr>
<td>VOT</td>
<td>ventral optic tract</td>
</tr>
<tr>
<td>Vp</td>
<td>postcommissural nucleus of the ventral telencephalon</td>
</tr>
<tr>
<td>Vs-l</td>
<td>lateral part of the supracommissural nucleus of the ventral telencephalon</td>
</tr>
<tr>
<td>Vs-m</td>
<td>medial part of the supracommissural nucleus of the ventral telencephalon</td>
</tr>
<tr>
<td>Vu</td>
<td>cuneate nucleus of the ventral telencephalon</td>
</tr>
<tr>
<td>Vv</td>
<td>ventral nucleus of the ventral telencephalon</td>
</tr>
</tbody>
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Abstract

The integration of reproduction and metabolism is necessary for the survival and continuation of a species. While the neural circuits controlling energy homeostasis have been well-characterized, the signals controlling the relay of nutritional information to the reproductive axis are not conclusively defined. The cichlid fish *Astatotilapia burtoni* is ideal for studying the neural regulation of feeding and reproduction because during their parental care phase, females undergo a two-week period of forced starvation while holding developing young in their buccal cavity. To test the hypothesis that candidate neuropeptides known to be involved in feeding and energy homeostasis in mammals show conserved distribution patterns, we performed immunohistochemistry or *in situ* hybridization to localize appetite-stimulating (neuropeptide Y, NPY; agouti-related protein, AgRP) and appetite-inhibiting peptides (cocaine and amphetamine-regulated transcript, CART; pro-opiomelanocortin, POMC) in the cichlid fish brain. NPY, AgRP, CART, and *pomc* somata were localized to the lateral tuberal nucleus (NLT), the putative homolog of the arcuate nucleus, as well as other brain regions, and fiber distributions were similar to other teleosts as well as to mammals. To test whether conserved neuropeptide-containing neurons varied with reproductive state, we also quantified neuron somata size in the NLT as a proxy for their involvement in regulating changes in energy status and reproductive condition. Our results show that gravid females had larger NPY and AgRP neurons in the NLT compared to brooding females, but brooding females had larger POMC neurons compared to gravid females. CART neuron size did not differ between the two reproductive states. Thus, larger appetite-stimulating neurons (NPY, AgRP) likely promote feeding while females are gravid, while...
larger POMC neurons may act as a satiety signal to inhibit food intake during mouthbrooding. Hypothalamic mRNA levels for *npy, agrp, pomic-1a, cart 2 and cart 4* were also measured, and while AgRP mRNA levels were higher in gravid compared to brooding females, the remaining gene products did not differ between reproductive states. Collectively, however, our data suggest a potential role for NPY, AgRP, POMC and CART in regulating food intake in *A. burtoni* females during varying reproductive states.
Introduction

Reproduction in vertebrates is an energetically costly process, especially for females, who invest in egg production and parental care (Grone et al., 2012; Clutton-Brock, 1991). Feeding and reproduction are tightly linked in most animals, and individuals must constantly sense and integrate cues from both the internal body and the external environment to make critical decisions about when to eat and when to reproduce. The brain plays a crucial role in maintaining these motivationally driven behaviors as well as homeostatic behaviors such as food intake and body weight (Sohn et al., 2013). Little is known, however, about which hormones and neurochemicals are involved in the complex regulation of feeding and reproduction, particularly in fishes, the largest and most diverse group of vertebrates with over 30,000 species.

Integration of reproduction and metabolism is an essential process for survival and continuation of a species that is linked through evolutionary processes (Roa 2013; Schneider 2004). During reproduction, energy is diverted to maintain fertility, pregnancy and nursing (Roa 2013; Wade et al., 2004). Reproduction is energetically costly, however, and during times of unfavorable conditions (e.g., famine, pathological conditions) the body must prioritize physiological processes essential for survival (Wade et al., 2004). Changes in energy stores result in the fluctuation of metabolic hormones (e.g., ghrelin, insulin, leptin), nutritional signals (e.g., glucose, lipids), and neuropeptides (e.g., Neuropeptide Y (NPY), Pro-opiomelanocortin (POMC), Agouti-related protein (AgRP), cocaine and amphetamine regulated transcript (CART)) that regulate metabolism and fertility through interaction with the hypothalamic-pituitary-gonadal (HPG) axis (Shahjahan et al., 2014; Roa 2013). While the neural circuits controlling
energy homeostasis are relatively well characterized in mammals (Roa 2013), the
signals controlling the relay of nutritional status to the reproductive axis are not well
understood in any vertebrate.

The integrative processes that regulate reproduction and metabolism are
controlled by hormones and neuropeptides in the brain (Tena-Sempre, 2007; Roa,
2013; Daniel et al., 2013; Navarro and Kaiser, 2013). While there are exceptions, many
of the hormones and neuropeptides that promote feeding also inhibit the reproductive
system and vice versa (Schneider, 2004). For example, in mammals, feeding signals
can stimulate the reproductive axis by promoting the release of gonadotropin-releasing
hormone (GnRH) from the hypothalamus and luteinizing hormone (LH) and follicle-
stimulating hormone (FSH) from the pituitary to promote steroid synthesis and gamete
production (Copeland et al., 2011). However, in mammals, some feeding signals, such
as ghrelin (a peptide hormone typically produced by the gastrointestinal tract to
stimulate hunger and feeding), inhibit the reproductive axis by suppressing the release
of LH (Tena-Sempre, 2007) and the activity of the melanocortin system (Tena-Sempre,
2007, Schioth and Watanobe 2002; Yang et al., 2011). The melanocortin system
encompasses melanocyte-stimulating hormone (MSH), adrenocorticotropic hormone
(ACTH), agouti related peptide (AgRP) and the central melanocortin (MC) 3 and 4
receptors, and plays a role in the hypothalamic regulation of energy balance (Schioth
and Watanobe, 2002). In teleost fishes, much like mammals, the reproductive axis is
connected with energy balance. For instance, the central melanocortin system in
teleosts functions similar to mammals in that up-regulation of the melanocortin receptor
antagonist, AgRP, occurs during fasting in goldfish (Cerda-Reverter and Peter, 2003)
and zebrafish (Song et al., 2003). The melanocortin system, more specifically the melanocortin 4 receptor, is also involved in the regulation of growth and reproductive hormones in zebrafish (Zhang et al., 2012). Thus, it appears that at least some of the neural control mechanisms regulating energetics and reproduction, including the melanocortin system, are conserved across vertebrates (Figure 1).

Metabolic status acts to regulate the production of neuropeptides to either promote or inhibit feeding behavior (Williams and Elmquist, 2012). In mammals, the arcuate nucleus of the hypothalamus is one of the best-studied brain regions for the neural control of feeding and appetite (Sohn et al., 2013). POMC, AgRP and NPY neurons are found in the arcuate nucleus and are key regulators of food intake and

Figure 1. Schematic representative drawing to illustrate the tight relationship between hypothalamic regulation of food intake and reproductive function. Peripheral signals enter into the brain where they have either stimulatory (+) or inhibitory (-) effects. Orexigenic neuropeptides (NPY, AgRP) have inhibitory effects on the reproductive hypothalamic-pituitary-gonad (HPG) axis while anorexigenic neuropeptides and the melanocortin system have stimulatory effects on the HPG axis.
energy expenditure (Kong et al., 2012). Yang et al. (2011) showed that the hormone leptin is involved in a “flip-flop” memory storage circuit with ghrelin (set signal), in which leptin (reset signal) inhibits orexigenic (appetite-stimulating) neurons expressing neuropeptides such as AgRP and NPY and stimulates anorexigenic (appetite-suppressing) neurons expressing neuropeptides such as alpha-melanocyte stimulating hormone (α-MSH) and CART. POMC, NPY, AgRP and CART are found in the lateral tuberal nucleus (NLT) of fishes (Copeland et al., 2011), the putative homolog of the arcuate nucleus in mammals (Liu et al., 2010). Due to the structural homology of these neuropeptides across taxa (Hoskins and Volkoff, 2005), comparative studies on their distribution, function, and how they may change under varying reproductive and metabolic states in diverse taxa will contribute to our understanding of the evolution of the regulation of feeding and energy expenditure.

The African cichlid fish Astatotilapia burtoni is an excellent model system to investigate which neuropeptides are involved in the integration of feeding and reproduction. Fishes are the most diverse group of vertebrates and have various modes of reproduction (e.g., broadcast spawning, substrate spawning) that include a wide range of behaviors (Gross and Sargent, 1985). Cichlid species provide parental care to their young and often do so in the form of mouthbrooding, in which the brooding parent can be male, female or both (Oppenheimer, 1970). Fishes that utilize mouthbrooding, including A. burtoni, undergo a dramatic physiological change linked to parental care: post-spawning they will consume little or no food while carrying the brood in their buccal cavity through the duration of the gestation period (Oppenheimer, 1970). During this brooding period, which typically lasts ~2 weeks in A. burtoni, the female does not eat
and as a result there is a noticeable decrease in body mass, delayed ovarian cycles, and delays in subsequent spawning (Fernald and Hirata, 1979; Grone et al., 2012; Renn et al., 2009). Thus, there are clear metabolic, physiological and behavioral differences between the gravid (ripe with eggs) energy investment phase, and the mouthbrooding energy consumptive phase. Little is known, however, about how feeding-relevant neuropeptides in the brain might be involved in regulating the cyclical transition between these reproductive-energy investment states. The goals of this study, therefore, were to 1) map the distribution of candidate orexigenic (AGRP, NPY) and anorexigenic (CART, POMC) neurons in the brain for the first time in a mouthbrooding fish species, and 2) quantify differences in somata sizes of neurons producing these neuropeptides and mRNA expression levels of these neuropeptides to test the hypothesis that the drastically different reproductive and energetic states of gravid and mouthbrooding females are associated with changes in orexigenic and anorexigenic systems.
Materials and Methods

Animals and tissue preparation

A laboratory stock of *A. burtoni* derived from wild-stock collected from Lake Tanganyika, Africa were housed in community aquaria kept at conditions similar to their natural habitat: pH 7.8-8.0, temperature 29-30°C and a 12 hour light: 12 hour dark cycle with full spectrum illumination and constant aeration. Aquaria contained gravel along the bottom and terra cotta pot halves that served as shelters and to facilitate the establishment and maintenance of territories by dominant males. Fish were fed crushed cichlid flakes (AquaDine, Healdsburg, CA) each morning and supplemented with brine shrimp (Sally’s Frozen Brine Shrimp, San Francisco, CA) twice a week. Fish care and experiments were approved and in compliance with Louisiana State University IACUC protocols.

