From Mom's Metabolism to Child Cannibalism: Feeding Regulation in the Maternal Mouthbrooder *Astatotilapia burtoni*

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Abstract

Renn laboratory maintains two genetically distinct lineages of mouthbrooding cichlid species *Astatotliapia burtoni*—an inbred laboratory stock that has been raised in captivity for over 30 years, and a wild-caught stock recently acquired from Lake Tanganyika, Zambia. Mothers from the lab stock frequently eat their offspring and display less maternal aggression than their wild counterparts; conversely, wild stock mothers of *A. burtoni* display a wide range of "good" maternal behaviors, including aggression towards conspecifics, and they do not eat their own fry. The inbred laboratory stock has been shown to lose more body mass while mouthbrooding than does the wild stock; this observation suggests an inability of laboratory stock fishes to appropriately decrease metabolic rate during the brooding period and implies a relationship between maternal care phenotype and the ability to regulate metabolism.

This study sets out to determine whether there are whole-organism differences in metabolism and feeding regulation between laboratory stock and wild stock *A. burtoni* females undergoing a period of starvation, and whether brooding-induced starvation affects female *A. burtoni* differently than non-brooding starvation. Gross weight changes, measurements of metabolic rate, and assays for differential expression of feeding-related neuropeptides provided three levels from which to zero-in on the mechanisms of metabolism during mouthbrooding. This experimental tack allowed for the diagnosis of metabolic irregularity and separated the reproductive context of brooding from the act of starvation.

The present study reveals that during conditions of brooding-induced or experimentally controlled starvation, laboratory stock *A. burtoni* are not able to appropriately reduce their metabolic rate and so expend more energy in the absence of energy intake. In addition, Neuropeptide Y (NPY), a strongly orexigenic (appetite stimulating) neuropeptide integral to feeding regulation, was found to be expressed at higher levels in laboratory stock fishes in these same conditions. The results of this study are foundational; they represent a logical starting-place to begin to tease apart the intricacies of feeding and its relationship to behavior.

Dedication

I dedicate this project to life; to living things; to being alive, wherefore, and how. More nearly true: this is for biology, for hunger, and for cichlid fishes. Really though, it's for 3 women and for 2 cats. My honey, Lindsay: the only person I have ever understood and the only person I will ever love... the Rayleigh (read: *Raley*) criterion ($\theta \approx \frac{1.22\lambda}{D}$) answers the question: "how close can two things get before they look like the same thing?" Our overlap is such that we cannot be resolved! My mother, whose parental care—though differing in kind from buccal incubation—has conferred cognate advantages, churning me from a neonate of wild stock to at least the wriggler stage, or its 22 year-old human equivalent. It is my sister, with whom I share 50% of my genetic self and so much more besides, who has had perhaps the greatest influence on my extended phenotype; finally, the Lady doe, the Jubdist man; each and every kitten plan, however wretched.

Introduction

Comprising more than 3,000 species of freshwater fishes distributed throughout eastern Africa, South America, and southern India, the family Cichlidae (Perciformes: Teleostei) is to date the most speciose vertebrate family scientifically described [Barlow, 2000]. The evolutionary time in which this diversification has occurred is more noteworthy still; by any measure, cichlid species-flocks in the Great Lakes of East Africa represent the most rapid radiations of any group of extant vertebrates. A great majority of these species—some 70% of all Cichlidae—belong to a single lineage, the taxonomic tribe Haplochromini [Salzburger et al., 2005]. Referred to colloquially as the East African cichlids, this lineage is endemic to the great East African lakes and their associated rivers; within the last million years, Lake Malawi and Lake Victoria have hosted independent radiations of 700 and 500 haplochromine species, respectively [Turner et al., 2001, Meyer, 2005], while during the last 8 million years Lake Tanganyika has seen the radiation of over 250 species [Salzburger et al., 2002].

Recent mitochondrial-DNA based phylogeographic analyses have revealed Lake Tanganyika to be the ancestral cradle of this lineage; it was this lacustrine birthplace whence characteristic behavioral and morphological innovations such as maternal mouthbrooding, egg spots, and color polymorphisms first arose. In association with the ecological diversity afforded by these large lakes and by smaller lakes throughout the region, said traits are thought to be responsible for the adaptive radiation and explosive speciation of these cichlids [Salzburger et al., 2005].

At some 23,500 species, the teleost fish are among the most numerically successful of the modern vertebrates, making Cichlidae the best of the best [Barlow, 2000]. Given the remarkable diversity—morphological, ecological, behavioral, genomic—of this group, and given the speed, recency, and coincidence of their diversification, East African cichlids provide a singular opportunity to probe the genetic basis of phenotype differences for the genetic mechanisms of adaptive evolution. Their unique evolutionary situation lends itself to comparative studies, and their amenability to captivity and to laboratory techniques makes them attractive model organisms.

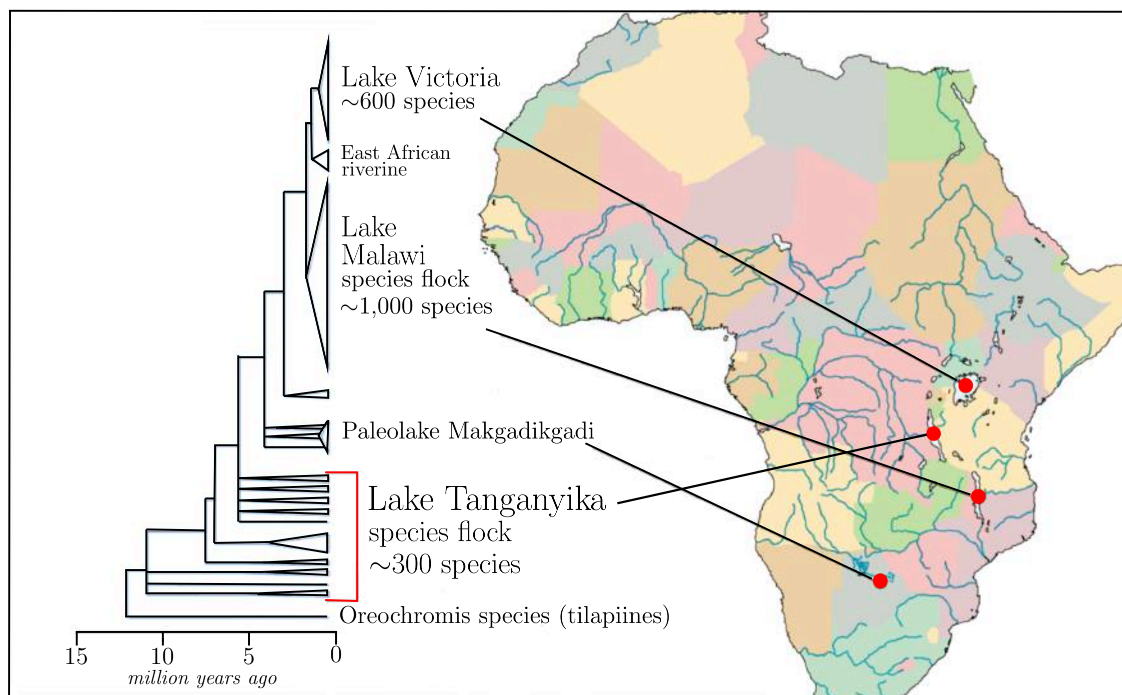


Figure 1. Cichlid radiation in the Great Lakes of East Africa. Zambia shown south of Lake Tanganyika in green; Tanzania, to the east in yellow, Democratic Republic of Congo, to the north west in pink. Note that the haplochromine lineage shares a common ancestor with the economically important Nile Tilapia (*Oreochromis niloticus*), diverging 10 to 15 million years ago [Klett and Meyer, 2002]. Figure created by the author using open-source LatexDraw software.

Breeding and parental care in haplochromines

The East African cichlids are known for the organization of their breeding systems and parental care behaviors [Taborsky, 2001], many of which have been reckoned among the "key innovations" that provided for the rapid evolution of this lineage [Salzburger et al., 2005]. Their mating is mostly polygamous and characterized by lekking—large, colorful, socially dominant male fishes establish small mating territories, often building species-specific sand bowers which egg-bearing females may visit. After spawning, a female will take the eggs up into her mouth, or buccal cavity, and hold them there; fertilization typically occurs when an egg-bearing female nips at "egg-spots" located along a male fish's anal fin (Figure 2), prompting sperm-release from his genital opening [Lehtonen and Meyer, 2011]. Taken into the female's mouth, the milt passes over the eggs and fertilizes them in the process; however, external fertilization is known to occur as well [Mrowka, 1987]. Generally, in this oral-oviparous fashion,

females typically breed with several males and abscond with the fertilized ova intact.

For most haplochromine species in Lakes Malawi and Victoria, a mother retains the eggs inside her buccal cavity in order to insure incubation and protection as they undergo development into free-swimming fry. In the earliest stages of development, mothers churn the eggs around orally, keeping them clean by sucking away any loose debris; this churning continues as the eggs grow into the "wriggler" stage where it serves the important purpose of enhancing respiration [Keenlyside, 1991]. The transition from the "wriggler" stage to the fry stage concludes the obligatory period of maternal care and is marked by the complete consumption of yolk sacs, the ability of fry to swim independently, and the release of developed fry from the mouth. This entire process, known as maternal mouthbrooding, lasts for about two weeks [Smith and Wootton, 1994] but mothers have been known to provide their fry with buccal protection for much longer [Fryer and Iles, 1972]. While forms of uniparental mouthbrooding—like the maternal mouthbrooding described above—are extremely common among Lake Tanganyika cichlids, they are not universal. Indeed, a broad spectrum of parental care behaviors, ranging from the ancestral practice of biparental substrate guarding, on through biparental mouthbrooding, up to the more recently evolved systems of uniparental mouthbrooding, are practiced among these species [Balshine-Earn and Earn, 1998].

While mouthbrooding offers extremely effective protection for developing young, it comes at a great price—their long-suffering mothers must go without food for the duration of the obligatory period. For these fishes, proper broodcare entails voluntary starvation, and though their metabolic rate is significantly reduced at this time, stunted growth and bodily atrophy invariably result [Keenlyside, 1991]. The extent to which metabolic rate is reduced in brooding females relative to non-reproductive females has been quantified for the East African haplochromine species *Pseudocrenilabrus multicolor*. The study, measuring relative rates of oxygen consumption, found that mothers holding their brood in the buccal cavity expended 13.8% more energy than starved non-reproductive females, while feeding non-reproductive females used more than twice as much energy as either brooding females or starved non-reproductive females [Mrowka and Schierwater, 1988]. Mouthbrooding, then, is a well-regulated and low-cost strategy characterized by low energy turnover; it profits from energetic investments made during the interval between brooding cycles—a period when food consumption and energy turnover are high. However, reductions in metabolic rate notwithstanding, brooding mothers still rapidly lose weight, and the physical atrophy sustained during mouthbrooding can have negative fitness con-

sequences later in life. A study by Smith and Wootton [1994] assessed the costs of mouthbrooding in terms of parental survival, fecundity, and breeding frequency, finding reduced growth and 33% longer latency to respawn in postpartum females (*Haplochromis argens*) compared to non-brooding females. These deficits, largely attributable to the mother's feeding cessation, may affect a female's future fecundity and serve to illustrate the collateral expense of buccal incubation.

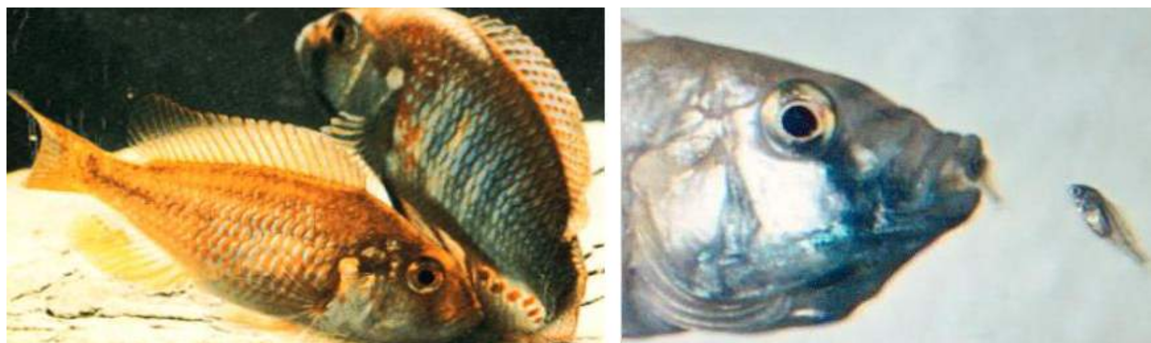


Figure 2. Fertilization and mouthbrooding in *A. burtoni*. Left: an egg-bearing *A. burtoni* female nips at her mate's egg-spots; Right: a mouthbrooding female taking up her fry. Image credit: Russ Fernald

Astatotilapia (syn. *Haplochromis*) *burtoni*

The East African cichlid *Astatotilapia* (syn. *Haplochromis*) *burtoni* is a maternal-mouthbrooding species that inhabits the shallower moving waters of Lake Tanganyika. At Reed College, the laboratory of Suzy Renn maintains two strains of *A. burtoni*—an inbred lab stock (LS) that has been raised in captivity for over 33 years, and a wild-caught stock (WS) recently acquired from the Zambian south end of Lake Tanganyika (see Figure 2). Because many fish of the same stock and generation are housed together in the laboratory's colony tanks, propagation of LS *A. burton*i has required the artificial removal of nearly-mature broods from their mother's buccal cavity before they are naturally released. In this way, LS fry are raised in the absence of maternal care and LS mothers have not been subjected to selective or ecological pressures against filial cannibalism for the duration of the stock's laboratory propagation [Renn et al., 2009].

With regard to maternal care, the two stocks differ in important ways. Mother *A. burtoni* from the LS frequently eat their offspring and are less aggressive than their wild counterparts during the brooding period, while WS mothers display a much wider range of natural maternal behaviors, including aggression towards conspecifics

during the brooding period, and do not eat their own eggs or fry [Carleton, 2009]. Interestingly, it has been shown in our lab that LS mothers lose more body mass while mouthbrooding than do WS mothers, which may be due to an inability of LS mothers to appropriately decrease their metabolic rate during the brooding period [Nguyen, 2009]. If so, these fish are experiencing something akin to cachexia, a condition of malnutrition that occurs when a high metabolic rate is maintained in the absence of nutrients.

These findings are suggestive of a relationship between maternal care phenotype and the ability to regulate metabolism, consistent with recent findings about the interaction between appetite-related and reproductive hormones [Volkoff et al., 1999]. In essence, these fish undergo a period of partial anorexia while brooding, during which time both acute and tonic regulatory mechanisms act to moderate the metabolic rate and thus maintain broodcare motivation. The ability to appropriately regulate metabolism appears to be compromised in the lab stock such that hunger may preclude natural maternal impulses toward brood care.

Starvation *vis-à-vis* broodcare motivation

Throughout the 1980s and into the early 1990s, Wolfgang Mrowka investigated many facets of parental care behavior in the mouthbrooding haplochromine *Pseudocrenilabrus multicolor* of Lake Victoria. His experiments on maternal hunger during the obligatory period revealed some instances of experienced *P. multicolor* mothers eating their own offspring, prompting him to define broodcare motivation as a mother's "readiness to take the brood up into [her] mouth and keep it there but not eat it" [Mrowka, 1984]. In *P. multicolor*, broodcare motivation is maintained up to 10-11 days after spawning; even if a mother has been made to release her brood prematurely, she will take them back into her mouth for the remainder of the term [Mrowka, 1987]. In the course of these studies, Mrowka demonstrated that a mother's broodcare motivation persists even after the artificial removal of her brood during the obligatory period. That is to say, mothers who had their broods stripped from them and removed from their tank during this period refused to eat when subsequently presented with food. This effect was universal, but the latency to resume feeding after brood-removal was found to vary depending on the amount of past experience the mothers had with spawning—first-time mothers refused to eat until several days after their brood was taken from them, while experienced mothers ceased to show broodcare motivation after a separation of only a few hours. This suggests that both first-time

and experienced mothers experience hunger during the obligatory period, and that first-time mothers experience somewhat less. While the physical stimuli of offspring held in the buccal cavity do contribute directly to the inhibition of food consumption, the persistence of broodcare motivation long after brood removal implicates deeper homeostatic feeding–regulatory mechanisms.

In a subsequent study, Mrowka [1986] was able to show that broodcare motivation could be restored to mothers who had lost it during an interval of separation from their offspring simply by satiation with food or by the administration of an anorexigenic compound; that is, when a separated mother and brood were reunited during the obligatory period, if the mother had not eaten anything or had not received an anorexigen during the separation she would either refuse to admit the brood back into her mouth or she would eat it outright, whereas if she had eaten to satiety after the loss of broodcare motivation, she would begin again to mouthbrood her developing fry. Similarly, the point of final fry-release—the total duration of the obligatory period—was found to depend only on the duration of brood separation and not on hunger or satiety, which was measured in terms of items of food consumed after brood-removal. He concluded from these studies that complete physiological anorexia is not needed to prevent filial cannibalism during mouthbrooding, but that a substantial decrease in the satiety threshold is sufficient. In conjunction with the felt presence of offspring in the mother's mouth, this internally-timed reduction in appetite serves to totally inhibit food consumption [Mrowka, 1984].

Metabolism and its regulation

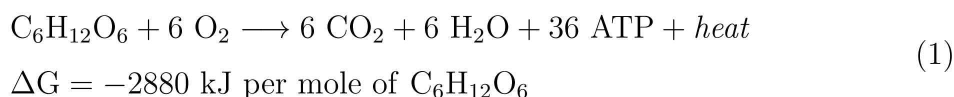
Like everything else in the physical universe, life itself is subject to the laws of thermodynamics. Energy cannot be created or destroyed, says the first law, and since each animal cell requires energy in order to perform its specialized functions—the local contributions necessary for homeostasis more globally—an external source must provide it. The second law demands that the entropy, or disorder, of any closed system increase over time. Indeed, the very fact of life seems to contravene this law and could even be defined in opposition to it, as the constant creation of order out of disorder. This is because organisms are open systems, constantly exchanging matter and energy with their environment; thus, a living system is a dissipative system, maintaining its complexity by causing larger increases in the entropy of its environment. The maintenance of organismal order means constantly struggling against a tendency universally entropic; with luck, life persists under this Sysiphean arrangement long

enough to commit the terms of its persistence (the organism's genome) to posterity. For this to happen, food intake ultimately supplies an energy source, in the form of chemical-bond energy, to each cell, and the process of eating must itself be regulated to maintain a favorable energy balance over time.

Cellular respiration

Life is private chemistry inside a lipid shell; since metabolism is the set of life-sustaining reactions that happen within a living organism's cells [Sherwood et al., 2005], it is clear that the two are intimately bound. The word metabolism is derived from the Greek $\mu\epsilon\tau\alpha\beta o\lambda\acute{\eta}$ – "metabolē", meaning "change", and it is apt insofar as the many processes it comprises are organized into metabolic pathways where one chemical is transformed into another by a series of steps and a sequence of enzymes. It is commonly divided into two categories: catabolism, or the energy-releasing reactions that degrade larger molecules into smaller units, and anabolism, the energy-requiring reactions that synthesize larger molecules from smaller units. The energy transfer linking catabolism and anabolism is mediated by adenosine-5-triphosphate (ATP), a nucleoside triphosphate coenzyme that functions as the universal unit of energy-currency when exchanged intracellularly; it is one of the end products of cellular respiration and is used by enzymes and structural proteins in many cellular processes, including biosynthesis, cell division, and motility.