To test for reproductive state differences in orexigenic and anorexigenic neuropeptide-containing neurons in the brain, we sampled both gravid reproductively receptive females and mouthbrooding parental females. Gravid females were initially selected based on body shape (distended abdomen), and gonadal status was subsequently confirmed based on oocyte size and gonadosomatic index (GSI) (GSI=[(gonad mass/body mass)*100]). Brooding females were collected 6-8 days after the onset of brooding, which is at or slightly past the mid-way point of their 12-14 day brooding period. Criteria were set for both gravid (GSI>5.0) and brooding (GSI<1.0; fry total length 5-10 mm) females to ensure that individuals within each group were of similar reproductive state. Individuals falling outside of these criteria were excluded from the dataset. Females were anesthetized in ice-cold fish water, sacrificed by rapid
cervical transection between the hours of 8-10 am, and their body mass (BM), standard length (SL), liver mass (LM) and ovary mass (OM) were immediately measured. Once the ovaries were removed the largest six oocytes were collected from the right ovary and the diameters of the long axis were measured and averaged. Liver mass was used to calculate the hepatosomatic index (HSI = [(liver mass/body mass)*100]) as a proxy for energy reserves, and ovary mass was used to calculate GSI as a measure of reproductive investment. Fulton’s condition factor was calculated using the formula K=100(W/L^3) where W is the whole body weight, L is the standard length in centimeters, and 100 is used to bring K close to 1. For mouthbrooding females, whole body weight without the presence of the brood in the buccal cavity was used to calculate K.

Following sacrifice by rapid cervical transection, brains were removed, fixed in 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS) overnight at 4°C, rinsed in PBS prior to cryoprotection, and cryoprotected in 30% sucrose in 1xPBS at 4°C prior to sectioning. Cryoprotected brains were embedded in Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek, Sakura) and sectioned in the transverse plane at 18 µm using a cryostat (Leica, CM1850). Sections were collected onto a series of four alternate slides (Superfrost Plus, VWR) so that all four-candidate neuropeptides could be stained and analyzed in each brain. Slides were allowed to dry overnight at room temperature and stored prior to staining (4°C for immunohistochemistry; -80°C for in situ hybridization).

Immunohistochemistry

To label NPY-, AgRP-, and CART-producing neurons in the brain, we performed standard immunohistochemistry. To minimize variability between individual IHC and ISH
runs for quantification purposes, each staining experiment contained representatives of both gravid and brooding fish. Slides were brought to room temperature (20-22°C) and a hydrophobic barrier (Immedge pen; Vector Laboratories) was applied around the sections and allowed to dry for about 40 minutes. The slides were then rinsed with phosphate buffered saline (PBS) (3x10 min), non-specific binding was blocked (NPY: 2 hours; AgRP, CART: 1 hour) with PBS containing 0.3% Triton-X-100 (Fisher Scientific), normal goat serum (NGS) (NPY: 8%; AgRP: 2%; CART: 8%) (NGS, Vector Laboratories), and bovine serum albumin (BSA) (NPY: 1.0%; AgRP: 0.2%; CART: 0.2%) (Sigma). Slides were then incubated with primary antibody (AgRP: 1:10,000, Phoenix Pharmaceuticals, H-003-53; CART: 1:5,000, Phoenix Pharmaceuticals, H-003-62; NPY: 1:12,000, Sigma, N9528; all polyclonal made in rabbit) overnight (~16-18 hours) at 4°C in a sealed humidified chamber. The primary antibody incubation was followed by PBS washes (3x10 min), incubation with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) made in PBS with NGS (NPY: 20%; AgRP: 3.3%; CART: 8.3%) for 45 min-1 hr, PBS wash (3x10 min), quenching of endogenous peroxidases with 1.5-3.0% hydrogen peroxide in PBS for 10 min, PBS wash (3x10 min), incubation with avidin–biotin–horseradish peroxidase complex (ABC Elite kit; Vector Laboratories) (NPY: 1 hour and 30 minutes; CART, AgRP: 2 hours), PBS wash (3x10 min), and reacted with a diaminobenzidine (DAB) chromogen substrate kit with nickel chloride intensification (Vector Laboratories) for 3 min. Slides were then soaked in distilled water for 10 min to stop the reaction, dehydrated in an ethanol series (50, 70, 95, 100%), cleared in xylene and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific).
Antibody characterization

To test for antibody specificity in the cichlid brain tissue, immunohistochemistry controls were performed by preabsorbing each antibody (anti-NPY/AgRP/CART) with its respective control peptide (NPY: 1.617 µM, Sigma; CART: 1.521 µM, Phoenix Pharmaceuticals, 003-62; AgRP: 706.6 nM Phoenix Pharmaceuticals, 003-53) overnight at 4°C prior to tissue application. These preabsorption controls eliminated all reaction products and resulted in no staining when run simultaneously with non-preabsorbed antibody (Figure 2A-F).

Figure 2. Photomicrographs showing antibody specificity for NPY, AgRP and CART, and specificity of probe binding for pomc-1a in the brain of Astatotilapia burtoni females. Representative coronal sections showing typical antibody (NPY, AgRP, CART) and in situ hybridization (pomc) labeling (left column; A,C,E,G) alongside adjacent preabsorption or sense control sections (right column; B,D,F,H) illustrating absence of staining. NPY in the NLTv (A,B), AgRP in the NLTv (C,D), CART in the VI (E,F) and pomc-1a in the NLTv (G,H). Scale bars = 25 µm in A-B, E-F and G-H; 10 µm in C-D.
Other procedural controls (omission of secondary antibody, ABC, and DAB) also resulted in no staining. Ideally, the absence of staining in transgenic knockout animals for each respective neuropeptide is a strong confirmation of antibody specificity. However, this option was not feasible as transgenic A. burtoni fish specific to these neuropeptides are not currently available. Since antibodies used in this study were polyclonal, our preabsorption controls show that the antibodies stain targets in the tissue that are at least cross-reacting with the original protein. These antibodies have also been used and validated previously in other fish species (Sakharkar, et al., 2005; Mirabella et al., 2004; Singru et al., 2007), providing further evidence of their specificity in the cichlid.

**Synthesis of riboprobes for in situ hybridization**

To localize pomc cells in the hypothalamus, we used a digoxigenin (DIG) in situ hybridization protocol with riboprobes for pomc-1a, pomc-1b and pomc2. Primers for pomc forms were designed from the A. burtoni sequences available in Genbank (Table 1), and then commercially synthesized (Life Technologies). Templates for the riboprobes were generated by PCR (PCR Platinum SuperMix, Life Technologies) from whole brain A. burtoni cDNA using the gene-specific primers. The reaction conditions were as follows: 95°C for 1 min, 40 cycles of: (95°C for 15sec, 55°C for 15sec, 72°C for 1 min), and 72°C for 1 min. Purified PCR products (MinElute PCR kit, Qiagen) were then used as the template in the transcription reaction to incorporate DIG- (DIG-labeling mix, Roche) labeled nucleotides into the nucleic acid sequence, followed by probe purification (GE Illustra Probe Quant G-50 microcolumns). Probes were transcribed from the T3 polymerase transcription initiation sequence (aattaaccctcactaaaggg) that
was added to the reverse (for anti-sense probes) or forward (for sense control probes) gene-specific primers. PCR products and final probes were checked on a 1% agarose gel after each step and verified to be single bands of the correct size. Probes were diluted with hybridization buffer and stored at -20°C.

Table 1. Primer sequences used for qPCR and to generate templates for synthesis of riboprobes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'→3')</th>
<th>Reverse (5'→3')</th>
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<tr>
<td>pomc-1a*</td>
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<td>AGCTCTGATGACCCGTAAC</td>
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<tr>
<td>pomc-1b*</td>
<td>CGCTCTCATTTCTTCATGCCTTCTTCTC</td>
<td>GCTGCTGTCCTTCTTTGT</td>
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<tr>
<td>pomc-2*</td>
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</tr>
<tr>
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</tr>
<tr>
<td>cart 4</td>
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<td>CCGTGCAGCTTATGAAAGA</td>
</tr>
<tr>
<td>lepr</td>
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<td>GCTCCAGCTTGGCTTCTT</td>
</tr>
<tr>
<td>ghs-r1</td>
<td>CGTGCTGTCAGGATAAGA</td>
<td>TACGAGCCACATGGAA</td>
</tr>
</tbody>
</table>

*Denotes a template primer for riboprobe synthesis. T3 transcription initiation sequence (aattaacctcactaaggg) was added to the reverse primer (for antisense probes) or forward primer (for sense control probes).

In situ hybridization

To identify the anorexigenic pomc-containing neurons, we performed chromogen-based in situ hybridization as a proxy for α-MSH. This approach was used because pilot experiments with several commercially available α-MSH antibodies failed to produce reliable staining. Following the teleost-specific whole genome duplication event, there are 3 forms of pomc in A. burtoni (pomc-1a, pomc-1b and pomc-2) (Harris et al., 2014). While we performed in situ hybridization to label all 3 pomc forms, we
chose to quantify *pomc-1a* neurons because phylogenetic analyses indicated that *pomc-1a* is the homolog to the mammalian form responsible for the synthesis and cleavage of α-MSH in the pituitary in response to satiety signals (Amano et al., 2005). Chromogen-based *in situ* hybridization (ISH) was performed as previously described (Grone and Maruska, 2015), and all solutions used during the protocol were RNase-free (0.25 µm filtered). Briefly, sections were brought to room temperature (20-22°C) and the hydrophobic barrier (Immedge pen; Vector Laboratories) was applied and allowed to dry for about 40 minutes. The slides were then washed in PBS (3x5min), fixed in 4% PFA (20min), washed in PBS (2x5min), permeabilized in proteinase K (10 µg/ml final concentration, 10 min), washed 10 min in PBS, fixed in 4% PFA for 15 min, washed in PBS (2X5min), rinsed in milliQ water for 3 min, incubated in 0.1M triethanolamine-HCl (pH 8.0) with acetic anhydride to 0.25% volume for 10 min, washed in PBS for 5 min, and incubated for 2.5 hours in pre-hybridization buffer at 60-65°C in a sealed humidified chamber. The pre-hybridization solution was removed, DIG-labeled probe was added, and slides were covered with hybrislips (Life Technologies) to hybridize overnight (12-16 hours) at 60-65°C in a sealed chamber. Following hybridization, the slides were washed at 60-65°C in pre-warmed 2X saline-sodium-citrate (SSC):50% formamide (2x30 min), 1:1 mixture of 2XSSC: Maleic acid buffer with 0.1% Tween-20 (MABT) (2x15min), and MABT (2x10 min). Slides were then washed at room temperature with MABT (2x10 min) and non-specific binding was blocked with MABT containing 2% BSA for 3 hours. Slides were then incubated with alkaline phosphatase-conjugated anti-DIG Fab fragments (Roche) diluted 1:5,000 in blocking solution (MABT with 2% BSA) at 4°C overnight in a humidified chamber. Following incubation, slides were rinsed in MABT
(3x30 min) at room temperature, washed in alkaline phosphatase (AP) buffer (2x5min), developed for 80-90 minutes in 4-Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3 indolyl-phosphate (NBT/BCIP) substrate solution (Roche) solution at 37°C. Slides were then washed with PBS (3x5min) to stop the reaction, fixed in 4% PFA for 10 min, washed in PBS (3x5min) and coverslipped with aqueous mounting media (Aqua-mount, Lerner Laboratories). Pomc-expressing neurons were identified with in situ hybridization, and therefore, only the distribution of pomc-1a, pomc-1b, and pomc-2 cells but not fibers are described.