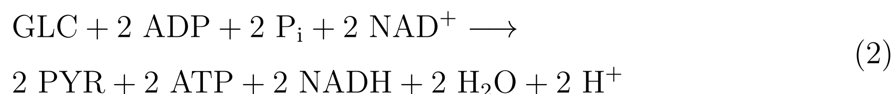
All living cells must cary out cellular respiration; this term refers to the set of catabolic processes whereby biochemical energy from nutrient substrates is transduced into cellularly-useful energy-rich compounds like ATP through a series of oxidation-reduction reactions. In eukaryotic cells, two types of cellular respiration occur. In aerobic respiration, O_2 acts as the oxidizing agent, accepting electrons and generating both carbon dioxide and water as waste byproducts; anaerobic respiration, a far less energy-efficient process more properly known as fermentation, occurs in the absence of oxygen, relies on substrate-level phosphorylation, and generates various other waste byproducts. More emphasis will here be placed on the former, which requires an appreciation of its basic biochemical processes. A very basic illustration of aerobic respiration is the canonical breakdown of glucose,



$$\begin{aligned} & C_6H_{12}O_6 + 6\ O_2 \longrightarrow 6\ CO_2 + 6\ H_2O + 36\ \text{ATP} + \textit{heat} \\ & \Delta G = -2880 \text{ kJ per mole of } C_6H_{12}O_6 \end{aligned} \tag{1}$$

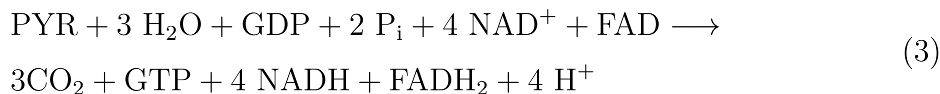
Glucose is the preferred substrate for glycolysis, the first of three metabolic

processes—glycolysis, the citric acid cycle, and oxidative phosphorylation—generally considered to fall under the compass of cellular respiration. Glycolysis, as its name suggests, refers to reactions that break glucose down to form pyruvate. The most common type of glycolysis is the Embden-Meyerhof-Parnas (EMP) pathway; while other pathways exist as well, the discussion here will be limited to the EMP pathway for brevity and clarity. In glycolysis, for every molecule of glucose, two molecules of pyruvate are formed, along with two of ATP, two of reduced nicotinamide adenine dinucleotide (NADH), two of water, and two protons [Sherwood et al., 2005]; this process occurs in the cytosol of the cell and does not itself require O_2. The stoichiometry of this reaction is given by:



$$\begin{aligned} &\mathrm{GLC} + 2\ \mathrm{ADP} + 2\ \mathrm{P_i} + 2\ \mathrm{NAD^+} \longrightarrow \\ &2\ \mathrm{PYR} + 2\ \mathrm{ATP} + 2\ \mathrm{NADH} + 2\ \mathrm{H_2O} + 2\ \mathrm{H^+} \end{aligned} \tag{2}$$

The citric acid cycle, also called the Krebs cycle or the tricarboxylic acid (TCA) cycle, is the second stage of cellular respiration, and in all eukaryotic cells it occurs within the mitochondrial matrix. It plays a central role in the catabolism of nutrients like glucose and other sugars, fatty acids, and some amino acids. Before these nutrients can enter the citric acid cycle, they must first be degraded into a two-carbon molecule called acetyl coenzyme A (acetyl-CoA). For example, after glycolysis, this occurs via pyruvate decarboxylation whereby a mitochondial enzyme complex oxidizes pyruvate to acetyl-CoA, producing NADH and CO_2 in the process. At this point, acetyl-CoA can enter the citric acid cycle, which consists of eight steps each catalyzed by a different enzyme (Figure 3). The final step of each 'cycle' regenerates oxaloacetate (OAA) and forms two molecules of CO_2. The stoichiometry of this reaction is given by:



$$\begin{aligned} &\mathrm{PYR} + 3\ \mathrm{H_2O} + \mathrm{GDP} + 2\ \mathrm{P_i} + 4\ \mathrm{NAD^+} + \mathrm{FAD} \longrightarrow \\ &3\mathrm{CO_2} + \mathrm{GTP} + 4\ \mathrm{NADH} + \mathrm{FADH_2} + 4\ \mathrm{H^+} \end{aligned} \tag{3}$$

Note that ATP is produced only once, in step 5. Most of the energy generated by the citric acid cycle is stored in NADH and reduced flavin adenine dinucleotide ($FADH_2$), to be released upon oxidation. So much energy is stored in these molecules, however, that it cannot be released all at once; instead, a series of reactions transfers electrons from an NADH to an electron acceptor (an enzyme complex), which then

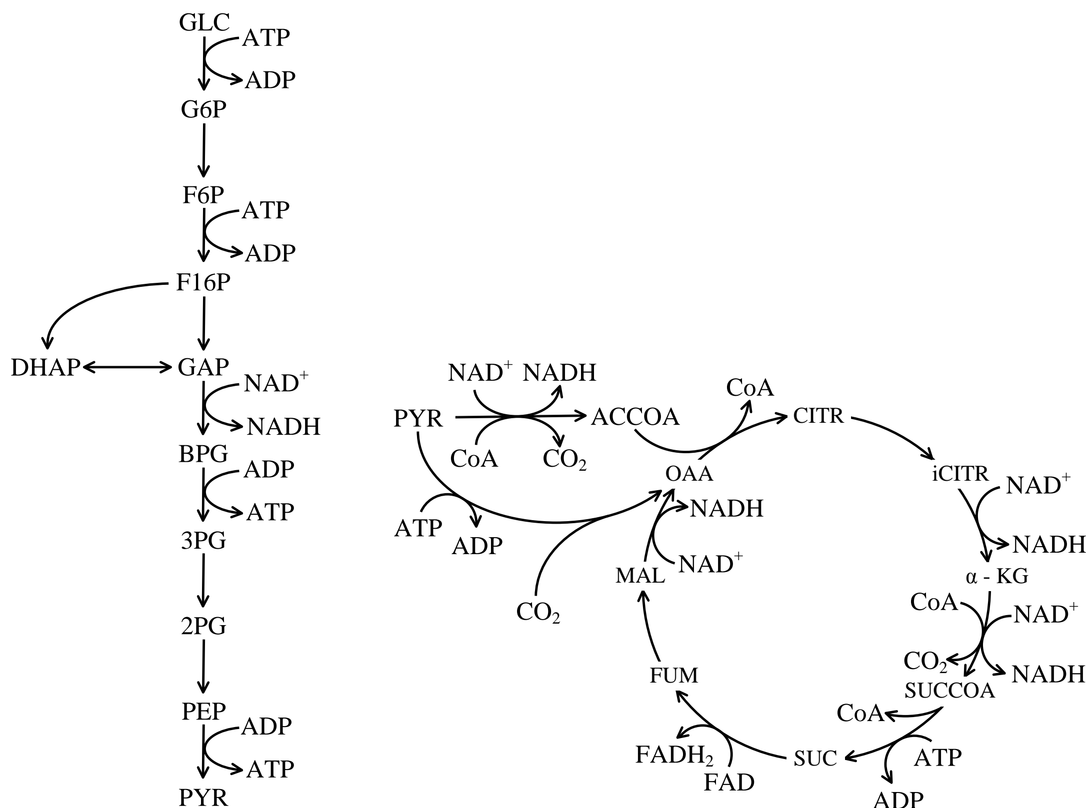


Figure 3. Glycolysis and the citric acid cycle. EMP glycolysis (left) and the citric acid cycle (right). In the EMP pathway, two phosphorylation reactions lead to the production of fructose-1,6-diphosphate (F6P) from glucose; then glyceraldehyde-3-phosphate (GAP) and dihydroxy-acetone phosphate (DHAP) are formed from F6P by an aldolase catalyzed reaction. An oxidation reaction converts GAP into 1,3-biphosphoglycerate (BPG); finally, conversions into 3-phosphoglycerate (PG) and phosphoenolpyruvate (PEP) yield pyruvate (PYR) as a final product. After PYR is converted to acetyl-CoA (ACCOA) through pyruvate decarboxylation, ACCOA enters the citric acid cycle. The eight steps of this cycle, clockwise from ACCOA, are as follows: (1) ACCOA reacts with the self-regenerative oxaloacetate (OAA), releasing coenzyme A (CoA) and forming citrate (CITR); (2) CITR is then rearranged, forming isocitrate (iCITR); (3) iCITR is decarboxylated and oxidized to form alpha-ketaglutarate (α-KG); (4) α-KG, in turn, is decarboxylated and oxidized to form succinyl CoA (SUCCOA); (5) SUCCOA is enzymatically converted to succinate (SUC); (6) SUC is oxidized to fumarate (FUM); (7) FUM is hydrated to malate (MAL); (8) MAL is oxidized to OAA, completing the cycle. Figure created by the author using open-source LatexDraw software

pass from other donors on to increasingly more electronegative electron acceptors until they make their way to O_2, the terminal electron acceptor in the chain.

The transfer of electrons down this gradient releases energy which drives proton pumps, creating a proton gradient across the inner mitochondrial membrane. The proton gradient is key, because it couples the respiratory chain to the process of oxida-

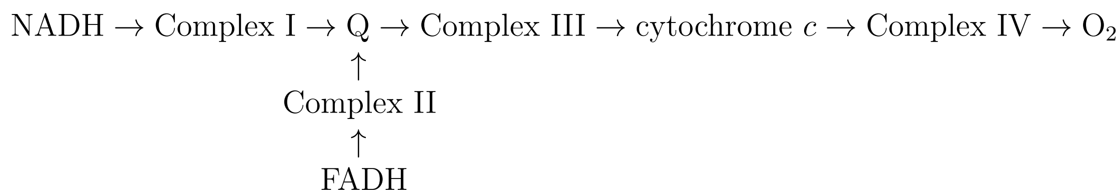


Figure 4. The respiratory chain. There are four membrane-bound enzyme complexes that receive electrons, each more electronegative than the next. In order, they are NADH dehydrogenase (Complex I); succinate dehydrogenase (Complex II); cytochrome bc_1 complex (Complex III); and cytochrome *c* oxidase (Complex IV). Complexes I, III, and IV are proton pumps. O_2 is the terminal electron acceptor. The electron donors are, in order, NADH; FADH (with electrons from succinate, not shown); ubiquinone (Q); and cytochrome *c*.

tive phosphorylation; ATP synthase allows proton flux back into the mitochondrion to drive the production of ATP. Ideally, this process results in the production of 34 ATP molecules per glucose molecule oxidized. Adding in the two ATP molecules from glycolysis of a single glucose molecule (2 pyruvate), and the two from the production of succinate during two complete turns of the citric acid cycle, the textbook-perfect total is 38 ATP. This maximum yield is never quite reached for a number of reasons, and current estimates range around 29 to 30 ATP per glucose molecule [Rich, 2003].

The biological universality of metabolism is striking—the basic metabolic pathways and their components are similar between even vastly different species [Pace, 2001]. The intermediates of the citric acid cycle are present in all known organisms, from unicellular bacteria to the quite-multicellular elephant [Smith and Morowitz, 2004]. The three pathways outlined above—EMP glycolysis, the citric acid cycle, and the respiratory chain—are perhaps the most important chemical mechanisms evolution has ever produced; a device for nutrient degradation; a device for oxidizing acetate to yield reduction equivalents; a device that makes these reduction equivalents move protons around, exploiting the constraints of ion transport and the electronegativity of environmental O_2 to create ATP. These pathways emerged during the origin of life, and unlike many other creature features, evolution's solution appears here to have been the best possible design from the very beginning [MelendezHevia et al., 1996].

Metabolic regulation

There are many hormonal and neurochemical signals that control the processes of metabolism, from the major, including cortisol, glucagon, and insulin, to the more

minor; the cytokines, orexin, and melatonin, many of which are discussed later. The speed of metabolism—the metabolic rate—is the rate at which energy is expended; it varies depending on many factors, including genetics, temperature, activity level, food intake, illness, and anxiety [Sherwood et al., 2005].

Basal metabolic rate (BMR) and postprandial thermogenesis (PPT)

Basal metabolic rate (BMR) is a concept that arose from the need to standardize measurements of metabolism so that accurate comparisons could be made between individuals; historically, the term has seen much redefinition in the hands of different researchers. These definitions range from the ideally conceptual to the rigorously operational; among the most succinct is "the minimal rate of energy expenditure compatible with life" [Mitchell, 1964], while experimentally strict definitions stipulate that BMR is the rate resulting from precisely controlled conditions, requiring subjects to be in a thermoneutral environment (22-26 °C), completely rested, fully awake, lying down, fasted for at least 10-12 hours, and free from emotional stress. If these conditions are met, the result is considered to represent the energy expenditure due to the basal life processes within the body of the organism in question.

Regardless of the precise definition, the idea remains the same; for the purposes of this document, basal metabolic rate is the rate of energy metabolism an organism needs to sustain in order to preserve the integrity of vital functions under conditions which as far as possible avoid the influence of the external environment [Henry, 2005]. In humans, between 50-80% of total energy expenditure is devoted to basal metabolic processes, demonstrating that even the basic demands of life are quite high [Weininger, 2012]. The cold-blooded counterpart to BMR, standard metabolic rate (SMR), is the metabolic rate of a resting, fasting, and non-stressed ectotherm at a particular temperature [Sherwood et al., 2005].

On top of BMR, and in addition to variations occasioned by short-term and long-term patterns of food intake, a phenomenon known as postprandial thermogenesis (PPT) causes a sustained rise in metabolism for several hours after eating. There are two components of PPT: obligatory PPT, resulting from the increased metabolic activity associated with the costs of obtaining and processing food, including gut motility and secretions, nutrient uptake, the biosynthesis of proteins, glycogen, and lipids, and the excretion of wastes; and regulatory PPT, manifested by increased heat production after a meal, largely for the purpose of removing excess nutrients. Obligatory PPT is known to occur in all animals; regulatory PPT is a known feature of all endotherms and may occur in other thermoregulatory modes as well. Recent

studies on PPT are beginning to elucidate the physiological processes that "burn off" excess calories. This has been found to occur through the activation of uncoupling proteins (UCPs) in some tissues. In bioenergetics, uncoupling refers to any process in which energy released from substrate catabolism in the mitochondria is not conserved. The last steps in the oxidation of substrate are the transfer of electrons from NADH and succinate, through the respiratory chain to oxygen, forming water. As discussed above, the energy here released is used by membrane-bound enzyme complexes to pump protons out of the mitochondrion; in most mitochondria, the majority of these reenter through ATP synthase. UCPs are proteins that function to allow the re-entry of protons by other means, releasing energy as heat instead of driving the ATP synthase [Nedergaard et al., 2005]. In mice, brown adipose tissue (BAT) is activated after a large meal and converts available lipid energy into heat using UCP-1, and in humans, white adipose tissue and skeletal muscle—containing UCP-2 and UCP-3—appear to be used to similar effect [Sherwood et al., 2005].

Measuring metabolism

Metabolic rate is the rate at which energy is expended; thanks to entropy, none of the energy transformations described above are completely efficient, and most energy expenditure in animals eventually dissipates as heat. As such, metabolism is most accurately measured in terms of an organism's heat production over time. Measuring the rate of heat production directly can be quite difficult in practice; fortunately, there exist other methods of reliably determining an organism's metabolic rate—techniques collectively known as indirect calorimetry.

A widely used method of indirect calorimetry is respirometry, which measures the organism's oxygen uptake per unit time. Food energy is liberated with the use of O_2 during aerobic respiration, so a direct relationship exists between the volume of O_2 used and the quantity of heat produced. This relationship also depends on the type of nutrients that are being oxidized, since fats, carbohydrates, and proteins have different calorie-to-gram ratios. In the absence of this information, respirometrically measured metabolic rates are reported either as liters of oxygen consumed per unit time—a reasonable approximation of heat production—or by using the ratio of CO_2 produced to O_2 consumed, a value known as the respiratory quotient (RQ),

$$\mathrm{RQ} = \left(\frac{\mathrm{CO_2}_{\text{ Produced}}}{\mathrm{O_2}_{\text{ Consumed}}} \right) \tag{4}$$

The RQ varies depending on the viand consumed. When a carbohydrate is being

used, the RQ is 1; that is, for every molecule of O_2 consumed, one molecule of CO2 is produced (see Equation 1). This ratio differs when other nutrients are used as metabolic substrates—for fat use, the RQ is 0.7; for protein, RQ is 0.8. As metabolic rate is scaled to body mass, the mass of the organism concerned must be considered as well.

Regulating metabolism

It has long been assumed that energy balance is homeostatically regulated; for instance, though day-to-day variations in food intake and energy expenditure can be dramatic for animals in the wild, most healthy mammals maintain a stable body weight throughout their adult lives [Coll et al., 2007]. This presupposes a homeostatic mechanism that exists to maintain a long-term balance between energy intake and expenditure at a set-point favorable to prevailing conditions. By the law of conservation of energy, necessity compels that the sum of energy inputs equal the sum of energy outputs for a given system, animal or otherwise:

$$\sum \mathrm{E}_{\mathrm{input}} = \sum \mathrm{E}_{\mathrm{output}} \tag{5}$$

This balance can only be maintained at a constant level by regulating the terms on either side; an exploded view of this equation, revealing the factors contributing to either side, is often called the animal energy equation:

$$\mathrm{E}_{\mathrm{intake}} - \mathrm{E}_{\mathrm{loss}} = \mathrm{E}_{\mathrm{BMR}} + \mathrm{E}_{\mathrm{activity}} + \mathrm{E}_{\mathrm{production}} + \mathrm{E}_{\mathrm{PPT}} \tag{6}$$

Term by term, intake is the energy consumed; loss is the energy lost to feces, urine, skin sloughing, and the like; activity is the energetic cost of neuromuscular efforts above BMR; production encompasses energy storage (e.g., in adipose tissue), the energetic requirements of growth during development, and the energy used for reproduction; PPT is the energy associated with postprandial thermogenesis.

For all this regulatory potential, animal energy balance has three main outcomes:

1. Energy balance can be neutral, ($\sum E_{input} = \sum E_{output}$) so net energy intake ($E_{input} - E_{loss}$) exactly equals the amount of energy expended.

2. Energy balance can be positive, ($\sum E_{input} > \sum E_{output}$), so net energy intake exceeds the amount of energy expended.

3. Energy balance can be negative ($\sum E_{input} < \sum E_{output}$), so net energy intake is

less than the body's immediate energy requirements.

In the neutral case, body mass remains constant. In the case of positive energy balance, extra energy consumed is not used immediately but stored (in the $E_{production}$ term of Equation 6), and body mass increases. In the case of negative energy balance, the animal must use stored energy, causing a decrease in body mass.

The body's energy balance can only be maintained at a constant level by regulating the components of Equation 6 [Sherwood et al., 2005]:

- Regulation of BMR or activity level in response to decreased food intake:

  ($\downarrow$ feeding $\rightarrow \downarrow$ BMR or $\downarrow$ activity)

- Regulation of BMR or PPT in response to increased food intake:

  ($\uparrow$ feeding $\rightarrow \uparrow$ BMR or $\uparrow$ PPT)

- Regulation of activity level in response to increased food intake:

  ($\uparrow$ feeding $\rightarrow \uparrow$ activity)

- Regulation of food intake in response to any change in energy expenditure:

  ($\updownarrow$ BMR or $\updownarrow$ activity or $\updownarrow$ production or $\updownarrow$ PPT $\rightarrow \updownarrow$ feeding)

The hypothalamus

The primary region responsible for regulating metabolism is the hypothalamus. This small, cone shaped structure is located on the diencephalon ventral to the thalamus where it forms the floor of the third cerebral ventricle [Utiger, 2012] (see Figure 5). Its most ventral aspect terminates in the infundibular stalk, a tube through which it makes both nervous and chemical connections to the pituitary gland, effectively linking the central nervous system (CNS) and the endocrine system. The CNS comprises the richly interconnected network of neural circuitry within the brain and spinal cord, wherein timely secretions of specific neurotransmitters and neuropeptides serve to organize and direct the function of the system as a whole. In this way, the connection of the hypothalamus to many other parts of the CNS allows signals from these regions, as well as signals from the periphery, to be funneled to it, and through it, on to the pituitary. Then, from the pituitary gland to the endocrine system at large, these CNS signals are able to exert their effects throughout the body.

As the nexus of the CNS and the endocrine system, the hypothalamus has fingers in many homeostatic pies; its chief functions include:

- The regulation of visceral activity through control and integration of the autonomic nervous system (ANS). The ANS is part of the peripheral nervous system (PNS) and is responsible for the contraction of smooth and cardiac muscle, as well as for the secretions of many endocrine glands; among other things, the ANS controls the heart rate, the respiratory rate, digestion and gut motility, contractions of the urinary bladder, and sexual arousal through the complementary actions of its adrenergic-sympathetic and cholinergic-parasympathetic divisions.

- The regulation of distant target organs through the actions of its releasing hormones on the anterior pituitary. The main releasing hormones secreted by the hypothalamus are:

  - growth hormone-releasing hormone (GHRH), responsible for the secretion of growth hormone (GH), which in turn stimulates growth, cell reproduction, and regeneration.
  - corticotropin-releasing hormone (CRH), an important component of the biological stress response which stimulates the production and release of corticosteroids, such as cortisol, from the adrenal cortex.
  - thyrotropin-releasing hormone (TRH), which regulates metabolism, growth, and the rate of function of many body systems through the endocrine function of the thyroid.
  - gonadotropin-releasing hormone (GnRH), which stimulates the release of two hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which stimulate processes responsible for development, growth, and reproduction.

- The regulation of water-electrolyte balance and thirst, via osmoreceptors in the hypothalamus adjacent to the third ventricle, and the production of arginine vasopressin (AVP), also known as vasopressin or antidiuretic hormone (ADH).

- The regulation of energy balance, which has been discussed in some detail above, and of food intake, to which the entirety of the next section is devoted.

Feeding regulation

Regulation of food intake is the most important factor in the maintenance of energy balance and body weight over long periods of time. Feeding is regulated on a short-

term basis too, which helps to control meal size and frequency.

In teleost fishes as well as mammals, short-term feeding regulation depends on hunger and satiety signals effected in large part by physical stimuli, such as the distention of various epithelia throughout the alimentary canal caused by food, or, in the case of haplochromine mouthbrooding, by offspring [Konturek et al., 2004]. Meal-to-meal vicissitudes in circulating levels of peripheral hormones and peptides—the relative abundances of acute hunger or satiety factors—are crucial mediators of this short-term regulation. In contrast, certain peripheral signals provide tonic information about how the body's energy reserves and expenditures are changing over time. The regulation of feeding in the long-term is under the control of the central melanocortin system, a collection of CNS circuits which function like an appetitive rheostat, integrating both acute and tonic inputs from hormones, nutrients, and afferent neural inputs to produce stable patterns of ingestive behavior and energy expenditure [Cone, 2005]. Recent studies have demonstrated that feeding-related neuropeptide function and the projection pathways involved in energy homeostasis are generally conserved among vertebrates [Forlano and Cone, 2007]. Indeed, an increasing number of homologs to mammalian appetite-regulating peptides are being characterized in fishes and appear to have selfsame effects [Volkoff et al., 2005, Gorissen et al., 2006].

Eating habits are also shaped by psychological, social, and environmental factors. Feeding is influenced by pleasure and reward, and the amount of pleasure derived from eating can powerfully motivate consumption [Sherwood et al., 2005]. This is strikingly evidenced by a recent experiment in which rats were fed a 'cafeteria-style' diet of highly palatable energy-dense 'human' foods. These rats overate by as much as 70-80% and became obese [Johnson and Kenny, 2010]. Interestingly, the development of this obesity was coupled with a progressively worsening deficit in neural reward responses, evinced by the downregulation of striatal dopamine D2 receptors crucial to mesolimbic dopamine signaling. Similar changes in reward homeostasis are seen in human drug addiction, and are thought to trigger the transition from casual to compulsive drug use. However, when rats were returned to their regular, monotonous diet of nutritionally-balanced rat chow, their obesity was gradually reversed as their food intake came back under the control of physiological, rather than hedonic, drives [Sherwood et al., 2005]. Stress, anxiety, and boredom have also shown to alter feeding behavior independent of an animal's energy requirements [Sherwood et al., 2005].

Even closely related species can vary greatly in the body mass they actually maintain. This observation has led to the proposal than the hypothalamus can have

different species-specific set-points for energy balance that are presumed to be evolutionarily advantageous in a given environment [Sherwood et al., 2005]. For instance, chronically lean animals do better in habitats where food is plentiful; their limited fat storage results in greater agility and increased success in predation, or escape therefrom. Thus, tropical carnivores such as tigers are lean despite being sedentary much of the time. Contrastingly, a high-adiposity set-point is clearly advantageous in environments were food availability is less predictable or where food abundance is limited to certain times of the year. Hibernating mammals are excellent examples of this—an arctic ground squirrel must reach about 50% body fat before winter or it will not survive the duration of its eight-month hibernation [Sherwood et al., 2005]. A propensity toward obesity or leanness is not necessarily an abnormal physiological state; for many species, extremes in metabolic set-point may indicate evolutionary adaptation for a particular habitat.

In the vein of interspecies differences, and because the preponderance of research on feeding and metabolism makes use of mammalian systems, a comparison between fishes and mammals is necessary here. In their most basic form these regulatory mechanisms are common to vertebrates of all description, but remembering that the most recent ancestor common to mammals and ray-finned fishes lived during the early Silurian, some 440 million years ago [Nelson, 1994], physiological differences between taxonomic classes almost guarantee that the control of feeding behavior involves certain mechanisms specific to each. To give an example, energy metabolism in fishes is quite similar to that of mammals and birds; however, fishes are poikilothermic, not expending any energy on thermoregulation, and being totally aquatic they require less energy for the excretion of nitrogenous waste than terrestrial homeothermic vertebrates.

On account of these differences, fishes may respond to environmental factors differently than mammals, and may even be subject to certain influences that mammals are not. For instance, there is a direct relationship between water temperature and food consumption in fishes except at biologically extreme values, which tend to decrease food intake. Also, the amount of available O_2 dissolved in the water affects feeding in fishes, having distinct appetite-suppressive effects. Even photoperiod—the relative lengths of day and night—has been shown to affect feeding activity in a number of fish species [Volkoff et al., 2009].

What's more, fishes need not only to maintain their basal metabolic rate, but they experience a trade-off in resource allocation between growth and reproduction throughout their lifetime [Heino and Kaitala, 1999]. Unlike most mammals who

achieve a fixed size, the growth of many fish species is indeterminate and may continue throughout life. Additionally, there are anatomical dissimilarities between mammals and fishes that bear directly on the regulation of feeding, not the least of which are differences in brain and gut morphologies. While some structural differences are functionally analogous, just as lungs and gills both subserve gas exchange, there are some organs present in fishes, such as the caudal neurosecretory organ, that have no counterpart in mammals at all.

Feeding and the brain (Hungry? It's all in your head!)

In the 1950s, it was first proposed that circulating substances produced in proportion to stored fat could act to regulate body weight through the modulation of energy intake and expenditure [Kennedy, 1953]. Around this same time, it was shown that targeted lesions in certain regions of the hypothalamus resulted in hyperphagia and obesity, while lesioning in other sites caused severe anorexia. In one striking demonstration by [Hervey, 1959], a group of rats received lesions to the ventromedial nucleus (VMN) of the hypothalamus and were then parabiosed, or surgically conjoined, to unlesioned animals so that blood would flow between them. The VMN-damaged rats ate voraciously, rapidly putting on weight, while their experimentally-consanguineous counterparts refused food altogether. This anorexia persisted until the animals died unless they, too, were given VMN lesions, in which case they also began overeating and quickly became obese. Experiments such as this one were the first to provide substantive evidence that a blood-borne satiety factor, or factors, required an intact hypothalamus for its activity, and the VMN quickly earned a reputation as the brain's 'satiety center'. Soon thereafter, similar experiments hailed the discovery of the 'appetite center' when lesioning the lateral hypothalamus (LH) resulted in the suppression of food intake while electrically stimulating it elicited feeding behavior [Berridge and Valenstein, 1991]. But in the past two decades the reality of the situation has proven far more complex. Presently, there is still evidence implicating the VMN in the control of satiety [King, 2006], and the LH in control of hunger [Williams et al., 2000], but other hypothalamic nuclei are now known to play roles of equal, if not greater, importance.

Despite the profundity of these findings, it wasn't until some 40 years later that the molecules mediating these processes were identified [Coll et al., 2007]. In addition, these recent studies have revealed multiple highly integrated and even redundant pathways, crisscrossing into and out of the hypothalamus, that are involved in controlling food intake and maintaining energy balance [Sherwood et al., 2005]. As such,

researchers no longer refer to 'centers' for this or that aspect of feeding regulation. Instead, progress is focused on discrete neuronal circuits, both within and without the hypothalamus, which generate integrated responses to a variety of signals—nervous, hormonal, and otherwise—that reflect the body's changing energy conditions. Integration of multiple molecular signals in this way ensures that feeding behavior is synchronized with both the immediate and long-term needs of the organism.

The central melanocortin system

Hormones and neuropeptides originating from the central nervous system (CNS) as well as the periphery—from adipocytes, the gastrointestinal (GI) tract, the pancreas, &c.—influence energy intake and expenditure through their actions on the autonomic nervous system (ANS) and ultimately the central melanocortin system. This last, the central melanocortin system, is a collection of CNS circuits which integrate these various inputs and ultimately signal the output of behavioral and autonomic responses necessary to the maintenance of energy homeostasis [Cone, 2005, Coll et al., 2007]. This system comprises three main types of functionally interconnected CNS pathways:

1. Neurons originating in the arcuate nucleus (ARC) of the hypothalamus that express orexigenic and anorexigenic neuropeptides, stimulating or suppressing appetite, respectively;

2. neurons originating in the nucleus of the solitary tract (NTS) of the brainstem that express primarily anorexigenic neuropeptides, and

3. the downstream targets of 1 and 2 which express the melanocortin receptors (MCRs) [Cone, 2005].

Its primary role is the integration of tonic information about the state of nutrient stores in the body, received primarily by the hypothalamus, with more acute information pertaining to hunger and satiety received primarily by the brainstem.

In fishes, the central melanocortin system functions much as it does in mammals, having important roles in the regulation of body weight, energy homeostasis, and pigmentation [Millington, 2007]. Many studies of the melanocortins, MCRs, and associated neuropeptides in fishes have found them to have high-percentage identity with their mammalian orthologs and to be similarly conserved in important functional domains [Cerda-Reverter et al., 2003]. The key-player peptides of the central melanocortin system are detailed in the following sections.

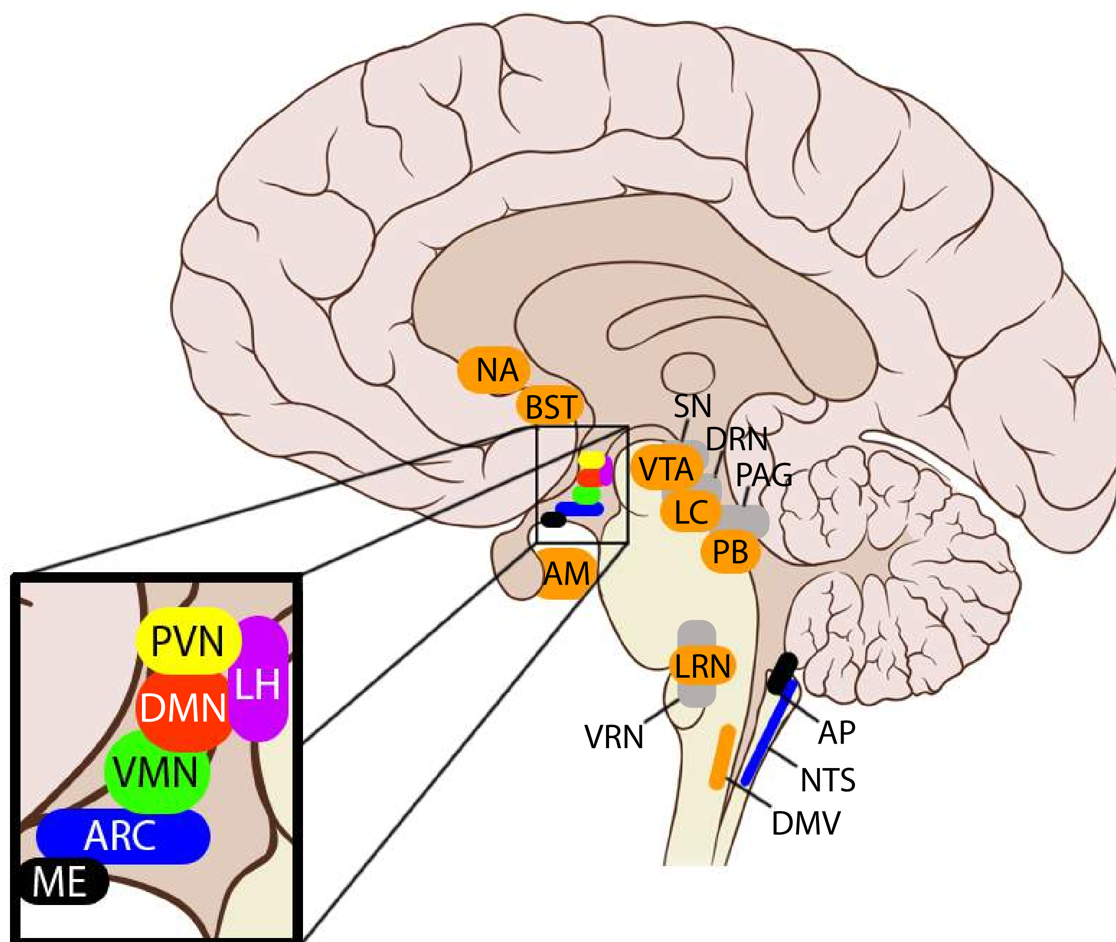


Figure 5. Hypothalamic nuclei and associated feeding regions. Cartoon of sagittal section of a human brain; all brain regions mentioned throughout this thesis are depicted. Hypothalamic and brainstem melanocortin regions—the arcuate nucleus (ARC) and the nucleus of the solitary tract (NTS), respectively—in blue. Hypothalamic nuclei in close-up: paraventricular nucleus (PVN), lateral hypothalamus (LH), dorsal medial nucleus (DMN), ventral medial nucleus (VMN), and the arcuate nucleus (ARC). Regions in orange are dually innervated by hypothalamus and brainstem melanocortin neurons: locus coeruleus (LC), parabrachial nucleus (PB), dorsal motor nucleus of the vagus (DMV), lateral reticular nucleus (LRN), bed nucleus of the stria terminalis (BST), nucleus accumbens (NA), and the ventral tegmental area (VTA). Regions characterized by an incomplete blood-brain-barrier in black: median eminence (ME) and area postrema (AP). Other regions, shown in gray: substantia nigra (SN), dorsal and ventral raphe nuclei (DRN, VRN), periaqueductal gray (PAG). Figure created by the author using Photoshop software (Adobe). Credit for background brain image: Patrick J. Lynch, medical illustrator, via wikimedia commons.

Hypothalamic division

Melanocortins

Since this group of neuropeptides is traditionally known for its importance in varying skin color, the pivotal role they have since been discovered to play in energy homeostasis is something of a surprise. The parent molecule of this group—the central melanocortin system's namesake—is a precursor polypeptide called pro-opiomelanocortin (POMC). Post-translational processing of this precursor produces two different classes of peptides, the melanocortins and β-endorphins, which have a host of dissimilar cellular functions. The melanocortin peptides include adrenocorticotropin (ACTH) and the α-, β-, and γ-melanocyte-stimulating hormones (MSHs), whose effects are mediated through five G protein-coupled melanocortin receptors (MC1R–MC5R).

The production of POMC occurs both peripherally and centrally—in the pituitary gland, the integument, and throughout the brain. In the periphery, pituitary-derived ACTH regulates secretion of glucocorticoids through adrenocorticotropin receptors (MC2Rs) in the adrenal cortex, while the MSHs regulate the production of certain pigments through MC1Rs. In the CNS, MSHs are agonists of MC3R and MC4R; the downstream consequences of stimulation at these sites are both catabolic and anorexigenic, resulting in decreased feeding behavior, increased lipolytic activity, and greater energy expenditure [Cerda-Reverter et al., 2003, Volkoff et al., 2005]. In the same way, obesity in mouse has been shown to result from the deletion or inactivation of genes encoding these receptors [Huszar et al., 1997], MC3R [Butler et al., 2000], or POMC [Yaswen et al., 1999]. Normal expression of functional POMC, MSHs, and MCRs is necessary to the regulation of satiety; any impairment thereof is likely to affect proper feeding. Importantly, the non POMC-derived neuropeptide agouti-related protein (AgRP, see pg. 24), is a potent, selective antagonist of these same MCRs, producing orexigenic effects that oppose those of POMC. Many CNS sites rich in the melanocortin receptors MC3R and MC4R are dually innervated by both agonistic-POMC and antagonistic-AgRP neurons, illustrating the potential of this system for signal integration.

Melancortins and their receptors have been identified in many fish species, including the familiar POMC-derived MSH and ACTH, the melanocortin receptors, and two endogenous antagonists of these receptors, AgRP and the agouti-signaling peptide (ASP) (see below). As in mammals, pigmentation is mediated through the MC1R, while MC4Rs are known to be integral to the central regulation of fish food intake and energy balance [Metz et al., 2006]. Cerda-Reverter et al. (2003) showed

that in goldfish (*Carassius auratus*), central administration of MC4R agonists inhibits food intake, while central administration of MC4R antagonists stimulates food intake. However, fasting in goldfish and zebrafish (*Danio rerio*) does not appear to significantly alter hypothalamic POMC mRNA, suggesting that this melanocortin precursor may not have direct actions [Cerda-Reverter et al., 2003]. In another goldfish study, central injections of α-MSH were shown to inhibit feeding, and CRH-signaling has been adduced as a mediating agent of these anorexigenic actions [Matsuda et al., 2008]. Notably, α-MSH and AgRP cells in the ventral PVN are more pronounced at five days post-fertilization, when zebrafish fry first begin to eat, further implicating these peptides in feeding behavior [Forlano and Cone, 2007]. An important difference though, is that whereas in mammals MC4Rs are expressed only in the CNS, fishes are also known to express these receptors peripherally [Takahashi and Kawauchi, 2006]. In both mammals and fishes, the major site of POMC expression in the CNS is the arcuate nucleus (ARC) of the hypothalamus. Typically, these POMC neurons also express another anorexigenic peptide, the cocaine- and amphetamine-related transcript (CART).

Cocaine- and amphetamine-regulated transcript (CART)

As its name suggests, the CART neuropeptide was originally identified as a gene-product found to be upregulated in the rat brain following acute administration of psychomotor stimulants. This gene, *CARTPT*, sees expression both centrally and peripherally; CART peptides are abundant, with particularly high concentrations in the VTA, hypothalamus, gut, and pancreas [Ekblad, 2006, Vicentic and Jones, 2007]. CART is strongly anorexigenic, inhibiting feeding in mammals. Additionally, CART modulates the mesolimbic dopamine system, affecting food- and drug-related reward and reinforcing behaviors [Vicentic and Jones, 2007] (see pg. 16). The putative receptor target for CART has not yet been identified, and its signalling pathways remain to be fully characterized; however, in vitro studies strongly suggest that CART binds to a specific G protein-coupled receptor (GPCR) which is itself coupled to Gi/o—a G protein subunit that inhibits the production of cAMP—resulting in extracellular signal-related kinase (ERK) activation [Lakatos et al., 2005].

In fishes, CART mRNA is present both in the brain and in peripheral tissues including the kidneys and gonads. Central injection of human CART inhibits feeding in goldfish and CART mRNA levels decline following a period of fasting in several species, attesting to an anorexigenic role for CART in fishes [Volkoff and Peter, 2000].

CART is known to interact with other feeding regulators. For instance, human CART has been shown to inhibit both NPY- (see below) and OX-A- (see pg. 40) stimulated feeding in goldfish, providing evidence for an inhibitory action of CART on both NPY and OX-A systems [Volkoff and Peter, 2000]. Indeed, immunoreactivity studies have revealed CART and NPY axons to be closely associated in the catfish brain (*Ictalurus punctatus*), much as they are in mammals [Singru et al., 2008].

POMC/CART neurons

The ARC contains two major subsets of neurons alike in their importance to feeding regulation, but opposing in their effects thereon. The first of these neurons, the POMC/CART neuron, is addressed here. The POMC/CART cells of the ARC project all throughout the hypothalamus, but particularly dense fibers are sent to the lateral hypothalamic (LH), paraventricular (PVN), and prefornical (PF) nuclei [Sherwood et al., 2005]. The LH, the site of the classical appetite center, produces neuropeptides known as orexins (OXs), which, true to their name, increase appetite (see below). In contrast, the PVN primarily releases anorexigenic factors, such as corticotropin-releasing hormone (CRH). Leaving the hypothalamus, dense projections run rostrally from POMC/CART cells in the ARC to the the bed nucleus of the stria terminalis (BST) as well as to the nucleus accumbens of the striatum. More caudally, projections extend to the amygdala and the periventricular region of the thalamus. Finally, two large POMC/CART-expressing neuron bundles are sent from the hypothalamus to the brainstem; one passes through the periaqueductal gray and dorsomedial tegmentum to innervate the nucleus of the solitary tract (NTS) and the lateral reticular nucleus, while the other runs through the ventral tegmental area to innervate the NTS, the ventrolateral medulla, and the spinal cord (Figure 5).