**Quantification of somata size**

To test for reproductive state differences in neuropeptide-expressing neurons, we performed blind unbiased measurements of somata sizes for NPY, AgRP, CART and pomc-1a neurons from transverse sections without knowledge of the SL, BM, GSI or reproductive state of the fish. Cell size was determined by measuring cell profile areas in the NLT from 10 randomly selected neurons for NPY (representing ~24% of all cells), AgRP (~22% of cells), and CART (~31% of cells), and 20 randomly selected neurons for pomc-1a (~25% of cells) per individual. Measurements were taken at a point where the labeled cell perimeter was easily discernible and a distinct nucleus was visible under the 40x objective lens. Somata areas for each neuropeptide-containing cell type were then averaged for each individual, and within each reproductive state (gravid and mouthbrooding) for statistical comparisons.

**Imaging and analysis**

To map the distribution patterns for NPY-, AgRP-, CART- and pomc-1a-containing neurons in the brain (pituitary was analyzed for pomc, but not for NPY, AgRP
or CART), stained slides were visualized on a Nikon Eclipse Ni microscope and photographs were taken with a color digital camera controlled by Nikon elements software. Neuroanatomical regions and specific brain nuclei were identified using atlases and cytoarchitecture previously described in A. burtoni (Burmeister and Fernald, 2009; Munchrath and Hoffman, 2010) as well as in other fish species (Imura et al., 2002). Images were sharpened and adjusted for contrast, brightness, and levels in Photoshop CS6 (Adobe Systems). Neuropeptide distribution maps were created by tracing the outline of the left side of a representative cresyl-violet-stained coronal section along with relevant nuclei and neuroanatomical structures. The traces were then duplicated and flipped horizontally to create a mirror image for representation of a full coronal section, and then the locations of cells and fibers for each neuropeptide were marked on the coronal section. Cells were defined as stated above, and immunoreactive fiber densities were defined as follows: few= 4 or less, low-density= greater than 4 but less than 20, moderately dense= 21-45, and dense= greater than 45 in the field of view using a 40x objective lens.

**Quantitative PCR (qPCR)**

To examine the mRNA levels of the same neuropeptides quantified for somata size and distribution, as well as several other relevant genes, we performed quantitative PCR on macro-dissected hypothalamic regions from a different set of females. Gravid (N=15) and brooding (N=15) females were sacrificed as described above and the brains were macro-dissected to first divide the brain into the telencephalon, diencephalon and mesencephalon, and rhombencephalon. The diencephalon was then further dissected
so that the hypothalamus (without pituitary gland) was removed and used for qPCR analysis (Figure 3).

Figure 3. Lateral view of the A. burtoni brain to illustrate macro-dissection of the hypothalamus for qPCR analyses. Numbers represent the order in which the cuts were made: pituitary (pit) (1), telencephalon with olfactory bulbs (OB) (2), cerebellum, hindbrain and spinal cord (3) and separation of hypothalamus (4). The area of interest (hypothalamus) used in qPCR experiments is highlighted in green. Rostral is to the left.

The brain was macro-dissected in this fashion to focus on the NLT, where somata sizes were quantified in a different set of animals, but also included other hypothalamic nuclei (e.g. ATN, NRL, NDILI, NRP, NCIL) known to be involved in feeding and reproduction. This approach also removed any NPY, AgRP, CART, and/or POMC populations in other brain regions, such as the preoptic area (POA). The hypothalamic tissue was weighed (2-6 mg per individual), rapidly frozen on dry ice, and then immediately stored at −80°C until RNA isolations were performed.
Hypothalamic tissue was homogenized and total RNA was extracted following the manufacturer’s protocol (RNeasy Mini Plus Kit, Qiagen). RNA yields were calculated using spectrophotometric values to ensure that a consistent amount of RNA (250ng/µl) was used as a template for cDNA synthesis (iScript, BioRad). The resulting cDNA was then re-measured via spectrophotometry to check the quality, and then diluted 1:5 prior to quantitative RT-PCR. Primers for all genes of interest (pomc-1a, agrp, ghrelin receptor (ghs-r1), leptin receptor (lepr), cart 2, cart 4, npy) were designed from A. burtoni sequences available in Genbank (Table 1) using PrimerQuest (Integrated DNA Technologies, Coralville, IA) and then commercially synthesized (Life Technologies). qPCR was performed on a CFX connect Real-Time system (Bio-Rad) with duplicate reaction volumes of 20 µl. Reaction parameters were as follows: 95°C for 30sec, 45 cycles of 95°C for 1sec and 60°C for 30sec, and 65°C for 5sec, followed by a melt curve analysis. Fluorescence thresholds for each sample were automatically measured (CFX Manager, BioRad) and then PCR Miner (Zhao & Fernald, 2005) was used to calculate cycle thresholds and reaction efficiencies for individual wells as described previously (Maruska et al., 2011; Maruska et al., 2013a; Maruska et al., 2013b). The relative amount of mRNA was then normalized to the geometric mean of the two housekeeping genes 18s and RPL32. Values of 18s (Mann-Whitney; U=73.00; p=0.421) and RPL32 (student’s t-test; p=0.939; t=0.0771; df=25) did not differ between gravid and brooding females, indicating they are appropriate reference genes for this study. Normalizing the mRNA levels to two reference genes, as opposed to one, allows for a more accurate quantification (Bustin et al., 2005; Mitter et al., 2009).
**Statistical analyses**

To test for differences between reproductive states in physiological variables (GSI, HSI, oocyte size, SL, BM) we used two-tailed unpaired t-tests or non-parametric Mann-Whitney tests when assumptions of normality and equal variance were not met (SigmaPlot, Systat, Inc., San Jose, CA). Pearson Product Moment Correlations were used to test for correlations between GSI and somata sizes (compared between each peptide), HSI and somata sizes, and between different orexigenic and anorexigenic somata sizes. To account for positive relationships between somata size and body size, we compared gravid and brooding somata sizes with analysis of covariance (ANCOVA), using standard length (for somata size comparisons) or hypothalamic mass (for mRNA level comparisons) as a covariate. Statistical significance was set at p=0.05 for all comparisons.
Results

Physiological differences between gravid and brooding females

A total of 24 female *A. burtoni* (12 gravid, 12 brooding) were used for the physiological analysis, quantification of somata sizes and distribution of neuropeptides in this study. Gravid females had higher GSI (Mann-Whitney; U=0.00; p<0.001) (Figure 4A) and larger ova diameters (student’s t-test; p<0.001; t=17.614; df=22) (Figure 4B) compared to brooding females. Gravid females also had higher HSI (Mann-Whitney; U=4.00; p<0.001) (Figure 4C) and condition factor (Mann-Whitney; U= 25.00; p=0.013) (Figure 4D) values compared to brooding females, indicative of greater energy reserves.

Figure 4. Physiological differences between gravid and mouthbrooding *Astatotilapia burtoni* females. Gravid females have greater gonadosomatic index (GSI) (A), ova diameters (B), hepatosomatic index (HSI) (C), and condition factors (D) compared to brooding females. In each graph, the bottom of each box is the 25th percentile, the top is the 75th percentile and the line in the middle is the median, whiskers (error bars) indicate min and max values and dots represent outside values. N=12 for both gravid and brooding females for A-D. Different letters indicate statistical differences at p<0.05.
Distribution of NPY, AgRP and CART-immunoreactive neurons

Neuropeptide Y (NPY)

Olfactory bulbs and telencephalon

NPY-ir cells were observed in the ganglion of the terminal nerve (gTN) at the junction between the olfactory bulbs and rostral telencephalon. In the olfactory bulbs, NPY-immunoreactive (-ir) fibers were present in the external cell layer (ECL), glomerular layer (GL), internal cell layer (ICL) and secondary olfactory layer (SOL) (Figure 5A).

NPY-ir cells were found in nuclei of both the dorsal and ventral telencephalon. NPY-ir cells in the dorsal telencephalon were found in the central part, subdivision 4 (Dc-4) (Figure 5D). In the ventral telencephalon, NPY-ir cells were found in the lateral part of the ventral telencephalon (VI) (Figure 5B-D, 6A) and central part of the ventral telencephalon (Vc) (Figure 5B-C, 6A), in the dorsal part of the ventral telencephalon, rostral subdivision (Vd-r) (Figure 5B-C), and in the ventral nucleus of the ventral telencephalon (Vv) (Figure 5B).

NPY-ir fibers were distributed in many nuclei throughout the dorsal and ventral telencephalic regions. In the dorsal telencephalon, low-density, scattered NPY-ir fibers were present in the ventral part of the lateral zone of the dorsal telencephalon, subdivision 1 (Dl-v1), dorsal part of the lateral zone of the dorsal telencephalon (Dl-d) and medial part of the dorsal telencephalon, subdivision 1 (Dm-1) (Figure 5A-C). Moderately dense NPY-ir fibers were present in the rostral ventral part of the lateral zone of the dorsal telencephalon, subdivision 2 (Dl-v2) and density increased caudally in the Dl-v2 (Figure 5B-D). Dense fibers were present in the nucleus taenia (NT) (Figure 5D) and dorsal part of the dorsal telencephalon (Dd) (Figure 5D-F). Dense fibers were
also present in rostral sections of the ventral area of the central part of the dorsal telencephalon, subdivision 1 (Dc-1). In more caudal regions of Dc-1, fibers remained dense in the medial area, while fiber density decreased to moderate levels in the lateral area of Dc-1 (Figure 5B-C). Moderately dense fibers were present in the unassigned subdivision of dorsal telencephalon (Dx) (Figure 5B), dorsal part of the dorsal telencephalon, dorsal subdivision (Dd-d) (Figure 5D-E), medial part of the dorsal telencephalon, caudal subdivision 2 (Dm-2c) (Figure 5D), medial part of the dorsal telencephalon, subdivision 3 (Dm-3) (Figure 5D-F), central part of the dorsal telencephalon, subdivision 3 (Dc-3) (Figure 5D-F) and granular zone of the lateral zone of the dorsal telencephalon (Dl-g) (Figure 5C-E).