As mentioned above, the POMC/CART neuron, which produces anorexigenic effects by expressing agonists of MC3R and MC4R, has an important functional counterpart in the central melanocortin system—neurons that coexpress neuropeptide Y (NPY) and AgRP, two strongly orexigenic neurotransmitters.

Neuropeptide Y (NPY) and related peptides

The neuropeptide Y (NPY) family of neuropeptides comprises NPY, peptide YY (PYY), pancreatic polypeptide (PP), and peptide Y (PY). NPY and PYY are found in all vertebrates, while PP is found only in the pancreas of tetrapods and PY is found only in some teleosts [Hoyle, 1999]. NPY is perhaps the most potent appetite stimula-

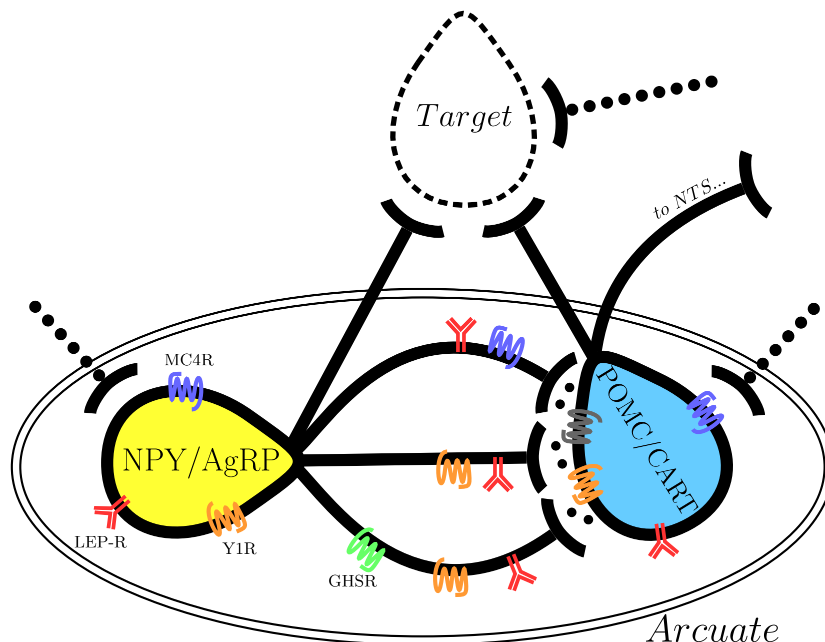


Figure 6. Diagram of POMC/CART and NPY/AgRP neurons. Schematic of the melanocortin system: POMC/CART and NPY/AgRP neurons in the ARC (blue and yellow, respectively). Solid arrows indicate direct projections; dotted arrows indicate secondary projections linking cells in the hypothalamus and brainstem to common effector sites. Receptors for MSHs, NPY, Ghrelin, and Leptin are MC4R (purple, GPCR); Y1R (orange, GPCR); GHSR (green, GPCR); and LEP-R (red, cytokine receptor), respectively. Gray colored receptor signifies generic receptor. Black dots at the end of terminals show neurotransmitter release, specifically NPY, GABA, and AgRP, onto the POMC/CART neuron. Target site could be any number of sites within or without the hypothalamus (Figure 5), including the LH and PVN. Note that POMC/CART neurons send direct projections to brainstem regions including the nucleus of the solitary tract (BST). Figure created by the author using open-source LatexDraw software.

tor yet discovered. Central administration of NPY stimulates feeding in mammals and the ablation of NPY neurons induces anorexia. During nutritional duress—starvation or low blood-glucose—NPY expression is upregulated [Volkoff et al., 2005]. NPY has also been shown to lower energy expenditure by suppressing outflow from the sympathetic division of the ANS, thus favoring more basal metabolic processes. NPY has five known receptors in mammals (Y1, Y2, Y4-Y6) [Kamiji and Inui, 2007]. It is abundant in the CNS, particularly in hypothalamic nuclei involved in the regulation of feeding such as the ARC, and again, it is one of the most potent orexigenic peptides known. Its orexigenic effects are mediated by Y1 and Y5 receptor subtypes [Gao and Horvath, 2007].

In fishes NPY is produced universally, and seven receptors for this ligand have

been discovered (Y1, Y2, Y4-Y8). However, while central administration of either mammalian or fish NPY increases feeding in fishes, there is uncertainty as to which of these receptor subtypes is mediating this behavior [Cerda-Reverter and Larhammar, 2000, Salaneck et al., 2008]. It has been recently demonstrated that brain NPY mRNA levels increase following fasting in tilapia [Peddu et al., 2009], further corroborating the role of this peptide in feeding regulation. Finally, NPY is known to interact with many other appetitive signals, such as CRF and glucocorticoids [Bernier et al., 2004], CART [Volkoff and Peter, 2001], leptin [Volkoff et al., 2003], melanin-concentrating hormone [Matsuda et al., 2008], orexins and galanin [Volkoff and Peter, 2001], growth hormone [Mazumdar et al., 2006], and ghrelin [Miura et al., 2006].

Agouti-related protein (AgRP)

Agouti-related protein is a orexigenic neuropeptide whose production in the brain is limited to the NPY-containing neurons of the arcuate nucleus. Indeed, as stated above, AgRP is co-expressed with NPY and acts as a highly specific antagonist of MC3R and MC4R, opposing the anorexigenic effects of POMC and CART. As a result, feeding increases under conditions of high AgRP expression. In mammalian models, fasting has been found to upregulate the expression of AgRP 5- to 10-fold [Cone, 2005], and transgenic overexpression of AgRP in mouse leads to pronounced obesity [Ollmann et al., 1997]. Indeed, humans suffering from anorexia nervosa have demonstrably higher levels of plasma AgRP than controls [Moriya et al., 2006].

AgRP has a demonstrably orexigenic effects in fishes. Transgenic zebrafish overexpressing AgRP exhibit marked obesity, adipocyte hypertrophy, and increases in growth [Song and Cone, 2007]. Also, in both goldfish and zebrafish, fasting has been shown to upregulate hypothalamic AgRP mRNA levels. A related peptide, Agouti-signaling protein (ASP) has been identified in fishes; it appears to be an inverse agonist at MC1R and MC4R, also acting as a melanization inhibition factor, but its role in regulating feeding in fishes is not yet known [Cerda-Reverter et al., 2005].

NPY/AgRP neurons

The second of the major feeding-regulatory neurons of the ARC is the NPY/AgRP neuron. Mirroring the location and projections of POMC/CART neurons, NPY/AgRP neurons primarily originate in the ARC and send fibers to many of the same hypothalamic and septal regions. In essence, the ratio of orexigenic NPY/AgRP to anorexigenic POMC/CART innervations determines the local activity of the hypotha-

lamic melanocortin system [Song and Cone, 2007]. The densest of the NPY/AgRP fibers innervate the PVN and the dorsomedial nucleus (DMN), while fibers are notably absent from brainstem, amygdalar, and striate regions. It is also noteworthy that the relationship between NPY/AgRP neurons and POMC/CART neurons is markedly unilateral—POMC/CART neurons only minimally affect NPY/AgRP neurons, if they do at all, while there is great regulatory potential for NPY/AgRP neurons on POMC/CART neurons (Figure 6).

The location of the ARC is noteworthy—it is positioned between the third ventricle and the median eminence (ME), an area with an incomplete blood-brain-barrier in which the portal vascular system transports releasing factors to the anterior pituitary. Arcuate POMC/CART and NPY/AgRP neurons have been shown to project to the ME, and the ME in turn may contribute to the sensing and response by these neurons to relevant hormones and nutrients in the blood [Cone, 2005].

Brainstem division

While the hypothalamus is the site of signal integration, maintaining energy balance and long-term control of food intake, the brainstem itself plays a crucial role in both the detection of and the response to hunger and satiety signals. Its own melanocortin signaling is remarkably well coordinated with that of the hypothalamic melanocortin system; like the ARC, the nucleus of the solitary tract (NTS) itself contains many POMC/CART-expressing neurons and these fibers project to many regions that are also innervated by hypothalamic POMC neurons [Palkovits et al., 1987]. These regions of dual innervation include the locus coeruleus (LC), the parabrachial nucleus (PB), the dorsal motor nucleus of the vagus (DMV), and the lateral reticular nucleus. In addition, levels of MC4R expression are particularly high in the DMV, NTS, and PB; the NTS is known to have dense neural projections extending directly to mediobasal hypothalamic nuclei, including the ARC [Kishi et al., 2003].

The NTS, which runs along the length of the medulla, is the primary site for innervation by vagus-nerve afferents from the GI tract [Schwartz, 2000]. These vagal afferents transmit information about three main stimuli to the NTS—the chemical contents of food in the lumen, the physical distention of GI epithelia due to presence or absence of food, and the neurotransmitters released by the stomach and the duodenum—which are important in the control of the initiation and cessation of eating [Sherwood et al., 2005]. Just ventral to the NTS lies the primary site of motor efferents to the GI tract, the DMV. This site is densely innervated by fibers from

the NTS, and together these two nuclei constitute the dorsal vagal complex (DVC), which coordinates responses to many GI stimuli. As with the POMC/CART and NPY/AgRP cell bodies in the ARC, the brainstem POMC cell bodies are positioned adjacent to a circumventricular organ, the area postrema (AP), implying that it too may be directly sampling the blood and cerebrospinal fluid.

Peripheral afferents to the melanocortin system

The central melanocortin system, with its hypothalamic and brainstem divisions, provides an ideal neural substrate for the integration of peripheral metabolic, endocrine, and neuronal signals; these include long-term metabolic signals, relaying information about the state of the body's energy reserves, as well as short-term hunger and satiety signals triggered by ingestive status. Peripheral hormones, for example, convey information to the central melanocortin system either via the vagus nerve or by crossing the blood-brain barrier to act directly on the brain [Brightman and Broadwell, 1976]. This signaling is modulated by other variables as well, including ontogeny, reproductive status, and environmental conditions. What follows is an exhaustive survey of these signals and their respective roles in the physiological processes that control food intake, each beginning with an overview of mammalian signaling—about which far more is known—and ending, where applicable, with a detailed consideration of fishes.

Adipostatic signals

In vertebrates, the ability to store a sufficient quantity of energy-rich triglycerides in adipose tissue allows survival during the frequent periods of food privation encountered throughout a lifetime in the wild. However, too much of this tissue can easily be maladaptive, as in the case of obesity. Hormones secreted by adipose tissue—particularly a hormone called leptin—are long-term signals integral to the regulation of energy balance.

Leptin

The original blood-borne molecular satiety signal—the one hinted at by pioneering research in the middle of the 20th century—was also the first to be identified and described in the mid 1990s [Sherwood et al., 2005]. This signal is leptin, from the Greek $\lambda\epsilon\pi\tau\bar{o}\sigma$, "leptos", meaning 'thin', an anorexigenic hormone produced largely, if not exclusively, in adipose tissue [Zhang et al., 1994].

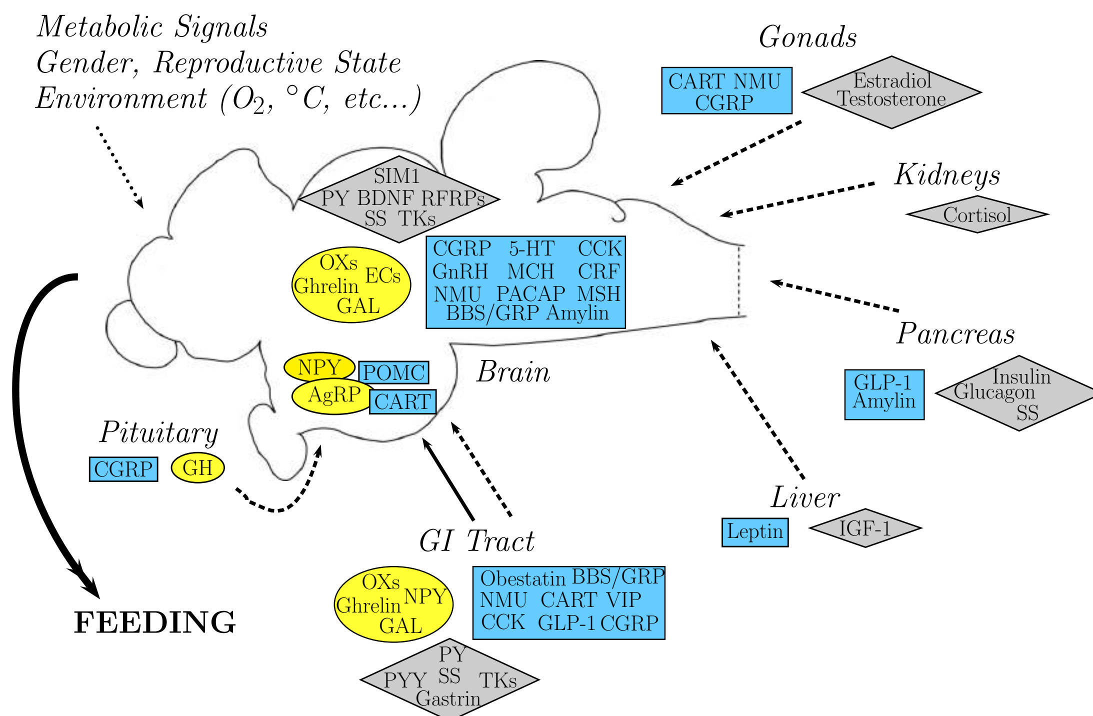


Figure 7. Summary of factors relevant to feeding in fishes. Diagram catalogs the staggering array of hormones, neuropeptides and other factors involved. Factors in yellow ovals are orexigenic; factors in blue rectangles are anorexigenic; factors in gray diamonds are thought to have a role in fish feeding, but which remains unclear. The small solid arrow indications a nervous connection; small dashed arrows indicate blood connection; the small dotted arrow indicates a factor neither peptidal nor hormonal. Orexigenic factors abbreviated: growth hormone (GH), neuropeptide Y (NPY), agouti-related protein (AgRP), orexins (OXs), endocannabinoids (ECs), galanin (GAL); anorexigenic factors abbreviated: calcitonin gene-related peptide (CGRP), pro-opiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), serotonin (5-HT), cholecystokinin (CCK), gonadotropin-releasing hormone (GnRH), melanocyte-concentrating hormone (MCH), corticotropin-releasing hormone (CRF), neuromedin U (NMU), pituitary adenylate cyclase-activaing peptide (PACAP), melanocyte stimulating hormone (MSH), bombesin and gastrin-releasing peptide (BBS/GRP), vasoactive intestinal protein (VIP), glucagon-like peptide 1 (GLP-1); uncertain feeding hormones abbreviated: singleminded homolog 1 (SIM1), peptide Y (PY), brain-derived neurotrophic factor (BDNF), RFamide-related peptides (RFRPs), somatostatin (SS), tachykinins (TKs), peptide YY (PYY), insulin-like growth factor 1 (IGF1). I'm sure I missed a couple. Note that this figure does not consider species specificity, depict precise locations, or illustrate interactions between factors. Figure created by the author using open-source LatexDraw software. Background outline of tilapia brain adapted from Pepels et al. [2004].

Circulating concentrations of leptin correspond quantitatively to fat-cell stores, increasing with increases in adiposity and decreasing during periods of starvation. In negative-feedback fashion, increased leptin from growing fat stores serves as a satiety signal. However, the exact mechanism whereby increased fat stores signal the adipose tissue to produce more leptin remains unknown; leptin is not stored in typical endocrine secretory vesicles, and the amount of leptin produced by an adipocyte appears to be regulated not only at the transcriptional level, but also at levels of translation, storage, turnover, and secretion [Lee and Fried, 2006]. Leptin suppresses appetite, and, to a lesser extent, increases metabolic rate—together, these effects promote weight loss [Sherwood et al., 2005]. Administration of leptin to starved mice reverses many of the neuroendocrine consequences of starvation, suggesting that the normal biological role of leptin may be to aid the organism in monitoring transitions between nourished and starved states [Friedman and Halaas, 1998]. The main receptor for leptin is LEP-R, a cytokine-type receptor that is highly expressed in the hypothalamus. Indeed, the POMC/CART and NPY/AgRP neurons of the ARC melanocortin system are thought to be the most important leptin-responsive neurons in the brain [Coll et al., 2007]. Leptin regulates these by directly inhibiting orexigenic NPY/AgRP neurons while at the same time stimulating anorexigenic POMC/CART neurons. In the VMN of the hypothalamus, it has been shown that leptin receptors on neurons that express steroidogenic factor 1 (SF1) are required for normal body weight [Dhillon et al., 2006]. Although the brainstem NTS has leptin-responsive, LEP-R–expressing neurons, as well as POMC/CART neurons, leptin signaling seems to occur entirely via the hypothalamus [Huo et al., 2006]. In addition to the canonical signaling pathways of the central melanocortin system, the ventral tegmental area (VTA) in the midbrain contains dopaminergic neurons which project to the striatum and play a role in the rewarding and motivational aspects of eating behavior (see pg. 16). Leptin has been shown to act directly upon these dopaminergic neurons to influence food consumption in this way [Fulton et al., 2006, Hommel et al., 2006].

Recently, leptin has also been shown to have effects on hypothalamic development and synaptic plasticity. Leptin appears to be a neurotrophic growth factor during hypothalamic development, as leptin deficiency severely reduces the density of projections from the arcuate nucleus to the PVN, LH, and DMN—all regions implicated in the control of energy balance [Bouret et al., 2004]. This suggests a possible 'critical period' for leptin-dependent hypothalamic development in the early neonatal period that may have major ramifications for energy balance later in life. Leptin-mediated plasticity has been demonstrated in the hypothalami of adult rats as well;

leptin deficiency rewires synaptic inputs to arcuate neurons, increasing excitatory inputs to NPY/AgRP neurons and decreasing them on POMC neurons [Pinto et al., 2004].

For fishes, the role of leptin in feeding regulation is controversial and remains poorly understood. In fact, leptin homologs were only recently characterized in fishes [Kurokawa et al., 2005], and in contrast to mammals, the liver appears to be the major site of their expression. In a study byVolkoff et al. (2003), both central and peripheral injections of murine leptin caused significant decreases in food intake in goldfish. This study also provided evidence that leptin potentiates the actions of CART and cholecystokinin (see pg. 30), while inhibiting the actions of NPY and orexin A (see pg. 40).

Interleukins 6, 1, and 18

Interleukins are a class of cytokines—small cell-signaling proteins that are not secreted by specialized endocrine glands but produced by numerous cell types—with various functions, particularly in immunoregulation and the inflammatory response. Their expression was first discovered in white blood cells, hence the *–leukin* suffix, but they are known to be produced by a variety of body cells. Under non-inflammatory conditions, interleukin 6 (IL-6) has been found to be expressed both peripherally, in adipocytes, and centrally, in the hypothalamus, and like leptin, IL-6 levels correlate with total body fat [Coll et al., 2007]. After exogenous treatment with IL-6, acute increases of circulating triglycerides and glucose have been observed, and IL-6 has been shown to stimulate the hypothalamic-pituitary-adrenal axis (see pg. 40) [Wallenius et al., 2002]. Interestingly, mice lacking IL-6 do not display hyperphagia but become obese in adult life as a result of disruption in energy expenditure; a combined deficiency of IL-6 and interleukin 1 (IL-1), however, does cause hyperphagia and results in more marked obesity [Chida et al., 2006]. It has also been demonstrated that the loss of interleukin 18 (IL-18) leads to hyperphagia and obesity; interestingly, the administration of IL-18 does not alleviate hyperphagia in these IL-18 knockouts if injected intravenously, but does if injected into the cerebral ventricles. This finding suggests that IL-18 might have a role in the regulation of food intake by the CNS. [Netea et al., 2006].

Gastrointestinal signals

The extent of GI distention determines the degree to which gastric stretch receptors are stimulated, and more distention has been shown to suppress food intake [Sherwood et al., 2005]. However, signaling related to gastric distention has more to do with controlling the rate of gastric emptying than it does with communicated satiety. Other signals, reflecting the depletion or availability of energy-producing nutrients are more important than stomach volume in controlling the initiation and cessation of meals. Several hormones and peptides produced in the GI tract have potent effects on appetite.

Cholecystokinin and gastrin

Cholecystokinin (CCK) is an anorexigenic gut peptide released from enteroendocrine I-cells of the duodenum and jejunum in response to the passage of fatty-acid-rich chyme through the pyloric sphincter during digestion of a meal to further facilitate digestion and absorption. In addition to stimulating the release of bile and digestive enzymes from the gallbladder and pancreas, respectively, CCK acts at receptors on peripheral vagal afferent terminals that transmit signals to appetite centers such as the POMC/CART neurons of the NTS. Since consumed fat stimulates CCK release, and fats are the last macronutrient to pass through the stomach to the duodenum after feeding, this peptide is known as a postprandial satiety signal [Chaudhri et al., 2006]. CCK shares a common C-terminal tetrapeptide sequence with another important digestive peptide, gastrin, and both peptides bind to the same receptor subtypes (CCK1R and CCK2R). Unlike CCK, which is found both in the brain and the GI tract, gastrin production is limited to peripheral G-cells which serve to stimulate the secretion of hydrochloric acid from parietal cells in the stomach [Raybould, 2007].

It has been found that CCK is ineffective in reducing food consumption in mice that are lacking MC4R, as well as in mice whose brainstem NTS melanocortin receptors had been pharmacologically blocked [Fan et al., 2004]. That is, mouse brainstem NTS POMC neurons are activated by CCK, and activation of MC4Rs are required for the anorexigenic effects of CCK. The dependence of CCK on melanocortin signaling exemplifies the integrative nature of the central melanocortin system—in addition to mediating the effects of long-term adipostatic signals, it is just as important in integrating short-term hunger and satiety signals, such as CCK.

Both CCK and gastrin have been identified in the gut and nervous system of several fish species [Volkoff et al., 2009]. As would be expected from mammalian

studies, CCK mRNA expression and binding sites are present in both brain and GI tract, whereas gastrin mRNA expression is found only to occur in the intestine [MacDonald and Volkoff, 2009, Kurokawa et al., 2003]. In fishes, the release of CCK and related peptides is triggered by the presence of food in the intestine, and they function to inhibit gastric emptying and to increase gut motility [Forgan and Forster, 2007]. Also, as in mammals, CCK acts as a satiety factor in fishes and has been shown to suppress food intake in goldfish following both ICV and IP injections [Thavanathan and Volkoff, 2006]; moreover, the expression of CCK mRNA in the brains of goldfish has been shown to increase following a meal [Peyon et al., 1999].

As suggested above, CCK might have a role in mediating the effects of leptin [Volkoff et al., 2003] and amylin [Thavanathan and Volkoff, 2006] on food intake in goldfish. At present, very little is known about gastrin's role in the regulation of feeding in fishes, though studies have suggested that it may affect gut motility [Forgan and Forster, 2007].

Peptide YY ($\mathbf{PYY_{3-36}}$)

Peptide YY (PYY_{3-36}) is a potent feeding inhibitor in the NPY-family of peptides. It is secreted postprandially by the enteroendocrine L-cells of the GI tract, especially those of the ileum and colon, and its inhibition of gastric motility contributes to its anorexigenic effects. Since NPY and PYY_{3-36} are found in all vertebrates; other members of this peptide family such as pancreatic polypeptide (PP) are found only in the pancreas of tetrapods, while pancreatic peptide Y (PY) is found only in the pancreas and nervous system of some teleost fishes [Hoyle, 1999, Cerda-Reverter and Larhammar, 2000]. While it shares much of its amino acid sequence with NPY, the two peptides have opposite effects on appetite—the anorexigenic effects of PYY_{3-36} are thought to be mediated by antagonism of the Y2 receptor subtype [Moriya et al., 2009], whereas the orexigenic effects of NPY occur primarily via the Y1R and Y5R (see pg. 23). PYY_{3-36} was recently discovered to be produced by a discrete population of brainstem neurons in the gigantocellular nucleus, which is innervated by neurons form the PVN, the LH, the periaqueductal gray, and the central nucleus of the amygdala [Glavas et al., 2008]. Circulating levels of PYY_{3-36} are low during fasting and, like leptin, the appetite suppressing effects of PYY_{3-36} appear to be mediated indirectly by the melanocortin system [Batterham et al., 2002], but some studies have found that its actions may result from an MC4R-independent mechanism [Halatchev et al., 2004]. PPY_{3-36} and PY have been identified in some fish species, including sea bass (*Dicentrarchus labrax*), yellow tail (*Seriola quinqueradiata*), and pufferfish

(*Takifugu rubripes*), but their roles remain unclear [Volkoff et al., 2009].

Bombesin (BBS) and Gastrin-releasing Peptide (GRP)

Bombesin (BBS) is a 14-amino acid peptide that was first isolated from the skin of a frog. In mammals, BBS and gastrin-releasing peptide (GRP) are structurally related, sharing a similar C-terminal sequence and having similar biological effects. In vertebrates generally, BBS and GRP peptides are widely distributed in the GI tract and CNS [McCoy and Avery, 1990]. Together with CCK, BBS is a major source of postprandial satiety [Yamada et al., 2000].

In fishes, BBS-like peptides regulate gastric acid secretion and motility [Thorndyke and Holmgren, 1990] and likely act as satiety factors: experiments in goldfish have shown that peripheral BBS injections suppress food intake [Himick and Peter, 1994]. Also, BBS treatments stimulate GH release and reduce pro-SS expression in the goldfish forebrain, and these effects are partially blocked by ghrelin, suggesting an intriguing interaction between these three peptides in fishes [Canosa et al., 2005].

Ghrelin

Ghrelin, discovered in 1996, is a 28-amino acid orexigenic peptide derived from the post-translational processing of the 117-amino acid prohormone preproghrelin. It is produced predominately by P/D1 cells in the fundus of the stomach, but also by certain cells of the hypothalamus—a region of ghrelin-positive neurons has been identified adjacent to the third ventricle between the PVN, DMN, VMN, and ARC [Cowley et al., 2003]. Ghrelin is unique among enteric feeding signals in that it is the only GI-derived hormone with confirmed appetite-stimulating properties [Olszewski et al., 2008]. This peptide is an endogenous ligand for the G protein-coupled growth hormone secretagogue receptor (GHSR1a) and thus a potent stimulator of GH release from the anterior pituitary. Highly dense regions of GHSR1a are present in the hypothalamus and the pituitary as well as vagal afferent cell bodies and their endings throughout the GI tract. Hypothalamic ghrelin-producing neurons send efferents onto important melanocortin feeding circuitry, including circuits within the ARC producing NPY, AgRP, POMC, and CRH [Currie et al., 2005, Cowley et al., 2003]. Ghrelin also interacts with other neurotransmitters and at other sites—for instance, when co-administered to the PVN, 5-HT has been shown to inhibit the effects of ghrelin [Currie et al., 2011].

Ghrelin mRNA expression and peptide secretion is increased by weight loss, fast-

ing, and insulin-induced hypoglycemia, whereas peripheral administration of ghrelin stimulates food intake [Williams and Cummings, 2005]. In addition to being a peripheral signal for meal initiation, the central actions of ghrelin have a role in controlling adipocyte metabolism. Centrally administered ghrelin partitions nutrients toward fat storage by favoring an increase in glucose and triglyceride uptake by increasing lipogenesis and inhibiting lipid oxidation [Currie et al., 2005]. Thus, changes in circulating ghrelin levels may initiate CNS processes that prepare the body to receive and appropriately process incoming nutrients.

Ghrelin has been identified in several teleosts [Xu and Volkoff, 2009] and elasmobranchs, a subclass of Chondrichthyes including the sharks, the rays, and the skates [Kawakoshi et al., 2007]. In fishes as in mammals, ghrelin is expressed mainly in the stomach, with lower levels of expression seen in the brain [Kaiya et al., 2008]. The ghrelin receptor GHSR1a has also been characterized in several fishes [Kaiya et al., 2008], and its high expression in the brain suggests that ghrelin has direct central actions [Chan and Cheng, 2004].

Riley et al. [2005] showed that peripheral administration of tilapia (*Oreochromis mossambicus*) ghrelin caused significant increases in food intake and significant elevations of liver fat content among treated tilapia. In a separate tilapia study, significant preprandial elevations in ghrelin and GHSR1a mRNA levels were observed [Peddu et al., 2009]. As tilapia are members of family Cichlidae, these findings are of particular importance to the present study.

Ghrelin is also known to interact with other appetite-related peptides. In goldfish, for instance, ICV injections of ghrelin result in increases in preproorexin (see pg. 40) and NPY mRNA expression in the brain. What's more, the orexin receptor 1A agonists abolish the effects of ghrelin, while NPY Y1 receptor antagonists attenuate its effects [Miura et al., 2006, 2007]. These results indicate that the orexigenic effects of ghrelin are mediated in part by NPY and orexin-dependent pathways.

Obestatin

Different posttranslational processing of ghrelin's parent compound, preproghrelin, yields obestatin, a peptide that acts in direct opposition to ghrelin in its effects on food intake (Zhang et al 2005). Obestatin is a circulating peptide detectable in the fundus of the stomach; by binding to GPR39, a G protein coupled receptor expressed in the stomach, intestines, and hypothalamus, it is thought to suppress food intake, inhibit jejunal contraction, and decrease body weight. Oddly, circulating concentrations of obestatin are far lower than those of ghrelin, and there appears to be no change in

circulating obestatin upon fasting or feeding in rodents [Nogueiras et al., 2007]. For the first time in fishes, Li et al. [2009] provided evidence of functional obestatin in zebrafish proghrelin peptides, but at present little else is known about fish obestatin.

The Secretin peptide family

The secretin peptide family comprises a group of evolutionarily related peptides that includes secretin, glucagon, glucagon-like peptides (GLPs) gastric inhibitory peptide (GIP), vasocative intestinal peptide (VIP) and oxyntomodulin (OXM) [Nelson and Sheridan, 2006]. True to its name, GIP has been found to have feeding-inhibitory effects comparable to those of PYY_{3-36} [Sherwood et al., 2005].

Glucagon, GLPs, and OXM are derived from the transcription product of the proglucagon gene (GCG) and have distinct functions in the regulation of metabolism [Irwin and Wong, 2005]. Also similar to PYY, GLP-1 is released from L cells of small intestine in response to food ingestion [Drucker and Nauck, 2006]. Some effects of GLP-1 on body weight result from inhibition of gastric emptying, but there is also evidence that GLP-1 can inhibit feeding behavior through actions at the NTS of the brainstem and at certain hypothalamic nuclei [Chaudhri et al., 2006], implicating the melanocortin system in this signaling. Indeed, both centrally-injected and peripherally-administered GLP-1 inhibits feeding in a number of species, and for both routes the site of action appears to be this brainstem-hypothalamus axis [Chaudhri et al., 2006].

OXM is released into the blood following the ingestion of food and in proportion to the calories consumed. Physiologically, it acts to reduce gastric motility and secretion, with recent experimental data providing direct evidence for the a role in appetite control. Both OXM ICV injections and OXM injections into the PVN of rats cause reduction in food intake compared to controls; peripheral injections of OXM have been shown to reduce feeding as well, and these effects have been successfully replicated in humans [Chaudhri et al., 2006, Cohen et al., 2003].

In fishes, a pancreatic glucagon gene produces both glucagon and GLP-1, whereas an intestinal gene encodes for glucagon, GLP-1, GLP-2, and oxyntomodulin [Navarro et al., 1999]. While there is no clear evidence for any involvement of glucagon in the regulation of fish feeding, it does affect metabolism. Glucagon treatment elevates plasma glucose and plasma fatty acid levels, and high glucose diets induce a decrease in glucagon circulating levels [Volkoff et al., 2009].

GLP-1 has been identified in several species, where it is produced in both the GI tract and the pancreas [Nelson and Sheridan, 2006]. In channel catfish, both ICV

and IP injections of GLP-1 decrease food intake and affect metabolism by promoting glycogenolysis and gluconeogenesis [Silverstein et al., 2001]. As for the other members of the secretin peptide family, only VIP has been shown to affect feeding in fishes; both central and peripheral injections of VIP decrease feeding in goldfish [Matsuda et al., 2005].

Endocrine pancreatic signals

In many vertebrates, satiety is signaled by increased glucose use; this often occurs during a meal as glucose is absorbed from the GI tract. On the other hand, after digestion is complete and no new glucose is entering the blood, the resultant reduction in glucose usage has been shown to activate LH neurons and arouse hunger [Sherwood et al., 2005]. The extent of glucose use appears to be more important in the short-term determination of meal timing than in the long-term regulation of metabolism. In other vertebrates, particularly birds, plasma glucose levels remain essentially unchanged regardless of whether the animal is fasting or feeding.

Insulin

Insulin is a hormone produced by β-cells in the endocrine regions of the pancreas known as islets of Langerhans. Its release is triggered by various stimuli, though signally by increases in blood glucose. Insulin has primarily anabolic effects, facilitating organismal growth; through its actions on insulin receptors, it stimulates the synthesis and deposition of glycogen, triglycerides, and proteins [Nelson and Sheridan, 2006]. The insulin receptor is a tyrosine kinase receptor (RTK) and is widely distributed in the brain, where it is most highly concentrated in the olfactory bulb, the hippocampus, the cerebral cortex, and the ARC. This pattern of insulin receptor expression density is in line with observations that insulin has clear anorexigenic effects when centrally administered and suggests integration through melanocortin signaling. Indeed, blunting the expression of insulin receptors in the hypothalamus is associated with increases in both NPY and AgRP expression and results in rapid and significant hyperphagia [Obici et al., 2002]. Oddly, insulin signaling at POMC neurons does not appear to play a role in feeding or energy homeostasis, and the physiological significance of insulin's appetite-suppressive effects remains unclear [Choudhury et al., 2005].

Insulin has been identified in the pancreatic islets of several fishes, and here as in other vertebrates it acts as an anabolic factor [Nelson and Sheridan, 2006]. How-

ever, because fishes have a very limited capacity for carbohydrate utilization, insulin production is only weakly stimulated by blood glucose, which suggests that the major insulinotropic factors in fishes are other compounds such as amino acids [Andoh, 2007]. The role of insulin in fish feeding regulation is unclear and warrants further study; recent experiments have shown that fasting in fish results in lower plasma insulin levels than those of fed fish, and the administration (both IP and ICV) of insulin inhibits food intake in rainbow trout [Soengas and Aldegunde, 2004].

Amylin

In mammals, the hormone amylin is cosecreted with insulin from pancreatic β-cells in response to the ingestion of food. It binds specific receptors in the CNS to suppress nutrient-stimulated glucagon secretion, slow gastric emptying, and promote satiety [Lutz, 2006]. While little is known about the physiological role of amylin in fishes, both central and peripheral injections of human amylin inhibit food intake in goldfish, suggesting an anorexigenic role here as well [Thavanathan and Volkoff, 2006].

Pancreatic polypeptide (PP)

Pancreatic polypeptide (PP) is a member of the NPY-related peptide family—including PYY and NPY—that to date has only been found in tetrapods. It is released predominately from F cells in pancreatic islets and in proportion to the amount of calories ingested, although some is also synthesized by acinar cells of the exocrine pancreas. PP is a high-affinity agonist of Y4 receptors and an intermediate-affinity agonist off Y5 receptors, the latter of which is known to mediate the orexigenic affects of NPY. When PP is injected intravenously in lean humans, it has been shown to result in a persistent decrease in appetite [Chaudhri et al., 2006].

Hepatic and thyroidal signals

C-reactive protein (CRP) is a blood-borne protein synthesized by the liver and related to the inflammatory response, binding dead or damaged cells to enhance phagocytosis; interestingly, CRP has been correlated with adiposity and levels of plasma leptin. CRP has recently been shown to bind leptin and to attenuate it action in vivo; leptin also stimulates the production of CRP from hepatocytes [Chen et al., 2006]. Thyroid hormones can influence feeding behavior, as tri-iodothyronine (T3), acting in the CNS, is able to increase food intake [Kong et al., 2004]. However, T3 induces a marked increase in basal metabolic rate, and thus any increase in food intake may

be a compensatory response to increases in energy expenditure.Thyroid activity is modulated by ANS signaling; thus, signals at the latter can potentially regulate the former.

Steroidal signals

A steroid hormone, or sterone, is a steroid that functions as a hormone. They are generally grouped into five classes based on the receptors to which they bind: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens. Several of these member hormones are known to be involved in feeding regulation.

Glucocorticoids

The PVN of the hypothalamus contains neurons that synthesize and secrete corticotropin-releasing hormone (CRH) and vasopressin. CRH is transported to the anterior pituitary via the portal blood vessel system of the hypophyseal stalk; vasopressin arrives at the anterior pituitary through axonal transport. Once there, these two peptides stimulate the secretion of adrenocorticotropic hormone (ACTH) into the bloodstream. This, in turn, stimulates the production of glucocorticoid hormones in the adrenal cortices, which have many effects, and ultimately act back on the hypothalamus and pituitary to suppress CRH and ACTH production. This system, known as the hypothalamic-pituitary-adrenal (HPA) axis, controls physiological stress responses and is involved in the regulation of digestion, immune function, mood, sexuality, and energy utilization.

Circulating levels of glucocorticoids derived from the adrenal cortex are able to profoundly affect appetite [Dallman et al., 2004]. Anorexia is a feature of the cortisol deficiency seen in primary adrenal failure, whereas an excess of glucocorticoids can cause hyperphagia. Glucocorticoids are known to have a significant impact on melanocortin system; adrenalectomy reverses the obese phenotype in leptin-deficient (loss-of-function) mice, normalizing both increased AgRP expression and decreased POMC expression found in these mice, thereby restoring melanocortin tone [Makimura et al., 2000]. In addition, the orexigenic effect of the melanocortin antagonist AgRP is absent in adrenalectomized rats but restored with supplementation of glucocorticoids [Drazen et al., 2003]

Sex steroids

In humans, steroid hormones derived from the gonads have been implicated in the modulation of eating behavior. Estradiol, an estrogen, has been shown to reduce body weight by increasing excitatory synapses upon POMC soma, resulting in both a reduction in energy intake and an increase in energy expenditure [Gao and Horvath, 2007].

In fishes, the effects of sex steroids on food intake remain unclear. Estradiol treatment has been shown to stimulate feeding in female perch [Mandiki et al., 2005] and to elevate hypothalamic MCH expression in goldfish. However, estradiol was shown to have no effect on food intake of sea bream (woo et al 1993), and to decrease food intake in sea bass [Leal et al., 2009].

Still, gender-specific differences have been reported in both the distribution and the expression level of several appetite regulators, including ghrelin [Parhar et al., 2003], GAL [Rao et al., 1996], and TKs [Peyon et al., 2000]. Crucially, there is a well-documented decline or complete cessation of feeding characteristic of certain reproductive behaviors, including courtship, spawning, brooding, and territoriality. However, evidence directly linking appetite-regulating factors to this peri-reproductive anorexia awaits further study [Volkoff et al., 2009]. Some evidence of a link has been suggested by research done in goldfish; central treatment with gonadotropin-releasing hormone (GnRH) results in decreased food intake, due in part to down-regulation of brain OX mRNA expression [Hoskins et al., 2008] Also, seasonal changes in NPY, UI, and circulating cortisol levels are well-correlated to gonadal cycles.

CNS signaling beyond the central melanocortin system

This section, while crucial for a complete understanding of feeding regulation in fishes, bears only obliquely on the research presented herein; those whose interests extend only as far as the present project could bypass this section without anywise imperiling comprehension.

Melanin-concentrating hormone (MCH)

First isolated from the pituitary gland of a teleost, melanin-concentrating hormone (MCH) is an orexigenic peptide that is produced in LH neurons which project widely throughout the brain. The expression of MCH has been shown to increase both in leptin gene-knockout mice and in fasting mice [Flier, 2004]. Mice lacking MCH through targeted deletion of the MCH gene are light-weight and lean due to hypophagia and

an inappropriately increased metabolic rate despite reduced amounts of leptin and POMC expression [Shimada et al., 1998], indicating that MCH has a critical role in energy balance and acts downstream of leptin and the melanocortin system.