In the ventral telencephalon, moderately dense NPY-ir fibers were present in the VI (Figure 5B-C), medial part of the supracommissural nucleus of the ventral telencephalon (Vs-m) (Figure 5D-F), Vv (Figure 5B-C), central part of the ventral telencephalon (Vc) (Figure 5B-C), and in the Vd-r. Fiber density increased in the Vd-r from moderately dense in rostral sections to dense in more caudal regions (Figure 5B-C). The postcommissural nucleus of the ventral telencephalon (Vp) (Figure 5F) and the cuneate nucleus of the ventral telencephalon (Vu) contained moderately dense to dense fibers in the rostral area of these regions. Dense fibers were also found in the intermediate nucleus of the ventral telencephalon (Vi).

Diencephalon and mesencephalon

Overall, NPY-ir cells and fibers were found in abundance throughout the diencephalon and mesencephalon of A. burtoni (Figures 5, 6). NPY-ir cells were found in the entopeduncular nucleus (E) (Figures 5E, 6B), the parvocellular preoptic nucleus,
anterior part (nPPa) (Figure 5E, 6C), parvocellular preoptic nucleus, posterior part (nPPp), lateral tuberal nucleus, dorsal part (NLTd) (Figure 5G, 6D-E), lateral tuberal nucleus, medial part (NLTm) (Figure 5G), anterior tuberal nucleus (ATn) (Figure 5G, 6D), and large cells were found in the nucleus of the medial longitudinal fasciculus (nMLF) (mean ± SD diameter; Gravid: 10.476 ± 2.001 µm; Brooding: 10.867 ± 1.471 µm) and periventricular nucleus of the posterior tuberculum (TPp) (Figure 5G).

NPY-ir fibers were located in many regions of the preoptic area including the nPPa, nPPp magnocellular preoptic nucleus, parvocellular division (nPMP), magnocellular preoptic nucleus, gigantocellular division (nGMp), and magnocellular preoptic nucleus, magnocellular division (nMMP) (Figure 5E-F). Low-density fibers were primarily present in the rostral nPPa with a moderately dense population of fibers just ventral to the anterior commissure (AC) in the dorsal nPPa (Figure 5E). Moderately dense to dense fibers were present in the area lateral to the rostral nPPa and ventral to the rostral E. Moderately dense fibers were also located in the nMMP, nPMP, nPPp, and nGMp (Figure 5F).

In the rostral hypothalamus, NPY-ir fibers were present in the NLTd, NLTm, lateral tuberal nucleus, ventral part (NLTv), lateral tuberal nucleus, intermediate part (NLTi), anterior tuberal nucleus (ATn), lateral part of the diffuse nucleus of the inferior lobe (NDILI), nucleus of the lateral recess (NRL), nucleus of the posterior recess (NRP) and posterior tuberal nucleus (NPT) (Figure 5G-J). Moderately dense varicose fibers were present in the ATN, which became denser towards the caudal ATN (Figure 5G). The rostral NLTd had fibers with large varicosities, and the NLTv, NLTi and NLTm had moderately dense fibers. In more caudal regions of these nuclei, the fibers were less
dense (Figure 5G). Low-density fibers bordered the medial edge of the rostral NDILI and as the NDILI became more caudal, there were scattered, low-density fibers positioned dorsally in the NDILI in the region ventral to the tertiary gustatory nucleus (TGN) (Figure 5G-I). The rostral NRL had moderately dense fibers and in more caudal regions the fibers were scattered around the recess of the NRL (Figure 5H-J). The caudal part of the diffuse nucleus of the inferior lobe (NCILc) had low-density fibers in the medial area dorsal to the NRL (Figure 5J). Dense fibers were also present in the NRP and in the NPT of the hypothalamus.

In the zone between the hypothalamus and thalamus, the rostral portion of the dorsal posterior thalamic nucleus (DP) contained dense fibers throughout, while the density was reduced in more caudal sections (Figure 5G). The central posterior thalamic nucleus (CP) had moderately dense fibers in rostral sections, but fibers were less dense and confined to the center of the nucleus in more caudal regions (Figure 5G). Moderately dense fibers were present in the ventral habenular nucleus (nHv) and dorsal habenular nucleus (nHd). Moderately dense fibers were also present in the TPp (Figure 5G) and in the ventromedial thalamic nucleus (VMn). Dense fibers were present in the PGn (Figure 5H), and moderately dense fibers in the dorsal preglomerular nucleus (PGd). Low-density fibers were found in the commissural preglomerular nucleus (PGc) and limited zone of the diencephalon (LZ). In the dorsal pretectum and thalamus, low-density fibers were present in the dorsal periventricular pretectal nucleus (PPd), dense fibers were present in the central pretectal nucleus (NPC) and moderately dense fibers were found in the anterior thalamic nucleus (A), superficial pretectal nucleus, medial division (PSm), and superficial pretectal nucleus, intermediate division
(PSi). Low-density fibers were found in the lateral tegmental nucleus (LT). Dense fibers were present in the central thalamic nucleus (CTn), interpeduncular nucleus (IP), nucleus corticalis (NC), and TGN.

In the mesencephalon, moderately dense NPY-ir fibers were found consistently in the tectum (T), including the periventricular gray zone of tectum (PGZ), deep white zone of tectum (DWZ), and superficial gray and white zone of tectum (SWGZ) (Figure 5F-J). Low-density fibers were found in the nucleus of the medial longitudinal fasciculus (nMLF), and oculomotor nucleus (III). Low-density fibers were also found in the torus semicircularis (TS), specifically the central nucleus of TS (TSc). Moderately dense fibers were found in the central part of the nucleus of the lateral valvulae (NLVc) (Figure 5I).

Rhombencephalon

NPY-ir cells were present in the central gray (CG) (Figure 5J) surrounding the fourth ventricle. In general, NPY-ir fibers were scarcer in the rhombencephalon compared to the telencephalon, diencephalon and mesencephalon. However, dense fibers were present in the rostral granular layer of the valvula cerebelli (VCeG), tectobulbar tract (TBT) (Figure 5J), and low to moderate density fibers were present in the granular layer (CCeG) (Figure 5J) and purkinje cell layer (CCeP) (Figure 5J) of the corpus cerebellum. Low-density fibers were present in the secondary gustatory nucleus (SGn) dorsal to the Rs and lateral to the CG (Figure 5J). Low-density fibers were found throughout the rostro-caudal CG and in proximity to the NPY-ir cells of the CG (Figure 5J), in the MLF tract (Figure 5H-J), and within the octavolateralis efferent nucleus (OEN) (Figure 5K). Dense fibers were also found in the region between the medial reticular nucleus (Rm) and secondary gustatory tract (sgt) (Figure 5K). Low-density fibers were
also present in the superior reticular formation (Rs). Moderately dense fibers were present in the descending octaval nucleus (DON) and medial octaval nucleus (MON). Moderately dense to dense fibers were found bordering the dorsal portion of the dorsal vagal lobe (VL) and moderately dense fibers were present within the VL. Moderately dense fibers were present in the inferior reticular nucleus (Ri) and in the area lateral to the Ri and DON. In the brainstem, low to moderately dense fibers were present in the medial funicular nucleus (MFN) and descending trigeminal tract (dtt).

**Agouti-related protein (AgRP)**

**Olfactory bulbs and telencephalon**

In the olfactory bulbs, there were no AgRP-ir cells, but there were a few scattered -ir fibers in the ventro-medial area of the ECL and in the medial area of the ICL (Figure 7A). Overall, while AgRP-ir fibers were present, they were not abundant in the olfactory bulbs of *A. burtoni*.

There were also no AgRP-ir cells found anywhere in the telencephalon, but AgRP-ir fibers were found in both dorsal and ventral telencephalon regions. In the dorsal telencephalon, low-density scattered fibers were present in the rostral portion of Dm-1 and the fiber density increased to moderately dense in the more caudal portion of Dm-1 (Figure 7A-B). Low-density AgRP-ir fibers were present within Dc-1, Dm-3 and Dl-d, and low-density, scattered fibers were present in Dl-v2 (Figure 7B-C). In the ventral telencephalon, moderately dense AgRP-ir fibers were present in the rostral Vs-m (Figure 7B), and scattered moderately dense fibers were in the Vd-c (Figure 7C). Low-density fibers were also present in the Vd-r (Figure 7B-C).
Figure 5. Localization of neuropeptide Y (NPY) immunoreactive neurons in the brain of *Astatotilapia burtoni*. Representative line drawings of transverse sections from rostral (A) to caudal (J) brain regions show the locations of NPY ir-somata (large black dots) and fibers (small black dots). Left side of brain shows NPY distribution and right side shows labels of nuclei and other neuroanatomical structures. Figure inset at the top right shows a lateral view of the brain indicating the approximate location of each transverse section. Scale bar = 250 µm. See list for abbreviations.
Figure 5 continued.

Dense fibers were found primarily in the medial area of the rostral portion of the Vv. However, there were also dense fibers in the lateral area of the rostral Vv. The caudal Vv also contained moderately dense fibers (Figure 7B-C).

Diencephalon and mesencephalon

The only AgRP-ir cells found in *A. burtoni* were present in the NLTv; the rostral portion of the nucleus contained cells along the lateral edge that were then located in a more medial position in the caudal portion of this nucleus (Figure 7F, 8B). The rostral NLTi also contained cells in the lateral area bordering the NLTv.
Figure 6. Representative photomicrographs of NPY-immunoreactive somata and fibers in the brain of A. burtoni. Coronal sections showing NPY-immunoreactive fibers in the Vc and VI (A) entopeduncular nucleus (B), and in the nPPa of the preoptic area (C). In the diencephalon, NPY-immunoreactive cells and fibers are found in the ATN and NLTd at low (D) and higher magnification (E) of the hypothalamus. Large NPY-ir cells and varicose projections in the nMLF(F). Scale bars = 10 µm in B; 250 µm in C; 25 µm in A, D, E and F. See list for abbreviations.
In the preoptic area, AgRP-ir fibers were present in the nPPa, nMMp, nPMp, nPPp and nGMp (Figure 7D-E, 8A). Dense fibers were present in the nPPa and nPMp (Figure 7D) while moderately dense fibers were present in the nPPp (Figure 7E). The rostral nMMp contained moderately dense fibers, which increased in density towards the caudal nMMp (Figure 7D).

In the hypothalamus, low-density fibers were found in the rostral NLTd, caudal NLTI and low-density varicosities were present in the rostro-caudal NLTv (Figure 7F, 8C). The caudal ATN and NLTv had light to moderate fiber densities (Figure 7F). Scattered fibers were present in some more caudal diencephalic regions such as the medial nucleus of the interior lobe (NMIL) bordering the ventral side of the glomerular nucleus (GN) (Figure 7G). Fibers were found scattered in the NRL and were present through the entire rostro-caudal extent of this nucleus (Figure 7G, 8D).

In the thalamic region, scattered fibers were present in the DP and CP (Figure 7F), nHv and nHd. Scattered, low-density fibers were also present in the TPp and VMn. In the mesencephalon, the tectum consistently contained AgRP-ir fibers located in the CZ bordering both the SWGZ and DWZ (Figure 7E-I). Moderately dense fibers were present in the PGn and scattered in the PGZ dorsally bordering the TSc (Figure 7H). Scattered fibers were also found in the rostral paratal tegmental nucleus (PTT) (Figure 7G).

Rhombencephalon

Scattered low-density AgRP-ir fibers were found in the rostral CG around the fourth ventricle. As the CG became more caudal there was an increase in density (Figure 7I), but the fiber density was again low in the most caudal portion of the CG.
(Figure 7J). Scattered moderately dense fibers were also present in the area between the CG and Rs, within the Rs, and in the TBT (Figure 7I). Low-density fibers were found ventral to the Rm. Low-density fibers were present in the CCEP. Low-density fibers were also found in the rostral ICo (Figure 7I). A moderately dense population of fibers was found in the region dorsal to the TBT and lateral to the IIIh. Scattered, low-density fibers were present in the medial portion of the IIIh (Figure 7G). Low-density scattered fibers were also present in the VL and in the MFN.

**Cocaine and amphetamine regulated transcript (CART)**

Olfactory bulbs and telencephalon

In the olfactory bulbs, CART-ir cells were present in the lateral margin of the rostral GL (Figure 7A). CART-ir fibers were also present in the lateral margin of the rostral GL, and scattered fibers were present in the medial area of the rostral ICL of the olfactory bulbs (Figure 7A).

In the telencephalon, CART-ir cells were present the rostral medial area of the ventral Dm-1 (Figure 7A), in VI (Figure 7B), and in the lateral part of Vc (Figure 7B, 9A). In the dorsal telencephalon, CART-ir fibers were present in low densities in DI-v1 just ventral to DI-d (Figure 7A), scattered fibers were present in the rostral dorsal portion of Dm-1 (Figure 7A) and the ventral portion of DI-v2 (Figure 7A), and scattered throughout the dorsal portion of DI-d (Figure 7A). Scattered fibers were also found in the dorsal area of the rostral Dc-1 just ventral to Dx (Figure 7A), and in caudal regions, the fibers were more scattered throughout this Dc-1 nucleus (Figure 7A-C).
Figure 7. Localization of AgRP (left half) and CART (right half) immunoreactive neurons in the brain of *Astatotilapia burtoni*. Representative line drawings of transverse sections from rostral (A) to caudal (J) brain regions show the locations of AgRP and CART ir-somata (large black dots) and fibers (small black dots). Left side of brain shows AgRP distribution and right side shows CART distribution, along with labels of nuclei and other neuroanatomical structures. Figure inset at the top right shows a lateral view of the brain indicating the approximate levels of the corresponding transverse sections. Scale bar = 250 µm. See list for abbreviations.
In the ventral telencephalon, scattered CART-ir fibers were present in low-densities in the rostral Vd-r and moderately dense in more caudal regions (Figure 7B-C). Low-density, scattered fibers were also present in the caudal Vv and caudal VI (Figure 7C). In the Vs-m, low-density fibers were found bordering the dorsal portion of the Vd-c. In the Vd-c, low-density fibers were present in the dorsal region bordering the ventral Vs-m (Figure 7C).
Figure 8. Representative photomicrographs of AgRP-ir somata and fibers in the brain of *Astatotilapia burtoni*. Coronal section of the diencephalon shows moderately dense fibers (arrowheads) in the nPPp (A). Immunoreactive cells were found in the NLTv (B). Immunoreactive fibers with large varicosities (arrowheads) were also observed in the NLTv of the hypothalamus (C). Scattered fibers (arrowheads) were also present in the NRL surrounding the lateral recess (D). Scale bar = 100 µm in A; 10 µm in B and C; 25 µm in D. See list for abbreviations.

Diencephalon and mesencephalon

CART-ir cells were present in the entopeduncular nucleus (E) (Figure 9B), in the medial area of the ventral nPPp (Figure 7E, 9C), the lateral area of the NLTv (Figure 7F, 9D) and in the NRP (Figure 9E). Large –ir cells (mean ± SD diameter: Gravid: 9.543 ± 1.146 µm; Brooding: 9.663 ± 1.180 µm) were also found in two areas of the nMLF: in the medial region of the nMLF dorsal to the mlf and along the midline in the ventral area of the nMLF (Figure 9F).
CART-ir fibers were present in the preoptic area of the diencephalon in the nPMp, nMMp, and nPPp (Figure 7D-E). Fibers in the nPMp were scattered in the ventro-medial portion of the nucleus in the region dorsal to the optic chiasm (OC) (Figure 7D). Moderately dense fibers were found in the medial portion of the nMMp along the midline (Figure 7D). The nPPp contained low-density fibers scattered throughout the nucleus (Figure 7E).

In the hypothalamus, fibers were present in the NLTv, NLTd, NLTi, NDILI, NRL and medial part of the diffuse nucleus of the inferior lobe (NDILm) (Figure 7F-G). The NLTv, NLTi and ATN had low to moderately dense fibers scattered throughout their rostro-caudal extents, while the rostral NLTd had only a few scattered fibers (Figure 7F). The NRL had low-density to moderately dense fibers located around the lateral recess with fibers primarily on the medial side in the region just dorsal to the NDILm (Figure 7G). The NDILI had a few scattered fibers throughout the nucleus while more dense fibers were found dorsally between the NDILI and the GN and NMIL (Figure 7G).

In the thalamus, the DP contained scattered low-density CART-ir fibers (Figure 7F). The PPd had scattered, low-density fibers confined to the dorsal portion of the nucleus that borders the PC. The nucleus of the thalamic eminentia (nTE) had low-density fibers confined to the medial portion of the nucleus. The TPp had a few scattered fibers throughout the nucleus (Figure 7F), and low-density fibers were found bordering the ventral area around the GN (Figure 7G). Fibers were scattered in the dorsal portion of the TGN and in the region between the aGn and ventral optic tract (VOT). The VOT also contained some scattered fibers. Low to moderately dense fibers was present in the region between the fasciculus retroflexus (fr) and the LT and nMLF.
In the mesencephalic tectum, fibers were consistently located throughout the SWGZ, DWZ, and CZ (Figure 7D-I). The SWGZ had scattered fibers while there were more numerous fibers at the borders between the SWGZ and CZ, and between the CZ and DWZ, and scattered fibers throughout the CZ (Figure 7E-I). The Iln contained a few scattered fibers with more fibers located just ventral to the nucleus (Figure 7G). The TL had a few scattered fibers in both the rostral portion of the nucleus as well as in the caudal part where the fibers were seen in the medial region bordering the ventricle. In the rostral TSc, fibers were scattered in the medial portion of the nucleus as well as bordering the dorsal portion near the TBT (Figure 7G-H). As the TSc became more caudal, moderately dense to dense fibers were confined to the dorsal area of the TSc and were present in the ventral area of the TSc (Figure 7G-H). Low-density, scattered fibers were also present in the TSvl (Figure 7G). Fibers were also scattered within the medial portion of nMLF.

Rhombencephalon

The rostral portion of the VCeG had some scattered CART-ir fibers in the lateral region bordering the ventricle. Fibers were also present in the VCeM around the fourth ventricle (Figure 7H). Low-density, scattered fibers were present in the NLVc in the region bordering the TMCa (Figure 7H). Low-density fibers were also present dorsal to the TBT and lateral to the IP (Figure 7H). Fibers in the rostral portion of the CCeG were confined to the ventro-medial region just bordering the ICo (Figure 7I). The CCeP (Figure 7J) and CCeM both contained a few scattered fibers. The rostral CG had a few scattered fibers bordering the fourth ventricle and moderately dense to dense fibers in the caudal CG (Figure 7I-J). The RS had low-density scattered fibers throughout the
area ventral to CG and MLF (Figure 7I-J). The MLF and SR had a few, low-density fibers confined to the medial area closest to the midline (MLF) and the medial area bordering the midline (SR) (Figure 7I). Moderately dense to dense fibers were found in the sensory trigeminal nucleus (STN) lateral to the CG and fourth ventricle. The facial motor nucleus (VIIm) also contained low-density, scattered fibers in the dorsal portion of the nucleus lateral to the CG (Figure 7J). A few scattered fibers were found in the area between the dorsal VIIm and ventral MON. Low-density fibers were found in the MON and scattered throughout the VL. Low to moderately dense fibers was found ventral to the sgt and lateral to the DON. Low-density, scattered fibers in the DON were present bordering the VL and along the lateral edge of the DON. In the brainstem, low to moderately dense fibers were present in the MFN and dt.