In fishes, MCH controls skin pigmentation by antagonizing the actions of a-MSH [Pissios et al., 2006]. Two fish MCH genes and fish MCH receptors have been characterized to date, and their role in the regulation of food intake remains controversial [Volkoff et al., 2009]. In goldfish, central treatment with mammalian MCH decreases feeding, while fasting induces a decrease in the number of hypothalamic MCH-like ir neurons [Matsuda and Maruyama, 2007], providing evidence for an anorexigenic role of MCH in fishes. Further, the actions of MCH in goldfish appear to be mediated by NPY and MSH but not by CRP, PACAP, or CCK [Matsuda et al., 2008].

Brain-derived Neurotrophic Factor (BDNF)

Brain-derived neurotrophic factor (BDNF) regulates development and plasticity in certain CNS neurons, supporting the survival, growth, and differentiation of these through actions on the receptor tyrosine kinase TrkB. BDNF has been implicated in energy homeostasis and is widely expressed in the brain [Volkoff et al., 2009]

In humans, the disruption of TrkB [Yeo et al., 2004] or BDNF [Gray et al., 2006] is associated with severe hyperphagia and obesity. Likewise, mice lacking BDNF from birth develop hyperphagia and obesity [Rios et al., 2001], and the administration of BDNF can ameliorate hyperphagia and metabolic disturbances in obese mice [Nakagawa et al., 2000]. The melanocortin receptor MC4R and BDNF/TRkB signaling may be coupled in the regulation of energy balance [Xu et al., 2003]. BDNF is highly expressed in the VMN, where its expression is regulated by nutritional status and MC4R signaling. Central administration of BDNF suppresses hyperphagia and excessive weight gain in mice with deficiencies in MC4R signaling.

Single-minded 1 (SIM1)

The hypothalamic transcription factor single-minded 1 (SIM1) is expressed in a number of regions known to be involved in energy homeostasis including the PVN and the LH; it has recently been suggested that Sim1-expressing neurons in the PVN regulate feeding in response to melanocortin signaling [Kublaoui et al., 2006].

Orexins (OXs)

The orexins (OXs, also called hypocretins) consist of two peptides, orexin-A (OX-A) and orexin-B (OX-B), which are produced from the cleavage of precursor peptide preproorexin. In mammals, OXs are produced primarily in the lateral hypothalamus (LH) and act through two G-protein coupled receptors (OX1 and OX2) to stimulate feeding, to control gastric secretion, and to regulate wakefulness [Korczynski et al., 2006]. There are possible links between OXs and leptin adipostatic pathways; administration of leptin decreases OX expression whereas fasting increases OX mRNA levels [Yamanaka et al., 2003].

In fishes, mRNA encoding preproorexin has been reported in several species [Faraco et al., 2006]; this mRNA is present in the hypothalamus and the pituitary, as well as in the gut [Xu and Volkoff, 2007]. In goldfish, OXs have been shown to stimulate appetite [Volkoff et al., 1999], and starvation increases brain preproorexin mRNA levels in both goldfish and zebrafish [Nakamachi et al., 2006, Novak et al., 2005].

OXs appear to interact with other neuropeptides involved in the regulation of feeding in fishes. In goldfish, CART and leptin have both been shown to inhibit OX-induced feeding [Volkoff and Peter, 2000]. Further, the blocking of goldfish OX receptors causes decreases in NPY-, GAL- (see below), and ghrelin-induced feeding. Vice versa, blocking NPY, GAL [Volkoff and Peter, 2001], or ghrelin [Miura et al., 2007] receptors has been shown to inhibit OX-induced feeding. Additionally, central administration of OX causes increased expression of both NPY and ghrelin mRNA.

Galanin (GAL)

Galanin (GAL) is a founding member of the galanin peptide family, which includes preprogalanin, galanin-like peptide, and alarin. It is widely expressed in the CNS and GI tract; among its many mammalian functions, it stimulates food intake and weight gain through actions on three G protein-coupled receptors: GAL1R, GAL2R, and GAL3R [Walton et al., 2006, Lang et al., 2007].

GAL has been isolated from several species of fishes [Volkoff et al., 2005], where it has been suggested to play an orexigenic role. Central, but not peripheral, injections of mammalian GAL stimulate food intake in goldfish [Volkoff and Peter, 2001], while fasting increases goldfish brain preprogalanin mRNA expression [Unniappan et al., 2004].

Corticotropin-releasing Factor (CRF) Family

The corticotrophin-releasing factor (CRF) family of neuropeptides includes corticotrophin-releasing hormone (CRH), urocortins (UCNs), and fish urotensin-I (UI), which differ in their binding affinities for two CRF receptor subtypes (CRF1 and CRF2) [Bernier, 2006]. These neuropeptides are important in the stress response, but are also distributed widely throughout the brain, suggesting diverse physiological and behavioral function. For instance, the prohormone CRH, expressed by the PVN, regulates the release of corticotrophin (ACTH) from the anterior pituitary via CRF1 receptors, thereby activating glucocorticoid release through the hypothalamic-pituitary-adrenal (HPA) axis (see section...). Suggestively, MCRs including MC4R are found on CRH neurons of the PVN, and it has been proposed that the melanocortin system has a role in regulating CRH production through the HPA axis to control feeding. Futhermore, the related ligand urocortin I (UCN1) has been shown to suppress feeding and to alter energy substrate utilization when centrally injected, and to inhibit the orexigenic effects of both ghrelin and NPY [Currie et al., 2011].

Transcripts encoding CRF, UI, UCNs as well as CRF1 and CRF2 have been reported for several fish species [Alderman and Bernier, 2007]. Peripheral injections of CRFs have shown no effect in fishes, but central treatment with CRF-related neuropeptides such as UI inhibit feeding in several species [Volkoff et al., 2009]. Furthermore, it has been reported that CRF-related peptides interact with other appetite regulated systems in fishes, such as serotonin [De Pedro et al., 1998] and NPY [Bernier et al., 2004].

RFamide-related peptides (RFRPs)

The RFamide-related peptides (RFRPs) are a diverse group of peptides characterized by a common N-terminal sequence, including prolactin releasing peptide (PrRP), metastin, and kisspeptin; their receptors have yet to be characterized [Dockray, 2004]. Of the vertebrate RFamides identified to date, all except kisspeptins have been demonstrated to modulate food intake in mammals [Bechtold and Luckman, 2007].

Very little is known about the structure and function of RFamides in fishes. In goldfish, hypothalamic PrRP expression significantly increases both after feeding and following seven days of food deprivation [Kelly and Peter, 2006], though the major role of this peptide in fishes seems to be the regulation of salt and water balance.

Tachykinins (TKs)

The tachykinins are a large family of small neuropeptides defined structurally by a common C-terminal amino acid sequence; they are distributed throughout the nervous system of chordates. The TKs include substance P, the neurokinins, and carassin (in goldfish), which are associated with inflammatory processes, vasodilation, and pain.

In fishes, TKs have been shown to rapidly induce contractions of gut smooth muscle [Volkoff et al., 2009], a fact from which their name derives. In several species, hypothalamic mRNA expression of preprotakykinin displays postprandial changes [Peyon et al., 2000], suggesting that TKs are involved in digestion and feeding processes.

Endocannabinoids (ECs)

Endocannabinoids (ECs) are endogenous phospholipid derivatives, which activate two cannabinoid receptor subtypes (CB1 and CB2). ECs are known to use retrograde signaling—they are released postsynaptically and have actions presynaptically. In mammals, ECs have central orexigenic effects through hypothalamic CB1 receptors [Despres, 2007]; among other functions, they also affect motivation and reward through the nucleus accumbens (see pg. 25 and Figure 5).

In goldfish, the ECs anandamide and arachidonoylglycerol are distributed widely throughout the brain [Valenti et al., 2005], and orthologues of CB1 and CB2 receptor subtypes have been recently identified [Elphick and Egertova, 2001, McPartland et al., 2007]. Still, the role of ECs in the regulation of food intake in fishes remains unclear. In goldfish, food deprivation has been shown to induce an increase in anandamide mRNA levels in the telencephalon [Valenti et al., 2005].

Neuromedin U (NMU)

Neuromedin U (NMU) is a multifunctional neuropeptide found throughout the brain; it has been implicated in appetite and energy homeostasis and has known anorexigenic effects in mammals [Maruyama et al., 2008]. In goldfish, fasting induces a decrease in brain proNMU mRNA levels and central administration of fish-NMU orthologs suppresses food intake [Maruyama et al., 2008], suggesting that NMU has a role fish appetite regulation as well.

Calcitonin gene-related peptide (CGRP) family

The calcitonin family of peptides includes CGRP, adrenomedullin (AM) and intermedin (IM). CGRP is produced by centrally and peripherally; it is a potent vasodilator, it plays a role in the transmission of pain, and its effects are mediated through G protein-coupled receptors [Brain et al., 1985]. All of these peptides have also been shown to be involved in food intake in mammals (book). A recent study in fish showed that central injections of either CGRP or IM, but not AM, significantly decreased food intake [Martinez-Alvarez et al., 2009].

Monoamine neurotransmitters

The mesolimbic dopamine "reward" pathway was described in some detail above; focus now shifts from the catecholamines to a tryptamine.

Serotonin (5-hydroxytraptamine, 5-HT)

Serotonin is a monoamine neurotransmitter derived from the amino acid tryptophan, found primarily in the CNS and GI tract of vertebrates. Indeed, about 90% of human serotonin is located in gut enterochromaffin cells where it regulates intestinal motility, while the remainder is made in serotonergic CNS neurons where it has various functions in the regulation of, among other things, mood, appetite, and sleep [Berger et al., 2009]. Serotonin, through actions at 5-HT1B receptors, has been shown to modulate the endogenous release of melanocortin receptor agonists and antagonists. Not only does serotonin inhibit NPY/AgRP neurons, but it activates POMC/CART neurons, leading to an increase in activity at MC4R in target sites and a reduction in food intake [Heisler et al., 2006]. Serotonin has also been recently shown to act within the PVN to block the orexigenic and substrate-utilization effects of ghrelin [Currie et al., 2010]. Little work has been done to explore the role of serotonin in fish feeding. In goldfish, central, but not peripheral, administration of serotonin resulted in significantly reduced food intake; this same study showed that this serotonin-induced feeding inhibition was partially mediated by CRF [De Pedro et al., 1998].

The growth hormone (GH) system

Growth hormone (GH) plays a critical role in the growth and metabolism of all vertebrates [Canosa et al., 2007]. The release of GH is directly regulated by the secretion of hypothalamic hormones into the hypophyseal portal system which leads to the an-

terior pituitary. Among these hormones are the somatotropes—the GH-stimulating growth hormone-releasing hormone (GHRH) and the GH-inhibiting somatostatin (SS, also known as growth hormone-inhibiting hormone)—as well as pituitary adenylate cyclase activating polypeptide (PACAP). Ghrelin, discussed in detail above, also stimulates GH secretion via its actions on GHSRs. Although the balance of these stimulating and inhibiting peptides determines GH secretion, it the balance is affected by many physiological factors such as exercise, stress, nutrition, and sleep. GH also appears to be involved in the regulation of feeding in fishes; feeding behavior has been shown to increase in GH treated teleosts [Johnsson and Bjornsson, 1994], and starvation induces changes in both pituitary GH mRNA and plasma GH levels [Volkoff et al., 2009]

Pituitary adenylate cyclase activating polypeptide (PACAP)

In fishes as in mammals, PACAP stimulates GH release from pituitary cells in vitro, and it too appears to be involved in the regulation of feeding. When injected either centrally or peripherally, PACAP suppresses food intake in goldfish, and these actions are thought to be mediated by POMC and CRF [Matsuda and Maruyama, 2007]. PACAP has also been shown to potentiate both feeding-induced pancreatic insulin release as well as the lipid-storing actions of insulin, suggesting that fish PACAP is involved in the regulation of energy metabolism in several tissues [Nakata and Yada, 2007].

Somatostatin (SS)

In mammals, SS exists in two biologically active forms—SS-14 and SS-28—that are produced by alternative cleavage of the same precursor, preprosomatostatin I (PPS I), and both of which inhibit pituitary GH secretion [Patel, 1999]. SS proteins, or mRNAs encoding PPS, have been isolated from over 20 fish species, and SS-14 has been shown to inhibit GH secretion in several of these species [Klein and Sheridan, 2008]. Little is known, however, about the effects of SS on food intake in fishes, but it is known to affect metabolism and energy homeostasis [Klein and Sheridan, 2008]. Not only does SS promote lipid mobilization and hyperglycemia, but its biosynthesis and secretion are both regulated by metabolite levels and by various appetite signals including NPY, CCK, and GAL [Volkoff et al., 2009]

Present study

Haplochromine cichlids in the Great Lakes of East Africa have undergone adaptive radiation in a singularly recent and extreme fashion, occuring within the last 10 million years and resulting in ~2000 unique species in this region alone. By comparison, another teleost fish model, the zebrafish, speciated some 110–160 million years ago. This alone makes a strong case for the widespread adoption of cichlid model organisms, but consider: the recent diversification of cichlid species has furnished genetic, morphological, behavioral, and ecological diversity *nonpareil.* This diversity can be thought of as a natural 'mutant screen' of interesting new phenotypes, many of which have likely contributed to their evolutionary success.

Maternal mouthbrooding is one such phenotype, receiving scientific attention for the fitness benefits likely to betide its fish practitioners—it is an extremely effective rearing strategy where brood-survival is all but ensured. On the other hand, though, it is a physically taxing parental care behavior that prescribes a mouth-full of fish-kids and 2-week term of anorexia; to effectively regulate hunger and metabolism during the brooding period clearly requires mothers of stout genetic constitution. The behavioral abnormalities of lab stock *A. burtoni* mothers during brooding and fry release were recognized by my experimental predecessors—Renn Lab members Carleton [2009], Nguyen [2009], and Ichikawa [2011]—and point compellingly to a case of compromised feeding regulation.

This study sets out in a top-down way to determine whether there are whole-organism differences in metabolic rate between laboratory stock and wild stock *A. burtoni* females undergoing a period of starvation, and whether brooding-induced starvation affects female *A. burtoni* metabolic rate differently than non-brooding starvation. Alongside a control group of *A. burtoni* females who are fed as usual, this experimental tack will allow for diagnoses of metabolic irregularity and will separate the reproductive context of brooding from the act of starvation, which could serve to rule out or confirm the involvement of reproduction-specific factors in the poor broodcare phenotype exhibited by labstock females.

Even in the absence of differential metabolism between the two stocks, feeding behavior—in turns motivated and discouraged by integrative decisions at the level of the melanocortin system as it 'weighs' countless orexigenic and anorexigenic inputs—could still be modulated by the exigencies of starvation. Accordingly, the second goal of this study is to assay for differences in gene expression for these decisive peptides between the stocks; that is, to determine if the POMC/CART and NPY/AgRP

standard-bearers of the central melanocortin system are functioning in the same way. Since the melanocortin system is the final destination of feeding signals broadcast bodily, like cholecystokinin and leptin, another objective is to home in on these more acute signals to try to determine the root of the regulatory impairment. This bottom-up aim was abandoned as untenable in the present study given prevailing constraints on time and effort, but the first two objectives are more foundational and a logical starting-place to begin to tease apart the intricacies of feeding and its relationship to behavior.

Hypotheses

The hypotheses proceed in order of the most biologically general to the most specific, beginning with:

1. Differences in weight loss will be observed between laboratory stock and wild-caught stock *A. burtoni* females under both brooding and starvation conditions, with greater weight loss—indicative of higher energy expenditure—on the part of the lab stock.

2. There will be metabolic-rate differences between laboratory stock and wild-caught stock *A. burtoni* females both *among* and *within* three experimental conditions: starved, fed, and brooding.

    - This entails at least two predictions: that (1) brooding females, starved females, and fed females will show condition-specific differences in metabolic rate (expected to increase in that order, with brooding females having the lowest and fed females having the highest) regardless of stock, and that (2) the lab stock will have a higher metabolic rate than the wild stock across all three conditions. The possibility that an interaction-effect between stock and condition is also influencing metabolic rate will be explored.

3. Between the first and second weeks of experimentation, the laboratory stock will not have reduced their metabolic rate to the same extent as the wild stock.

    - This would be indicative of an inability to respond metabolically to acute starvation stresses and their associated signaling.

4. Regulation of feeding at the level of the central melanocortin system will be different between lab stock and wild stock females both among and within the

experimental conditions at the time of sacrifice; gene expression profiles will be consistent with the metabolic results.

- Specifically, the orexigenic neuropeptide NPY will be upregulated in laboratory stock females compared to wild stock females after two weeks of starvation or brooding conditions, presumably causing intense feelings of hunger that override broodcare motivation; conversely, it is predicted that the anorexigenic neuropeptide POMC, associated with feelings of satiety, will see higher levels of expression in wild stock females than in lab stock females.

Materials and Methods

Provenance of *A. burtoni* stocks

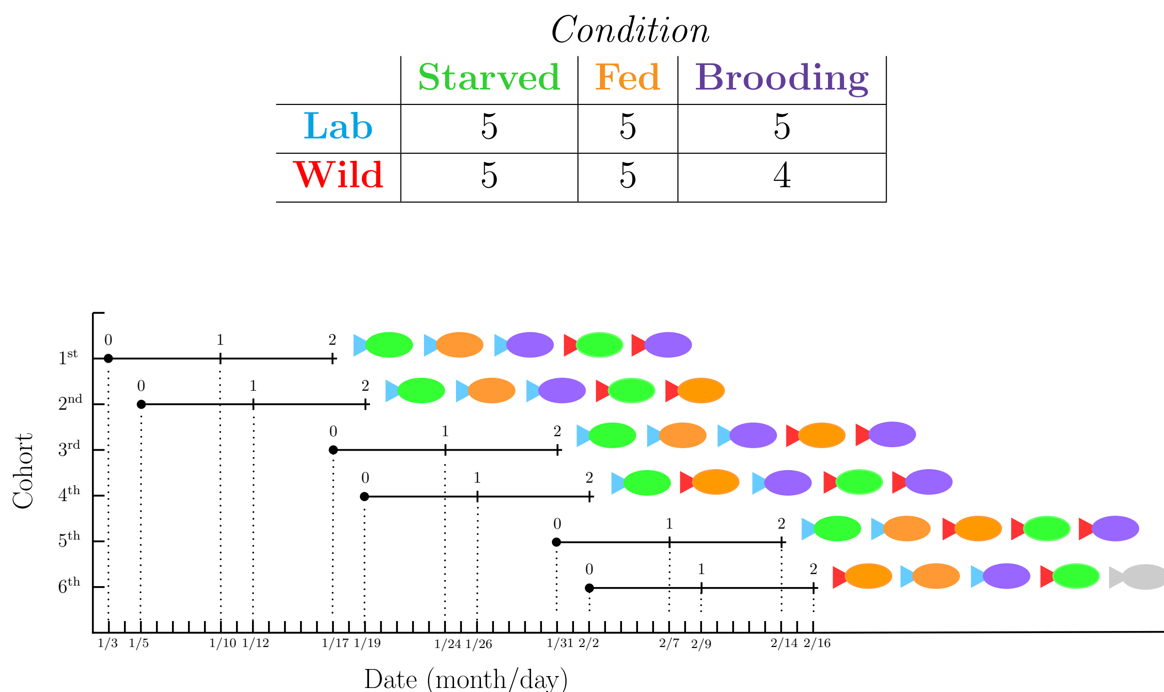
All fish used in this experiment were isolated from colony tanks measuring 90 x 45 x 30 cm (110 L), which housed either the laboratory stock (LS) *A. burtoni* or second-generation wild stock (WS) *A. burtoni*. The LS *A. burtoni* were originally collected by Russ Fernald in 1977 from a site at the north end of Lake Tanganyika, Burundi (Fernald et al., 1977) and have been inbred in a laboratory environment for over 30 generations. The WS *A. burtoni* were collected by Suzy Renn in 2005 from a site at the south end of Lake Tanganyika, Zambia, and have been inbred ever since.