Pro-opiomelanocortin (pomc)

All three forms of pomc were located in the rostral pars distalis (RPD) and pars intermedia (PI) of the pituitary (Figure 10A). The distribution of pomc-1a and -1b cells was restricted to the medial NLTv (Figure 10A-C) and some large cells in the rostral portion of the ATN (mean ± SD diameter: Gravid: 8.375 ± 3.164 µm; Brooding: 9.056 ± 2.729 µm) (Figure 10A). Pomc-2 cells were the most widely distributed of the three forms and were found in the Dm-1, DI-d, DI-v and DI-g of the rostral telencephalon, and in the nPPa, NLT, and ATN of the diencephalon. Pomc-2 cells were also found in the tectum at the dorsal edge of the PGZ beneath the DWZ. Several scattered pomc-2 cells were also found in the commissural nucleus of Cajal (NCC) of the brainstem.
Figure 9. Representative photomicrographs of CART-ir somata and fibers in the brain of *Astatotilapia burtoni*. Coronal sections of the telencephalon show NPY-immunoreactive cells (arrowheads) in the Vc (A) and entopeduncular nucleus (B). In the diencephalon there are immunoreactive cells (arrowheads) in the nPPp (C), the NLTv (D), and the NRP (E). Large immunoreactive cells were also present in the nMLF (F) of the mesencephalon. Scale bars = 100 µm in B and C; 25 µm in A,D,E and F. See list for abbreviations.
Figure 10. Representative photomicrographs of pomc-1a somata in the brain of Astatotilapia burtoni. Sagittal section of the brain showing pomc-1a cells in the ATN, NLTv and pituitary (Pit) stained via in situ hybridization. (B,C) Transverse sections illustrating pomc-1a cells in the NLTv at low (B) and high magnification (C). Scale bars = 250 µm in A,B; 10 µm in C.

Reproductive state differences in orexigenic and anorexigenic somata size

NPY-ir (ANCOVA; F=24.531; df=18; p<0.001) (mean ± SE; Gravid: 19.792 ± 0.869 µm²; mouthbrooding: 13.057 ± 0.918 µm²) and AGRP-ir (ANCOVA; F=20.529; df=19; p<0.001) (Gravid: 16.035 ± 0.636 µm²; mouthbrooding: 11.672 ± 0.636 µm²) mean somata sizes in the NLT were larger in gravid females compared to brooding females (Figure 11A,B). Pomc-1a mean somata sizes were larger in brooding compared to gravid females (ANCOVA; F=19.401; df=17; p<0.001) (Gravid: 22.898 ± 1.197 µm²; mouthbrooding: 30.702 ± 1.197 µm²) in the NLT. However, mean CART-ir NLT somata sizes (ANCOVA; F=0.967; df=21; p=0.337) did not differ between brooding and gravid females (Figure 11C, D). Correlations between somata sizes, GSI, and HSI are summarized in Table 2. There was a negative correlation between pomc-1a somata size and GSI. Pomc-1a somata size was also negatively correlated with both NPY-ir
and AgRP-ir somata sizes. NPY-ir and AgRP-ir somata sizes both showed a positive correlation with GSI. HSI was not correlated with any of the neuropeptide somata sizes.

**Hypothalamic mRNA expression differences between gravid and brooding females**

A total of 28 (15 gravid, 13 brooding) females were used in the hypothalamic mRNA analysis. Similar to the females used for neuropeptide-containing somata quantification analyses described above, gravid females had higher GSI (mean±SD; 10.08 ±1.35) (Mann-Whitney; U=0.00; p<0.001) and larger ova diameters (2.35 ± 0.30 mm) (Mann-Whitney; U=0.00; p<0.001) compared to brooding females (0.71±0.23 mm). Gravid females also had higher HSI (1.66 ± 0.61) values compared to brooding females (0.86 ± 0.33) (Mann-Whitney; U=19.00; p<0.001), and higher condition factors compared to brooding females (gravid: 3.90 ± 0.41; mouthbrooding: 3.06 ± 0.43) (Student’s t-test; p<0.001; t=5.346; df=26), indicative of greater energy reserves and overall fitness. Standard length (Student’s t-test; p=0.152; t=-1.475; df=26) and body mass (Student’s t-test; p=0.0796; t=1.824; df=26) did not differ between gravid and brooding females. Macro-dissected hypothalamic mRNA levels for *agrp*, *cart 2*, *cart 4*, *ghrelin receptor (ghs-r1)*, *leptin receptor (lepr)*, *npy and pomc-1a* were compared between gravid and brooding females. *Agrp* mRNA expression was significantly higher in gravid females compared to brooding females, but there were no differences between gravid and brooding females for any of the other mRNA levels measured (Table 3). Correlations between hypothalamic mRNA levels are summarized in Table 4. *Pomc-1a* mRNA levels positively correlated with *agrp* mRNA levels in the hypothalamus. Interestingly, *npy* mRNA levels positively correlated with mRNA levels of the receptors, *ghs-r1 and lepr*.
Figure 11. Reproductive state differences in somata sizes for orexigenic and anorexigenic neurons in *A. burtoni* females. NPY (A) and AGRP (B) somata sizes are larger in gravid compared to brooding females, while *pomc-1a* (C) neurons are larger in brooding compared to gravid females. CART-ir somata sizes (D) did not differ between gravid and brooding females. In each graph, the bottom of each box is the 25th percentile the top is the 75th percentile and the line in the middle is the median, whiskers (error bars) indicate min and max values and dots represent outside values. Sample sizes are indicated within each plot. Different letters indicate statistical differences at p<0.05.

Table 2. Correlations between somata sizes, gonadosomatic index (GSI), and hepatosomatic index (HSI) for combined gravid and brooding *A. burtoni* females

<table>
<thead>
<tr>
<th></th>
<th>NPY</th>
<th>AgRP</th>
<th><em>pomc-1a</em></th>
<th>CART</th>
<th>GSI</th>
<th>HSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>0.653</td>
<td>-0.7821</td>
<td>&lt;0.001*</td>
<td>0.119</td>
<td>0.659</td>
<td>0.405</td>
</tr>
<tr>
<td></td>
<td>0.001*</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgRP</td>
<td></td>
<td>-0.696</td>
<td>-0.162</td>
<td>0.482</td>
<td>0.581</td>
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<tr>
<td></td>
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<td></td>
<td>0.001*</td>
<td></td>
<td>0.005*</td>
<td>0.09</td>
</tr>
<tr>
<td><em>pomc-1a</em></td>
<td></td>
<td></td>
<td>-0.0567</td>
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<td>0.032*</td>
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<tr>
<td>CART</td>
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<td></td>
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<td>-0.196</td>
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<td></td>
<td></td>
<td>0.395</td>
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Correlation coefficients (top value) and p-values (bottom value) are shown. *Denotes significance at p<0.05 from Pearson Correlation tests.
Table 3. Hypothalamic mRNA expression levels for gravid and mouthbrooding *A. burtoni* females

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gravid females</th>
<th>Mouthbrooding females</th>
<th>F</th>
<th>df</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>agrp</td>
<td>0.0146 ± 0.0153</td>
<td>0.00393 ± 0.00298</td>
<td>4.926</td>
<td>23</td>
<td>0.037*</td>
</tr>
<tr>
<td>cart 2</td>
<td>1.15x10(^{-7}) ± 6.20x10(^{-4})</td>
<td>1.67x10(^{-7}) ± 1.14x10(^{-4})</td>
<td>2.284</td>
<td>25</td>
<td>0.143</td>
</tr>
<tr>
<td>cart 4</td>
<td>4.0x10(^{-7}) ± 8.00x10(^{-7})</td>
<td>1.00x10(^{-7}) ± 2.00x10(^{-7})</td>
<td>1.851</td>
<td>24</td>
<td>0.186</td>
</tr>
<tr>
<td>ghrelin receptor</td>
<td>0.00226 ± 0.000953</td>
<td>0.00345 ± 0.00213</td>
<td>0.023</td>
<td>24</td>
<td>0.880</td>
</tr>
<tr>
<td>leptin receptor</td>
<td>0.00226 ± 0.000953</td>
<td>0.00345 ± 0.00213</td>
<td>3.380</td>
<td>23</td>
<td>0.079</td>
</tr>
<tr>
<td>npy</td>
<td>1.99x10(^{-8}) ± 9.60x10(^{-9})</td>
<td>3.32x10(^{-8}) ± 2.79x10(^{-8})</td>
<td>3.099</td>
<td>25</td>
<td>0.091</td>
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<tr>
<td>pomc-1a</td>
<td>0.00166 ± 0.000783</td>
<td>0.00172 ± 0.00132</td>
<td>0.001</td>
<td>25</td>
<td>0.974</td>
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</tbody>
</table>

Macro-dissected hypothalamic mRNA levels are normalized to the geometric mean of 18s and *RPL32*. Values are reported as mean ± SD. Comparisons were made with ANCOVA, using hypothalamic mass as a covariate.

Table 4. Correlations between hypothalamic mRNA levels for combined gravid and brooding *A. burtoni* females

<table>
<thead>
<tr>
<th></th>
<th>agrp</th>
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<th>lepr</th>
<th>npy</th>
<th>cart2</th>
<th>cart4</th>
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<tbody>
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<td>0.547</td>
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<td>0.233</td>
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<tr>
<td>*<em>0.004</em></td>
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<td>0.124</td>
<td>0.243</td>
<td>0.631</td>
<td>0.498</td>
<td></td>
</tr>
<tr>
<td><strong>agrp</strong></td>
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<td>0.212</td>
<td>0.917</td>
<td>0.371</td>
<td>0.869</td>
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<tr>
<td>0.0678</td>
<td>0.0822</td>
<td>0.917</td>
<td>0.371</td>
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<td></td>
</tr>
<tr>
<td><strong>ghs-r1</strong></td>
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<td>0.506</td>
<td>0.007*</td>
<td>-0.370</td>
<td>-0.265</td>
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<tr>
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<tr>
<td><strong>lepr</strong></td>
<td>0.501</td>
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<tr>
<td><strong>npy</strong></td>
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<tr>
<td><strong>cart 2</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.188</td>
<td>0.348</td>
<td></td>
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</tbody>
</table>

Correlation coefficients (top value) and p-values (bottom value) are shown. *Denotes significance at p<0.05 from Pearson Correlation tests.*
**Discussion**

Our results provide the first localization maps of the orexigenic neurons NPY and AgRP and the anorexigenic neurons CART and *pomc* in the brain of a mouthbrooding teleost fish. We demonstrate that these neuropeptides are found in brain regions that regulate feeding, energy status, and reproduction that are conserved with those of higher vertebrates (Figure 12). Further, we show that somata size of *pomc-1a*, AgRP and NPY-producing neurons differs between gravid and mouthbrooding females, suggesting that changes in the synthesis, release, or activity of these neurons is important for regulating cyclical variations between these different energetic and reproductive states.

Figure 12. Schematic summary representation of the locations of NPY, AgRP, CART and *pomc-1a* cells in the brain of the cichlid *Astatotilapia burtoni*. Cell populations for each neuropeptide and areas of co-regionalization are represented on a lateral view of the brain (rostral is to the left) and coded by color: *pomc-1a* (red), CART (dark blue), NPY (yellow), *pomc-1a* and AgRP (purple), CART and NPY (green), and NPY and *pomc-1a* (orange). Analysis for –ir cells and fibers was not done in the pituitary for NPY, AgRP and CART . See list for abbreviations.
Comparisons of NPY, AgRP, CART and pomp-1a with other teleosts

Neuropeptide Y (NPY)

Moderate NPY-immunoreactive fibers were detected in all of the layers of the olfactory bulb (ECL, ICL, GL, SOF) in A. burtoni, which is similar to other fish species including tilapia (Sakharkar et al., 2005), goldfish (Pontet et al., 1989), and the ayu (Chiba et al., 1996). NPY-ir cells were not detected in the olfactory bulbs of A. burtoni, which is similar to the Senegalese Sole (Rodriguez-Gomez et al., 2001), but differs from other species such as the goldfish (Pontet et al., 1989), senegal bichir (Reiner and Northcutt, 1992) and ayu (Chiba et al., 1996), in which NPY-ir cells were present in the ECL of the olfactory bulbs. The presence of NPY-ir fibers in the olfactory system suggests a modulatory role in olfactory processing, possibly related to appetite and hunger level of the animal, similar to that described in the axolotl (Mousley et al., 2006).

In the telencephalon of A. burtoni, NPY-immunoreactive cells were found in the Vd-r, Vv, VI, Vc and Dc-4, and –ir fibers were present throughout the dorsal and ventral telencephalon. The NPY-ir fiber pattern observed in A. burtoni is generally similar to that reported in other teleosts (Kah et al., 1989; Pontet et al., 1989; Batten et al., 1990; Danger et al., 1991; Subhedar et al., 1996; Chiba et al. 1996; Gaikwad et al., 2004; Sakharkar et al., 2005). NPY-immunoreactivity in A. burtoni was also present in various regions that are homologous to mammalian nuclei involved in feeding and reproduction. For example, NPY-ir cells were present in the entopeduncular nucleus (E), which is consistent with findings in goldfish (Pickavance et al., 1992), rainbow trout (Danger et al., 1991), catfish (Gaikwad et al., 2004), killifish (Subhedar et al., 1996) and carp (Marchetti et al., 2000). While the definitive homolog of the entopeduncular region is not
known in teleosts, the mammalian entopeduncular region is part of the amygdala (O’Connell and Hoffmann, 2011). However, the entopeduncular nucleus is also thought to play a role in reproduction. In castrated male tilapia, for example, depletion of NPY immunoreactivity in E neurons was evident post-castration but was restored following testosterone treatments (Sakharkar et al., 2005), suggesting a reproductive role for the NPY-ir cells in the E. The prevalent NPY immunoreactivity in the telencephalon of teleosts suggests conserved functions in reproduction, reproductive and social behavior and the mesolimbic reward system. In the preoptic area of A. burtoni, NPY-ir cells were present in the nPPa and fibers were found through the nPPa, nPPp, nPMp, nGMP, and nMMP. In teleosts, the importance of the preoptic area in reproduction is well known because it contains GnRH1 neurons that project directly to the pituitary to stimulate the synthesis and release of gonadotropin hormones (Maruska, 2014). In A. burtoni, NPY-ir cells were present in the NLTd and NLTm, the teleost homolog to the mammalian arcuate nucleus (Cerda-Reverter and Peter, 2003; Liu et al., 2010) that is involved in the hypothalamic control of energy balance and feeding behavior. Thus, the present study suggests that NPY’s role in regulating feeding and reproductive functions is likely conserved.

**Agouti-Related Protein (AgRP)**

AgRP-immunoreactivity in the olfactory bulb of A. burtoni was restricted to a few fibers in the ICL. This is similar to zebrafish where fine fibers were found in the olfactory bulbs, however, they were not restricted to a particular cell layer (Forlano and Cone, 2007). Therefore, the presence of AgRP in the olfactory bulbs of A. burtoni suggests a conserved neuroanatomical distribution, but examinations in more species are needed.
Furthermore, the presence of AgRP in the olfactory bulb could suggest a role in olfactory processing, specifically in conjunction with the melanocortin system. α-MSH-ir fibers are present in the olfactory bulbs of zebrafish and the MC4 receptor was present in the olfactory bulb of the spiny dogfish (Ringholm et al., 2003), suggesting that AgRP may modulate olfactory processing.

AgRP-ir cells in A. burtoni are found in the lateral tuberal nucleus of the hypothalamus. This is consistent with findings in goldfish (Cerda-Reverter and Peter, 2003), zebrafish (Forlano and Cone, 2007), and sea bass (Agulleiro et al., 2014), where AgRP is mainly expressed in the caudal portion of the lateral tuberal nucleus. Similarly, the NLTv is the primary region expressing AgRP cell bodies and fibers in A. burtoni. This region of the brain is associated with feeding and energy expenditure in teleosts (Forlano and Cone, 2007), and is considered the homolog of the arcuate nucleus in mammals because it expresses the specific arcuate markers, AgRP and pomc. In goldfish (Cerda-Reverter and Peter, 2003) and zebrafish (Song et al., 2003), fasting up-regulates mRNA levels of AgRP, suggesting it also functions as a feeding modulator in teleost fishes. Our study provides further evidence that the neuroanatomical distribution of AgRP is conserved, and suggests that the mechanisms regulating energy homeostasis may be similar across vertebrates.

**Cocaine and amphetamine-regulated transcript (CART)**

CART-immunoreactivity was absent from the SOF and gTN of A. burtoni, but CART-immunoreactive cells were found in the GL and fibers were observed in the ICL, ECL and GL of the olfactory bulb of A. burtoni, which is consistent with previous findings in catfish (Singru et al., 2007) and zebrafish (Akash et al., 2013) suggesting a role for
CART in the processing of olfactory information in teleosts. Similarly, in rats, low-density CART-ir fibers were found in the inner plexiform layer of the olfactory bulb and CART-ir cells were present in the cells of the mitral layer and outer plexiform layer (Koylu et al., 1998; Couceyro et al., 1997). Further, CART peptide was found in the mitral cells of the rat olfactory bulb (Couceyro et al., 1997), suggesting a role for CART in the processing of olfactory signals in mammals as well (Koylu et al., 1998). Taken together, these findings suggest a role for CART in the processing of olfactory information in vertebrates that warrants further investigation with a comparative approach.

CART-immunoreactivity was also present in neuroendocrine regulatory areas of the telencephalon and diencephalon in A. burtoni. For example, CART-ir cells were present in the rostral entopeduncular nucleus of A. burtoni, which is congruent with studies done in the catfish (Singru et al., 2007; Mukherjee et al., 2012) and zebrafish (Akash et al., 2014). In contrast, however, CART-immunoreactivity was not observed in the entopeduncular nucleus of the frog species Rana esculenta (Lazar et al., 2004). This suggests that there may be species or taxa-specific differences across vertebrates. CART-ir somata were also present in the nPPp of A. burtoni, and fibers were present in the nMMP, nPMp, nGMp and nPPp subdivisions of the preoptic area, which is consistent with previous findings in teleosts. In zebrafish, in situ hybridization experiments showed CART 2 and CART4 cells in the nPPa and nPPp (Akash et al., 2014), and immunostaining experiments demonstrated a dense CART-ir fiber network in the preoptic area (Mukherjee et al., 2012). In catfish, dense immunoreactivity was also seen in the preoptic area that varied seasonally and with reproductive state (Barsagade et al., 2010). The preoptic area of teleosts is well recognized as being important in the
regulation of reproduction (Yu et al., 1991), and contains neurons that innervate the pituitary gland, such as GnRH1 cells, which regulate hypophysiotropic functions such as gonadotropin hormone secretion (Akash et al., 2014; Zohar et al., 2010). CART-immunoreactive fibers were observed in close proximity to GnRH cells in the preoptic area of the Siberian hamster (Leslie et al., 2001) and pig (Bogus-Nowakowska et al., 2011). In mammals, the CART-expressing neurons in the arcuate nucleus and premammillary ventral nucleus communicate with GnRH-containing neurons in the POA. It is thought that CART modulates GnRH secretion through the regulation of luteinizing hormone as well as facilitates leptin’s effect on GnRH secretion (Subhedar et al., 2014; Rondini et al., 2004). A more recent study by True et al. (2013), showed that CART had extensive connectivity with GnRH and kisspeptin-expressing neurons in the POA, and electrophysiology experiments showed that CART postsynaptically depolarized both GnRH and kisspeptin cells. In addition, CART mRNA expression and the number of CART-immunoreactive cells decreased during calorie restriction suggesting that CART expression in animals of depressed metabolic conditions could inhibit the reproductive axis (True et al., 2013; Subhedar et al., 2014). Overall, this conserved neuroanatomical distribution from fishes to mammals suggests a potential mechanism by which nutritional status is conveyed to the reproductive system in the preoptic area of vertebrates.

CART-immunoreactivity is also observed in regions associated with the regulation of feeding, energy balance and gustation in teleosts (Copeland et al., 2011). These areas include the E, NLT, NRL, and NDIL, which all showed CART immunoreactivity in A. burtoni. This is consistent with previous findings that have shown
CART-ir somata in the lateral tuberal nucleus of zebrafish and catfish (Subhedar et al., 2014; Akash et al., 2014). In zebrafish, starvation led to a significant reduction in CART 2 mRNA-expressing neurons of the E and NRL, and CART 4-expressing neurons in the NLT (Akash et al., 2014). Thus, CART may have a central role in the regulation of energy balance and feeding in teleosts, similar to that described in mammals (Lin et al., 2000; Volkoff et al., 2005; Subhedar et al. 2014). Due to the whole genome duplication event in teleosts, multiple cart genes have been reported in zebrafish (Akash et al., 2014) and in A. burtoni. In the present study, the antibody used for immunohistochemistry likely labels multiple forms of CART and further studies are needed to examine differential expression patterns of the different forms.