Animal husbandry

LS and WS female *A. burtoni* were isolated from their respective colony tanks and were housed separately in zebrafish tanks (9 L) for a two-week period. All WS fishes were second generation (F2). Brooding fishes were isolated as close to the onset of the brooding period as possible; this was gauged by examining egg-size. No attempt was made to identify reproductive stage of fishes in *fed* and *brooding* conditions beyond confirming the absence of brood in the buccal cavity. Experimental-housing tanks contained just enough gravel to completely cover the floor as well as two terra cotta potshards, overturned in order to provide territory for the fish. The conditions which the fishes were housed mimicked those of their natural environment. The photoperiod was 11.5 h:11.5 h light:dark, with an additional half hour of dim light between transitions in order to simulate dawn and dusk. Water temperature was maintained at 28 ± 0.5°C by ambient heat. Water conductivity was kept between 630-650 μS/cm (salinity = 403-416 ppm) and pH was kept between 8.1-8.5. Nutritionally-balanced cichlid flake food was provided once a day in the morning ad libitum to fishes in the *brooding* and *fed* conditions. Fishes in the *starved* condition received no food.

Experimental design

Assays for metabolic differences and differential gene expression between LS and WS females in brooding, starvation, or controlled-feeding conditions necessitated a somewhat complicated experimental schedule. A thoroughgoing explanation of the experimental time-course, the composition of each cohort, and the sample size is given graphically in Figure 8. The cohort design was expedient because weekly fluctuations in the number of brooding females and equipment limitations allowed a maximum of 5 fish to be simultaneously tested for metabolic rate.



Condition

	Starved	Fed	Brooding
Lab	5	5	5
Wild	5	5	4

Figure 8. Experimental timetable. Lab stock and wild stock fishes in starved, fed, and brooding conditions, coded by color according to the sample-size table. Design based on 6 cohorts of 4-5 fish each; a single cohort of fishes undergoes experimental conditions and metabolic assays parallel. Each cohort depicted by a horizontal bar on the vertical axis. Time in days depicted by ticks along the horizontal axis. Black dots labeled '0' on cohort bars correspond to the day of isolation; tick-marks labeled '1' or '2' on bars correspond to the day of respirometry and are seven days apart; experimentation for each cohort lasted 2 weeks, indicated by the length of each cohort bar. Fish sacrifice occurred at the right-most tick-mark ('2') after the second repsirometry measure. Color-coded fish-shapes describe the make-up of each cohort in terms of stock and condition of each experimental animal; tail color indicates the stock(lab/wild), while body color indicates condition (starved/fed/brooding). Total duration of experimentation was 29 days, beginning with the isolation of the 1st cohort on January 3, 2012 and ending with the sacrifice of the 6th cohort on February 16, 2012. Respirometery sessions lasted from between 10-11 AM to between 3-4 PM. Figure created by the author.

Respirometry

Each aquatic respirometery system consisted of USB respirometry hardware and its supported software (RESP-EDU, LoligoSystems), a cylindrical acrylic fish chamber (800 mL), a galvanic dissolved-oxygen probe (MINI-DO, LoligoSystems), a thermometer, and two water pumps connected to the fish chamber by plastic tubing. Glass aquaria (15 gallons) were used to house each of the five respirometer systems and to contain the ambient water necessary for intermittent-flow respirometry. Airstone bubblers were employed to ensure the consistent oxygenation of ambient water during respirometry. Respirometry-system software was supported by Microsoft operating systems only; Apple MacBook laptop computers (5), equipped with Microsoft Windows XP through Boot Camp (Apple), were used to run the program.

Figure 9. Respirometry set-up. The experimental workspace used in the present study. Originally, each cohort was to consist of six animals run in parallel; pump failure during the first session took one of the respirometers offline. The O_2 probe, thermometer, and water pumps were connected to each respirometer (blue/gray box beside each computer), which was itself connected to the computer. Figure inset in lower left corner depicts an animal inside the chamber; the location of the O_2 probe within the chamber is indicated in white text.

In an intermittent-flow respirometry paradigm, water flux through the chamber is under the control of two pumps—a recirculation pump and a flush pump—that work in series to determine the time-course of respirometry. The recirculation pump effectively creates a closed system within the chamber, sealing it off to outside water while functioning to recirculate water contained within so as to ensure an even distribution of dissolved O_2 throughout the system. It is during this period that the O_2 consumption of the animal is measured. After 15 minutes of measurement, the recirculation pump shuts off and the flush pump is activated. The flush pump replaces O_2–depleted water in the chamber with freshly-oxygenated ambient water from the aquarium tank.

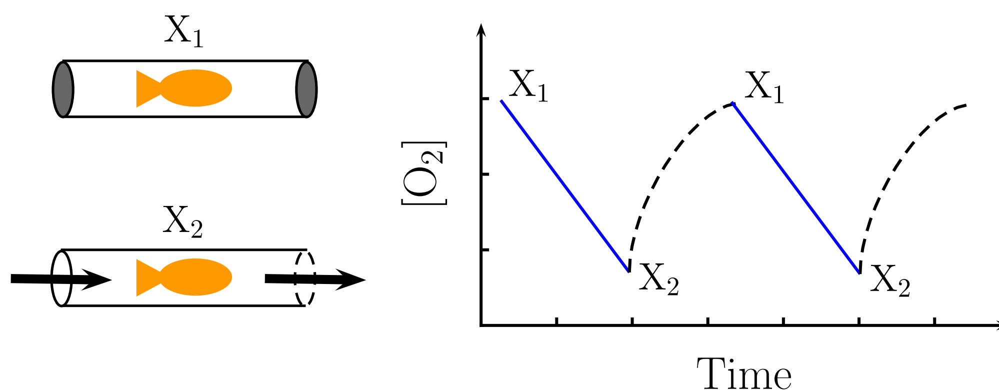


Figure 10. Diagram depicting intermittent-flow respirometry. Blue lines indicate that the respirometer is measuring the $[O_2]$ of the water while the flush pump is turned off and no water is circulating (X_1); the slope of these lines corresponds to the rate at which the experimental animal is consuming O_2. Dashed lines indicate the rise in $[O_2]$ within the chamber as deoxygenated water is pumped out while freshly-oxygenated water is pumped in (X_1). It is imperative that measurements take place in a closed system; that is, with no O_2 entering or leaving. Intervals (time $X_1 \rightarrow X_2$ and $X_2 \rightarrow X_1$) were 15 minutes long in the present student, providing enough time for accurate measurement while maintaining overall $[O_2] > 90\%$. Figure created by the author using LatexDraw.

Respirometry is only as effective as the instrument used to measure dissolved O_2. The galvanic O_2 probes used in this experiment measure the partial pressure of O_2 in the water as an indicator of the total amount present. These instruments rely on electrodes (a base-metal anode and noble-metal cathode in electrolyte medium) surrounded by a membrane that is partially permeable to O_2 but not to charged species. As O_2 diffuses through the membrane it becomes reduced at the cathode, generating a measurable current corresponding to the total amount of O_2 present in the water sample. For galvanic probes, this circulation of water throughout the chamber ensures accurate rate measurements. Additionally, the RESP-EDU software

prompts the user to input the barometric pressure (hPa) and the salinity (‰) when initiating each session to ensure proper function of the probe. Barometric pressure data was gathered from a nearby weather station (West Hills, Portland; accessed online through *wunderground.com*).

Sample collection

All animal husbandry and sacrifice was carried out in accordance with the approved protocol (Renn, IACUC 103.2009). After the second respirometry session at the end of the second week, fish were sacrificed between 2:30–4:30 PM. Fish were anesthetized by placing them in a 1 L beaker of water containing MS-222 (tricaine methanesulfonate, 100 mg in 500 mL conditioned fish water, pH 7-8) for 1–2 minutes or until fish were unresponsive to touch. Fish were decapitated and the brain was carefully removed from the braincase and transferred to an Eppendorf tube containing RNA later (1 mL, Ambion). The maximum time elapsed between removal of a fish from the chamber of the respirometer to placement of the brain in RNAlater was 15 minutes and the procedure was unlikely to affect gene expression. Brain samples in RNAlater were stored at 4°C until RNA isolation.

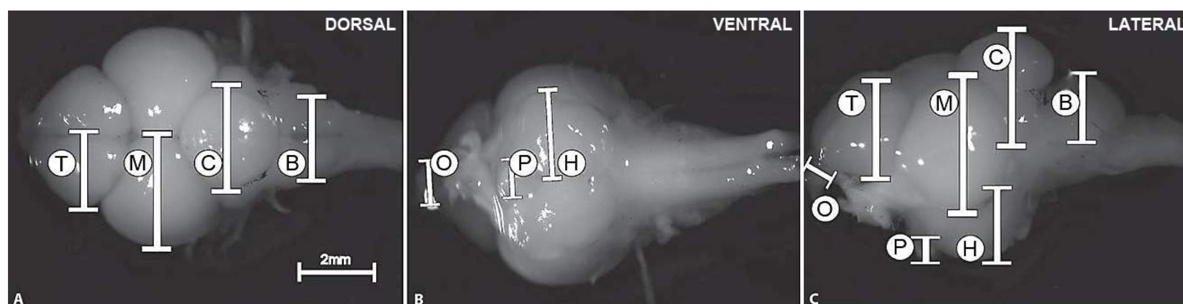


Figure 11. Cichlid brain with structures labeled. A Lake Tanganyika haplochromine cichlid brain (*sp. Enantiopus melanogenys*) viewed dorsally (A, left), ventrally (B, center), and laterally (C, right). Bar in bottom-right corner of dorsal view indicates scale (2 mm). Others bars serve to indicate the boundaries of the structures shown: telencephalon (T), midbrain/optic tectum (M), cerebellum (C), brain stem/medulla (B), olfactory bulb (O), pituitary gland (P), hypothalamus (H). Image adapted from Pollen et al. [2006].

Isolation of RNA from brain tissue

Whole-brain RNA extractions were performed using TRIzol LS Reagent (Invitrogen), a phenol- and guanidium thiocyanate-based reagent, diluted 3:1 with nuclease-free water following the manufacturer's protocol and adapted for use with phase lock gel tubes (PLG, 5-Prime). To begin the process of cell lysis, brains were removed from RNAlater using sterile forceps and transferred to 15 mL polystyrene falcon tubes containing TRIzol (1.5 mL). Brain tissue was immediately homogenized, or mechanically lysed, using a sterilized TissueTearor instrument (Midwest Scientific) for 30 seconds or until tissue was completely homogenized The homogenate was incubated at room temperature for 5 minutes. The homogenate was then split between two PLG tubes so that each contained 750 μL of the sample. Chloroform (150 μL) was added to each PLG tube and the mixture was shaken vigorously for 1 minute before incubation at room temperature for 15 minutes. The PLG tubes were then centrifuged (12000g, 4°C) for 15 minutes, causing the aqueous and organic phases to become separated by a gel layer. The organic lower phase and DNA-containing interphase were discarded; the aqueous upper phase was transferred to a new 1.7 mL microcentrifuge tube and isopropanol (480 μL) was added to precipitate RNA. The isopropanol mixture was incubated at room temperature for 10 minutes before the RNA was pelleted by centrifugation (15 min, 12000g, 4°C). The pellet was then washed with ethanol (75%, 1mL) by centrifugation for 8 minutes (12000g, 4°C). The ethanol was completely removed and pellets were allowed to air dry for 5 minutes before drying in a 554°C dry-bath for 3 minutes. RNA was resuspened in 15 μL of RNA Storage Solution (containing 1 mM sodium citrate, pH 6.4, Ambion) by incubation at 554°C for 10 minutes. Concentration of RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed by 260/280 and 260/230 absorbance ratios (Appendix 1).

Purification and reverse-transcription of RNA isolate

The phase-separation step in the RNA isolation procedure is vulnerable to DNA contamination; if the white DNA interphase is not completely separated from the aqueous upper RNA phase after centrifugation, the RNA phase will inevitably contain DNA as well. In order to combat this, all resuspended RNA was purified with a mini-kit containing recombinant DNase I (DNA-*free*, Ambion), which digested genomic DNA. In brief, 10x DNase I buffer (0.1 volume) and recombinant DNase I (1 μL) was added to each RNA sample. This mixture was the incubated at 37°C (30 min) before

treatment with DNase Inactivation Reagent (2 μL). Centrifucation (1.5 min, 10000g, $\sim$23°C) resulted in a pellet containing the inactivation reagent and any digested genomic DNA. An RNA sample for each fish was not treated with DNase and set aside to serve as a control for potential genomic contamination during qPCR.

Purified RNA (1 μg) was reverse-transcribed to cDNA by RNase H+ MMLV-derived reverse transcriptase in oligo dT primed cDNA synthesis reaction using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad). In brief, 4 μL of 5x iScript reaction mix (containing buffer, RNase inhibitor, oligo (dT) primers, $MgCl_2$, and stabilizers) and H+ reverse transcriptase (1 μL) was added to the RNA template, and nuclease-free water was added to bring the total reaction volume to 20 μL. The reaction mix was incubated at 25°C (5 min), 42°C (30 min), and 85°C (5 minutes). The precise measurement of RNA template(1 μg) provides for the equality of concentrations among the cDNA samples going into qPCR. All cDNA product was stored at 4°C.

Quantitative polymerase chain-reaction (qPCR)

By the *central dogma of molecular biology*, the DNA makes the RNA makes the protein. Gene expression, and therefore turnover of gene products (DNA $\rightarrow$ RNA), is regulated within the cells of all organisms, and the number of copies of these products—messenger RNA (mRNA) transcripts—in a given cell, region, or tissue is determined by relative rates of gene expression and transcript degradation. The present experiment relies heavily on this principle and so requires a method for quantifying the expression of particular genes between organisms receiving differential experimental treatment.

In a normal polymerase chain-reaction (PCR), a double-stranded DNA template containing a nucleotide sequence of interest— a 'target site' corresponding to a particular gene or mRNA transcript, say—is amplified by a DNA polymerase through a series of 20-40 repeated temperature changes, each consisting of $\sim$3 discrete temperature steps. The 3 fundamental steps that make up a single cycle of the PCR are responsible for:

1. the **denaturation** of the double-stranded DNA template to single-stranded DNA;

2. the **annealing**, or binding, of primers—little complementary strands of nucleotide designed to correspond to specific sequences flanking your gene or mRNA region of interest—to one or another strand of the denatured DNA;

3. the **extension**, or elongation, step, where temperature is held at a level optimal for DNA polymerase activity; it binds to where the primers have annealed to the template and synthesizes DNA copies of the gene or mRNA 'target site'.

After the first two three-step cycles, DNA copies of just the 'target site' exist, and each subsequent cycle causes the amount of these copies to double; that is to say, each cycle results in a regular, exponential increase in the amount of 'target site' DNA transcripts. If you had only one strand of DNA to start with, 40 cycles of PCR would yield $\sim 2^{39}$ copies—over half a trillion!

Quantitative polymerase chain-reaction (qPCR) exploits PCR's regular amplification of almost imperceptibly (let alone quantifiably) small concentrations of nucleic acid template by the simple addition of a DNA-binding dye, which binds *all of these these double-stranded templates* and begins to fluoresce. A direct relationship therefore exists between the amount of dye-bound DNA product during PCR amplification cycles and the intensity of fluorescence, which is detectable in real-time by a qPCR instrument.

Data collected over the course of the reaction is graphically plotted by the qPCR instruments on a logarithmic scale; fluorescence is plotted on the vertical axis, while cycle number is plotted on the horizontal axis. A threshold for detection of DNA-fluorescence above background noise is set by the instrument, and the number of cycles where fluorescence exceeds this threshold is called the cycle threshold (C_t) value for a given sample. To quantify gene expression, the C_t value for the gene of interest is divided by the C_t value of a "housekeeping" gene in the same sample to normalize for variation in the amount and quality of nucleic acid template between different samples. This common technique, usually referred to as the $\Delta\Delta C_t$-method, permits a comparison of gene expression among different samples. However common, there are two important caveats with regard to normalization with a reference gene:

- The expression of the reference gene needs to be very similar across *all* of the samples.

- The efficiency of amplification often varies considerably from primer-set to primer-set; these efficiencies must be assessed experimentally through the creation of a standard curve showing the change in C_t with each serial dilution.

To address the first of these concerns, care must be taken in the choice of a housekeeping gene. These so-called "housekeeping" genes must be constitutively expressed in all cells of an organism across conditions. These genes code for proteins that are

constantly required by the cell for basal processes and are usually essential to cellular survival and function.

Initially, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was going to be used as the reference gene in this experiment; past Renn Lab researches had designed and optimized a GAPDH primer set for use in *A. burtoni*. For the present purpose, however, the choice of GAPDH is questionable; this gene codes for the enyzme that catalyzes the 6th step of EMP glycolysis, converting the metabolic intermediate glyceraldehyde 3-phosphate into to 1,3-bisposphoglycerate (Figure 3). Since metabolic regulation is the crux of the study, and expected to be influenced by experimental conditions, this gene was abandoned in favor of β-actin, a cytoskeletal actin isoform involved in cellular structure and motility, expressed at relatively constant levels within and between animals' cells and household name among housekeeping genes. The expression of β-actin was deemed unlikely to be affected by experimental conditions.

Primers for qPCR

Primers for neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) were designed in Suzy Renn's laboratory using web-based "Primer 3" software by a former lab members (Nguyen, 2009). Primers for β-actin were designed in Suzy Renn's laboratory this year by current lab member Chris Galvin using Geneious software (Biomatters). Experimentally-determined primer efficiencies were: NPY, 1.78 (78%); POMC, 1.98 (98%); β-actin, 1.91 (91%).

Table 1. Primers for β-actin, NPY, and POMC used during qPCR.

Target gene	Amplicon size (bp)	Primer sequences
β-actin	89	**F**: TCCCCTCCATCGTCGGTCGC
		R: GCTCTGGGCCTCATCACCAACG
NPY	115	**F**: GCCAAATACTACACTGCCCTGAGAC
		R: TGTTATCGCCACCAAACAGCAG
POMC	209	**F**: TTCTCAGGCCAAGCGCTCCT
		R: TCGGCCTCCTCGACCATCTC

Conducting qPCR

All qPCR reactions were conducted in duplicate for each individual using a LightCycler 480 (Roche). Since each qPCR plate has a 96-well capacity, a single primer set was used for each of three plates (29 individuals x 2 replicates + 29 noDNase/noRT controls = 87 wells). Due to sample shortages, the noDNase/noRT control (the original RNA isolate) was only included in the NPY-primed reaction. Reaction mixtures were set up so that final volume equaled 10 μL. For each primer set, a 'master mix' was prepared using 5x ImmoMix (5 μL, Bioline)—a complete reaction mixture including a hot-start (7 min, 95°C) DNA polymerase—50x SYBR Green I dye (0.15 μ), 5 μM mixed foward-and-reverse primers (1 μL), and nuclease-free water (3 μL) per reaction. The addition of the cDNA template (1 μL) brought the total reaction volume to 10 μL. Master mix (9 μL) was pipetted into each experimental well; template cDNA (1μL) was then added to each of these wells.

The program of thermal cycles employed in this reaction were:

- **Hot start**: ImmoMix activation, 7 minutes, 95°C
- **Amplification**: 40 cycles with three temperature steps
  - *Denaturation*, 30 seconds, 95°C
  - *Annealing*, 30 seconds, 61°C
  - *Elongation*, 1 minute, 72°C
- **Final elongation**, 2 minutes, 72°C
- **Melting curve generation**, with continuous acquisitions (5/°C)
  - 15 seconds, 95°C, ramp rate 4.8 (°C/second)
  - 15 seconds, 61°C, ramp rate 2.5 (°C/second)
- **Cooling**, 2 minutes, 40°C

Data handling and statistical analyses

Metabolic measures

RESP-EDU intermittent-flow respirometry software (LoligoSystems) outputted data for [O_2] $\left(\frac{mg}{l \cdot hour}\right)$ and temperature (°C) inside the chamber at a rate of 1 datapoint/second into a .txt file. Each of 58 respirometry sessions (2/animal) lasted 5 hours on average; this yielded just over 1,000,000 lines of data spread evenly among 58 .txt files.

Microsoft excel was not able to handle all of this data at once; indeed, this quantity exceeded its inbuilt limit on row number. However, a single day's worth of measurements (∼17000 lines) at a time proved manageable. In Excel,

- The first 3600 lines of data, equivalent to the first hour of measurement (3600 sec/hr), were discarded for all experimental animals. This was done to allow the animal sufficient time to become comfortable with the apparatus and to rule out the potential confound of handling stress.