Pro-opiomelanocortin (pomc)

The pro-opiomelanocortin gene (pomc) produces several bioactive peptides (i.e., ACTH, β-endorphin, α-MSH, β-MSH, and γ-MSH) that play key physiological roles in vertebrates (de Souza et al., 2005: see review Cerda-Reverter et al., 2011). Studies done in other species such as the pufferfish, Tetraodon, used radioactive in situ hybridization and found that pomc-1 was expressed in the lateral tuberal nucleus of the hypothalamus, as well as in the rostral pars distalis (RPD) and pars intermedia (PI) of the pituitary, whereas pomc-2 was expressed in the preoptic area of the brain and weakly in the pituitary PI. This is consistent with our findings using in situ hybridization in A. burtoni where pomc-1a was located in the NLTv, ATN and the RPD and PI of the pituitary. However, pomc-2 was more widely distributed as it was found in the forebrain, preoptic area, tectum, pituitary and caudal brainstem. Due to the teleost whole genome duplication, higher teleost species such as Tetraodon (Tetraodon nigroviridis), Fugu
(Takifugu rubripes), and zebrafish (Danio rerio), possess two paralogous genes and here we report the distribution of three forms of pomc in A. burtoni (Harris et al., 2014). The pomc genes have many roles in vertebrate physiology including the regulation of serum glucocorticoid levels, pain sensitivity, food intake and the control of skin pigmentation (de Souza et al., 2005). The expression patterns of pomc-1 and 2 suggest subfunctionalization due to the need for multiple forms. For example, in Tetraodon, pomc-1 mRNA was detected in the PI and RPD of the pituitary and NLT of the hypothalamus. Pomp- 2, however, was located in the preoptic area, with weak expression in the pituitary (de Souza et al., 2005). Pomp expression in Tetraodon is similar to A. burtoni, and the similarities in expression could suggest that multiple forms and functions of the pomc gene may be a shared characteristic across teleosts.

Reproductive state differences in somata size and mRNA expression

Neurons are responsible for the coordination and regulation of gene expression in response to functional needs of the organism (Ransdell et al., 2010); however, there are not always straightforward relationships between somata size, cell number, mRNA expression, and protein levels within individual neuron types. For example, a study in the European starling bird showed that changes in the number of GnRH-I and gonadotropin-inhibitory hormone (GnIH) cells throughout the breeding season (Calisi et al., 2011), overall, mirrored changes in neuronal soma size and estimated peptide concentration. That study, therefore, showed a positive relationship between cell number, cell size and peptide concentration. Therefore, an increase in cell size could be due to either an increase in mean production or a reduction in peptide release and increased storage, but it is not possible to distinguish amongst these possibilities via
staining techniques alone. In our study, we tested whether somata size and gene expression for candidate orexigenic and anorexigenic neurons varied based on reproductive and energy consumption states. Our results showed that somata sizes varied between reproductive states with NPY-ir and AgRP-ir somata sizes being larger in gravid females and pomc-1a somata sizes being larger in brooding females. One explanation for the changes in cell size between gravid and brooding females could be the need for a mechanism for quick adaptive changes in the neuroendocrine system without cell addition or cell death (Francis et al., 1993). For example, somata that increase in volume can support more complex dendritic inputs, which can have important functional implications for the response properties of individual neurons within the circuit (Roberts et al. 2006, Mainen et al. 1996). This is potentially important in mouthbrooding fish such as A. burtoni, as females will undergo a fairly rapid transition from a gravid (energy investment) to a brooding (energy consumptive) state when her clutch of eggs is effectively transferred from her abdominal cavity into her buccal cavity during spawning. During this “motivational switch” from a self-promoting to an offspring-promoting phenotype, it is important that the neurons involved in regulating the accompanying behaviors and physiology demonstrate plasticity, especially when food intake is no longer physically possible. There is also a precedent for rapid changes in somata size in male A. burtoni, since GnRH1 neurons enlarge within the first 24 hrs. of a transition from reproductively-suppressed subordinate to reproductively-active dominant social status (Maruska and Fernald 2013). Thus, rapid changes in cell size may be a common mechanism mediating phenotypic plasticity within different neuroendocrine systems for both males and females of this species.
To further investigate the involvement of these candidate neuropeptides in the regulation of the neural control of the mouthbrooding parental care strategy, including the period of necessary food-deprivation, RT-qPCR was performed on macro-dissected hypothalamic tissue. Our initial prediction for lepr and ghs-r1 in the hypothalamus was that lepr mRNA expression would be higher in brooding females, and ghs-r1 mRNA expression would be higher in gravid females. While the females are brooding, they are physically incapable of eating so we predicted that lepr mRNA expression would be higher during this reproductive stage, thereby increasing the sensitivity of the hypothalamus to the leptin signal to prevent the females from cannibalizing or releasing their brood. On the other hand, gravid females have to maintain their current metabolic state as well as undergo vitellogenesis, so they need to increase their caloric intake. We therefore predicted that there would be higher levels of ghs-r1 mRNA during this reproductive state to maintain feeding motivation and food intake. In the current study, however, mRNA levels of ghrelin receptors and leptin receptors did not differ between gravid and brooding females, so it is possible that fasting/feeding does not influence transcription but may modify the density or turnover of receptors in certain neurons (Cerda-Reverter et al., 2011). During fasting, for instance, the availability of receptors could be reduced, thereby decreasing sensitivity to hormones such as ghrelin, to orexigenic neuropeptides such as NPY and AgRP, or to constitutive signaling of hormones and neuropeptides.

AgRP was the only gene product to differ between gravid and brooding females. The difference in AgRP mRNA levels could be explained by its role as a melanocortin receptor antagonist, and therefore, higher AgRP mRNA levels in gravid females may
contribute to regulation of the melanocortin system by inhibiting the binding of α-MSH to the MC4 receptor, maintaining high food intake during the consumptive gravid phase. This is important, as the females need to build up metabolic stores in addition to vitellogensis to ensure a successful brood in which the female carries to term and does not pre-release or cannibalize her fry. Previous studies have shown that during changes in feeding/fasting, POMC hypothalamic mRNA levels show little to no change (Cerda-Reverter et al., 2011), which is similar to the lack of differences in pomc-1a mRNA levels between gravid and brooding females in our study. A previous study done in A. burtoni by Grone et al. (2012) compared whole brain mRNA levels of several neuropeptides and receptors in gravid, starved and mouthbrooding females. That study found that whole brain npy expression did not vary between gravid (fed), starved or brooding (starved) females. This is generally consistent with our findings that npy expression did not vary within the hypothalamus of gravid and brooding females. However, this could be due to the fact that npy is a neuromodulator known to be involved in the regulation of many other behaviors including stress and aggression.

CART 2 and CART 4 mRNA levels also did not differ between gravid and brooding females, which is consistent with the lack of somata size differences between reproductive states. CART 2 and CART 4 were selected as potential candidates regulating energy balance and feeding behavior due to their previously described roles in zebrafish. For example, starvation in zebrafish led to a reduction in the population of CART 2 mRNA-expressing cells of the E and NRL, and in the CART 4 mRNA-expressing cells of the NLT (Akash et al., 2014). However, our observations that pomc and cart mRNA levels do not differ between gravid and brooding females could suggest
that regulation of the MCR is primarily due to an increase in AgRP synthesis rather than a decrease in \textit{pomc} and \textit{cart} synthesis. It is important to note, however, that mouthbrooding is an extreme form of parental care and forced starvation, and there may be additional regulatory circuitry within the hypothalamus that is fundamentally different from that found in non-brooding fish species in a fasted condition.

In both mammals and teleosts, there is a link between energy balance and reproduction that works in unison to improve reproductive success (Schneider 2004). In the current study we chose to use two drastically different reproductive and energy states to investigate potential differences between orexigenic and anorexigenic neuropeptides in the hypothalamus of \textit{A. burtoni} females. Changes in soma sizes between the two states were observed, but differences in mRNA were only observed for AgRP. One possibility for this discrepancy could be that gene expression does not track positively with soma size, suggesting a complex relationship between feedback of cell size and transcript numbers (Ransdell et al., 2010). Another explanation is that 2 different sets of animals were used in our experiments, one for somata size measurements, and another for mRNA expression, which precludes direct inferences on relationships between mRNA levels and somata size within the same individuals. Further, another explanation could be due to the timing of collections and when they fell in the reproductive cycle. A previous study in \textit{A. burtoni} showed that plasma levels of 17β-estradiol (E), testosterone (T), progestins (P), and prostaglandin F2a (PGF2) fluctuate during the 28-day female reproductive cycle (Kidd et al. 2013). Therefore, additional time points are needed to account for fluctuations in circulating hormone levels that may influence anorexigenic and orexigenic neurons. In addition, we only
used females that were either gravid (feeding state) or brooding (parental state with forced starvation), but did not include a non-brooding starved state. In mammals, a neural circuit has been identified from the parabrachial nucleus (PBN) to the central nucleus of the amygdala (CeAlc) that mediates appetite suppression during unfavorable conditions, which is different from the circuit that controls appetite suppression after feeding (Carter et al., 2013). Therefore, it would be important in the future to include a starved female group (non-brooding state) with a brooding female group (unfavorable condition). Grone et al. (2012) found that the food deprivation accounted for the physiological changes seen in the brooding females (delayed ovarian cycles, delays in subsequent spawning, decrease in body mass) but that starvation alone did not account for the neurological changes. Thus, the starved female group (non-brooding state) could help to test whether the conditions of the starvation state influences somata sizes and gene expression within specific brain nuclei and whether or not they are due to the parental behavioral state. Nevertheless, since these candidate neuropeptides and their functions are relatively well conserved, our work provides insights for understanding the neural control of feeding and reproductive circuits across vertebrate taxa.

In this study, we provide the first complete distribution patterns of candidate anorexigenic (CART, \textit{pomc}) and orexigenic (NPY, AgRP) neurons in the brain of a mouthbrooding teleost fish. The similarity in localization of these neuropeptides amongst teleosts, as well as with that of mammals, suggests that the neural circuitry regulating the interaction of feeding and reproductive circuits are conserved. Reproductive-state plasticity in the somata size of NPY, AgRP and \textit{pomc}-expressing neurons in the \textit{A. burtoni} brain also suggests that these neuropeptides are involved in
regulating phenotypic and metabolic changes associated with the transitions between gravidity and mouthbrooding parental care stages in *A. burtoni*. Future studies should focus on the functional roles these neuropeptides may play in regulating phenotypic and metabolic changes during the transitions between gravid and mouthbrooding states.


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Vita

Danielle grew up in Coral Springs, Florida and later attended the University of Alabama (UA) where she graduated with a Bachelor of Science degree in Biology in 2012. While attending UA, Danielle worked in the lab of Dr. Matthew Jenny studying the effects of the Deep Horizon Oil Spill on the starlet sea anemone, *Nematostella vectensis*. After completing her undergraduate degree, Danielle joined the lab of Dr. Karen Maruska at Louisiana State University. Her research has been focused on looking at the distribution and correlation of orexigenic and anorexigenic neuropeptides between gravid and brooding females of the African cichlid, *Astatotilapia burtoni*. Her research has been presented at two national conferences, and will aid in the better understanding of neuropeptides that may be involved in feeding and reproduction. Danielle plans to continue to study reproductive endocrinology to better understand the role metabolic state has in influencing reproductive potential.