- Erroneous 'blips' in the [O_2] data were manually excluded. During the course of measurement in a given respirometry experiment, the fish in the chamber would occasionally bump into the O_2 probe, causing a brief 2-5 second period of artificially high data points; These were very obvious in a plot of [O_2] over time; their removal was in line with good experimental practice and found to have only a marginal affect.

- The slope of the least squares line of best fit was found for each 15 minute measuring period. Since the respirometer cycled between 15-minute cycles of 'measure' and 'flush', the flush data was omitted. Generally, this resulted in 8 oxygen concentration slopes ($\frac{d[O_2]}{dt}$) per animal per respirometry session; R^2 values were noted for the least squares fit line, and all 8 of the slopes and R^2 values were averaged to give a single value for $\frac{d[O_2]}{dt}$ per animal per session. Given that several respirometry sessions produced unusable data owing to experimenter error (failure to notice bubbles trapped around O_2 probe), a sensible R^2 threshold for the least squares line fit to the $[O_2]/time$ data ($R^2 \geq 6$) was determined for inclusion in final analysis.

- The metabolic rate (MO_2) was determined for animal using the following equation:

$$MO_2 = \frac{d[O_2]}{dt} \cdot \frac{volume}{wet\ weight} \tag{7}$$

  In this equation, MO_2 is oxygen consumption $\left(\frac{mg}{kg \cdot hour}\right)$; $\frac{d[O_2]}{dt}$ is the rate of change of $[O_2]$ $\left(\frac{mg}{l \cdot hour}\right)$; *volume* is the respirometer volume minus the volume of the fish (l); and *wet weight* is the wet weight of the fish (kg). These values were then opened in JMP 8 software (SAS institute) to perform formal analyses.

All of the following statistical tests were performed using JMP 8 unless otherwise noted. In JMP,

- One-way, fixed-effect model Analyses of Variance (ANOVA) were performed to compare mean percent weight changes between and within *stocks* and *feeding conditions*.

- One-way ANOVAs were likewise performed to compare mean metabolic rate (MO_2) between and within *stocks* and *feeding conditions*.

- A two-way ANOVA was used in to determine whether the simultaneous influence of variables *stock* and *feeding condition* was influencing MO_2 in a non-additive way; that is to say, to determine presence or absence of an interaction effect.

- For all ANOVAs, F-tests are used to test the null hypothesis that group means are equal; results were said to be significant (group means unequal) when the probability of obtaining a F-test statistic at least as extreme as the one observed was less than 5%, or $\alpha = 0.05$. Since F-tests are extremely sensitive to non-normality, a Shapiro-Wilk test was used to test the null hypothesis that data fit the normal distribution.

Gene expression measures

Before each qPCR reaction, the contents of each well (target gene, reference gene, or control) and the efficiency of primers were specified using the Roche LightCycler 480 software. The 'relative quantification' was specified under the 'analysis' menu and the calculation of normalized target gene/reference gene expression ratio for each individual was carried out with efficiency correction automatically by the software (Appendix 2). These expression ratios were taken into JMP where one-way fixed effect model ANOVAs were performed to compare the mean expression ratios for NPY and POMC within *stocks* and *feeding conditions*.

Finally, a derivative plot of the melting curve data (melting peaks) was examined to compare peaks in order to determine the production of contaminating reaction byproducts such as primer dimers, which would appear as an additional peak separate from the main peak corresponding to the desired amplicon (Appendix 2).

Results

Metabolic measures: weight changes, respirometry

Having been weighed both upon isolation from colony tanks and just prior to sacrifice, the percentage change in overall body weight for each animal was determined. The group means for these changes are depicted in Figure 12.

The Analysis of Variance F-test operates on several assumptions; foremost among this is the assumption that the data are normally distributed. In the Shapiro-Wilk goodness-of-fit (W) test, small p-values reject the null hypothesis that the data are normally distributed. When performed on the distribution of percent weight changes, $W=0.9566$, $p=0.2525$. Thus, at the 5% confidence level the conclusion that all MO_2 data come from the normal distribution is supported.

Overall, percent weight changes were significantly different between the three conditions ($F(2,27)=34.268$, $p<0.0001$); fed fishes had a slight net increase in percentage weight change, while starved and brooding fishes had large percentage decreases in weight change.

Lab stock and wild stock fishes in either the fed or the brooding condition did not differ significantly from each other in percent weight change. Between the two stocks in the starved condition, however, the percent weight changes were significantly different ($F(1,8)=8.563$, $p=0.033$). This indicates that over the course of the experiment, laboratory stock fishes lost more weight than wild stock fishes. The brooding fishes of each stock do show a similar trend in this respect, but the difference here was not significant.

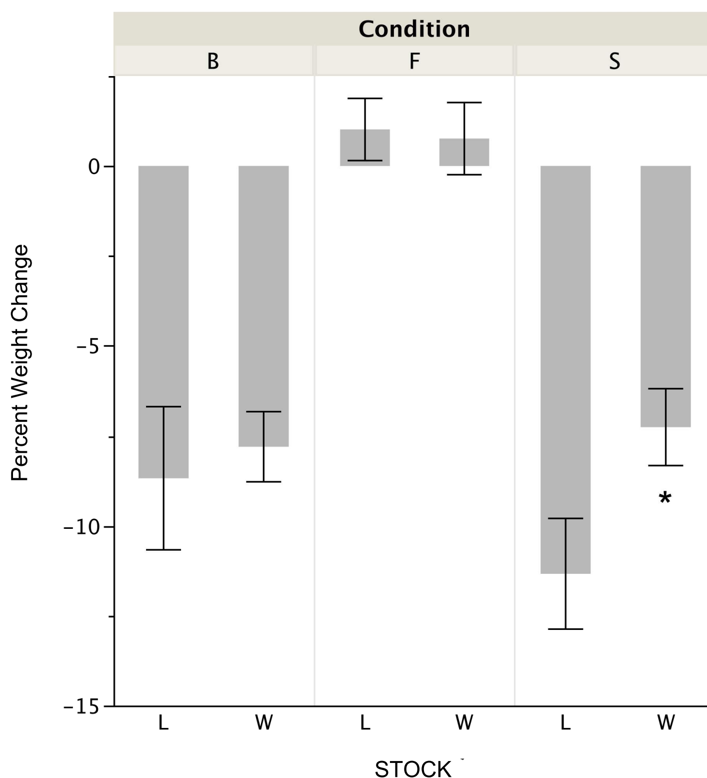


Figure 12. Mean percent weight changes between *stock*, *condition*, and *week*. Here and in the data presentation that follows, letters **B, F,** and **S** represent conditions 'brooding,' 'fed,' and 'starved', while letters **L** and **W** represent 'laboratory stock' and 'wild stock'. Error bars indicate one standard error. Asterisks indicate significance, where $\alpha = 0.05$.

For the respirometry data, Shapiro-Wilk goodness-of-fit (W) tests were used to see whether the MO_2 data are normally distributed. The first week MO_2 measures were found to come from the normal distribution (W=0.9450, p=0.358); the same was concluded for the second week MO_2 measures (W=0.9247, p=0.157).

Descriptions of these data will proceed from the general to the more specific. Overall MO_2 measures between the stocks, averaged between the two weeks, were significantly different (F(1,17)=5.5042, p=0.0322). This indicates that lab stock and wild stock fishes had different metabolic rates regardless of condition. Overall MO_2 measures between the conditions, averaged between the two weeks (F(2,17)=5.2858, p=0.0183), indicates that fishes had different metabolic rates between conditions when the stock of the fish is not considered. Overall percent change in MO_2 was significantly different from week 1 to week 2 (F(1,17)=6.754, p=0.0194) indicating a significant change in MO_2 from week 1 to week 2 regardless of stock or condition.

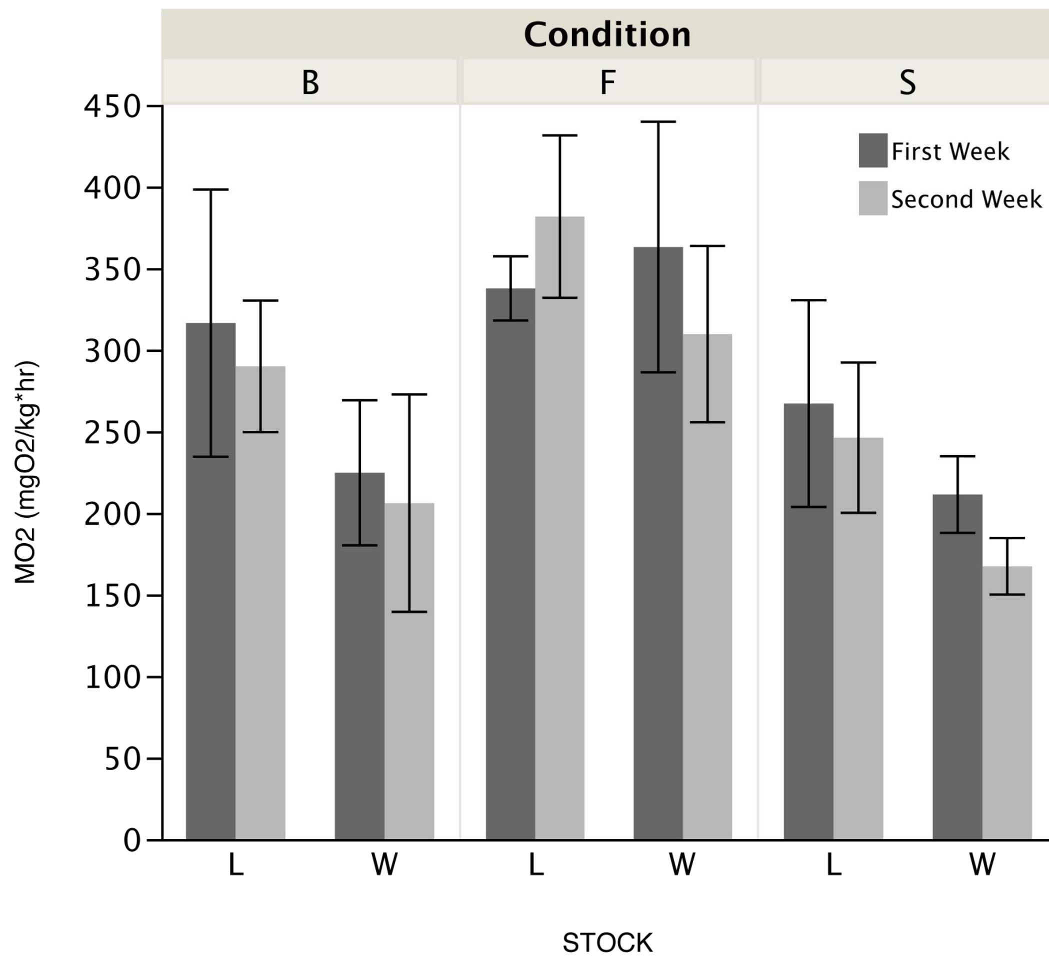


Figure 13. Metabolic rate between *stock*, *condition*, and *week*. Graph encompasses all respirometry results. Graph is partitioned into thirds by *condition* (**B/F/S**); within each condition, *stock* (**L/W**) is shown (paired bars). Dark bars represents mean MO_2 at week 1; light bars represent mean MO_2 at week 2. Error bars indicate one standard error.

Lab stock and wild stock fishes in the starved condition (depicted in the rightmost third of Figure 13) differed importantly in their MO_2 ($F(1,6)=3.26$, $p=0.131$). Though not significant at the 5% level this trend appears graphically in Figure 13, indicating that wild stock fishes have lower metabolic rates than lab stock fishes under starvation conditions. Put another way, there is a 83% chance that the variation in mean MO_2 between starved lab stock and starved wild stock fishes is statistically meaningful. A similar trend is seen for lab stock and wild stock fishes in the brooding condition (depicted in the leftmost third of Figure 13), but neither is it statistically significant ($F(1,5)=1.20$, $p=0.3536$).

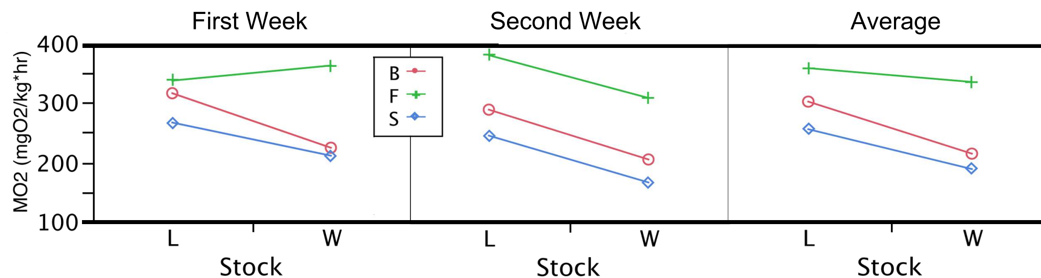


Figure 14. Metabolic rate interaction-effect plots for *condition* and *stock*. Least-squares mean plots for week 1, week 2, and averaged-weeks MO_2 measures.

A two-way ANOVA was used to examine the influence of both independent variables, stock and condition, on MO_2 measures. These data are presented in Figure 14 for MO_2 measures during the first and second weeks, and then MO_2 measures when the weeks are averaged. There was determined to be no significant interaction effect between condition and stock on MO_2 regardless of week. The value for the first week is reported here, F(2,15)=0.7731, p=0.4833.

Zooming in on the the changes in MO_2 between weeks 1 and 2 under the starvation condition (Figure 15), an important difference becomes apparent. Between weeks 1 and 2, mean MO_2 for wild stock fishes in the starved condition decreased by a significantly greater percentage ($\sim$ 25%) than that of lab stock fishes in the starved condition ($\sim$ 6.5%) (F(1,5)=6.914, p=0.0466). This indicates that the wild stock fishes were able to lower their metabolic rate significantly more than lab stock fishes in response to starvation conditions.

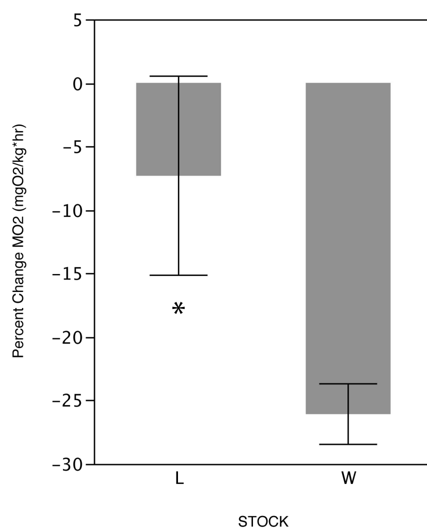


Figure 15. Metabolic rate changes between *stocks* in the *starved condition*. Error bars indicate one standard error. Asterisks indicate significance when $\alpha = 0.05$.

qPCR: differential expression of NPY, POMC

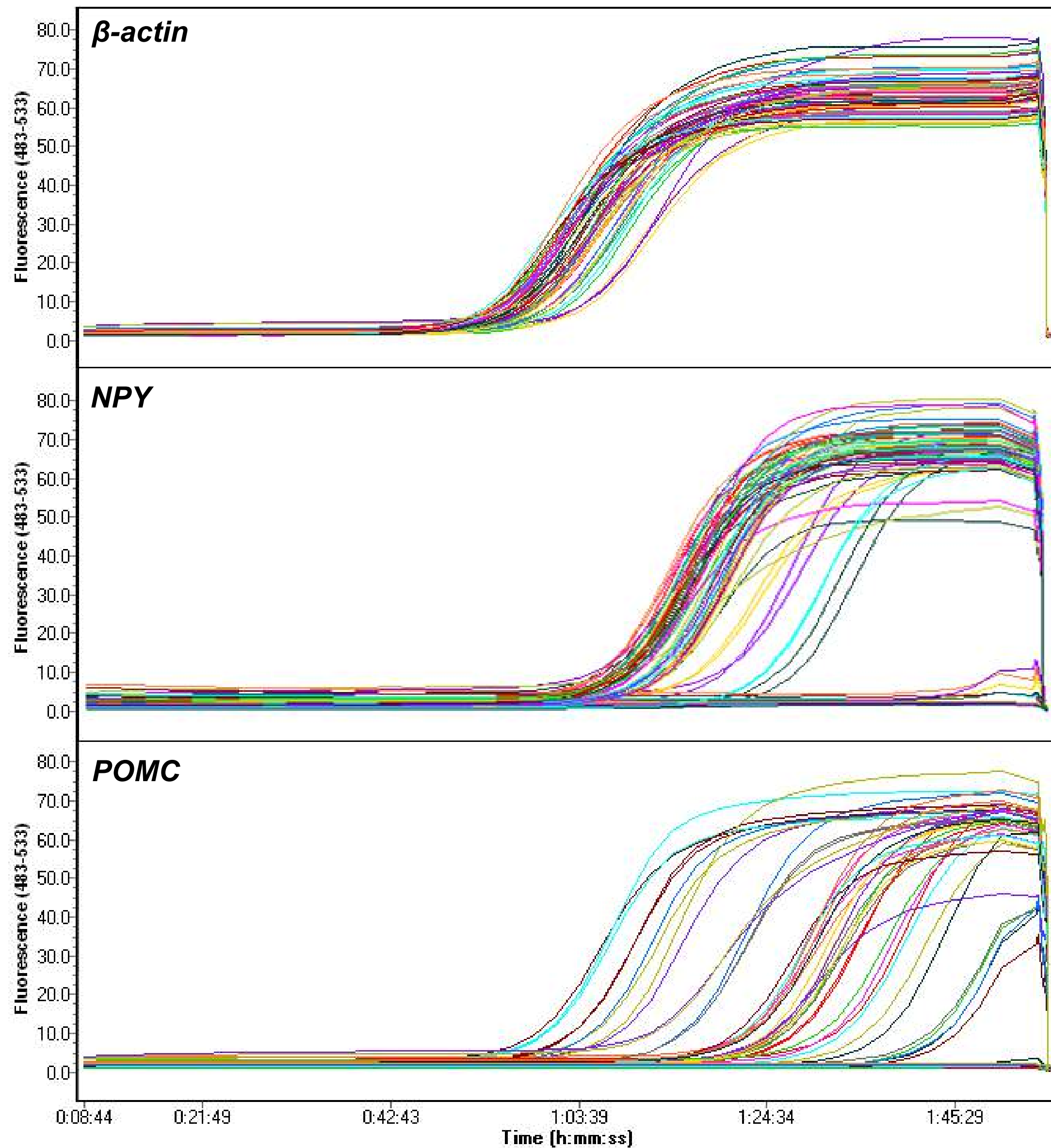


Figure 16. The fluorescence time-course of qPCR for target and reference genes. Three gene products: β-actin (top), NPY (middle), and POMC (bottom). Fluorescence (vertical axis) is plotted against reaction time (horizontal axis) on a logarithmic scale; during the exponential amplification phase, the target nucleic acid doubles with every cycle. The fluorescence that appears at bottom of the NPY plot indicates noRT controls; however, that which appears along the bottom of the POMC plot indicates failed reactions.

RNA isolation yields were reasonable in quantity and quality (see Appendices). qPCR was used to compare changes in gene expression ratios—ratios of the two target genes, NPY and POMC, to the reference gene β-actin—between fishes of different stocks and in different conditions. The Roche LightCycler 480 calculated these ratios and corrected for different primer efficiencies as described in the Materials and Methods section. Logarithmic plots of fluorescence by reaction time for each neuropeptide primer over cDNA for all experimental fishes are shown in Figure 16. Visual judgments indicate that β-actin saw the highest expression levels, followed by NPY, with POMC seeing the least expression.

Differences between stocks and conditions in their average expression ratios of NPY and POMC were calculated using one-way ANOVAs (Figures 17 and 18). These analyses revealed higher NPY expression in starved lab stock fishes compared to starved wild stock fishes bordering on statistical significance (Figure 17: $F(1,9)=4.3025$, $p=0.068$). Lab stock and wild stock fishes in the brooding condition exhibited a similar, if less statistically compelling, trend (Figure 17: $F(1,8)=1.7097$, $p=0.238$). NPY expression ratios between the stocks in the fed condition did not differ significantly. Indeed, NPY expression did not differ between brooding, starved, or fed lab stock fishes, suggesting that during brooding and starvation, lab stock NPY expression is the same as during fed conditions.

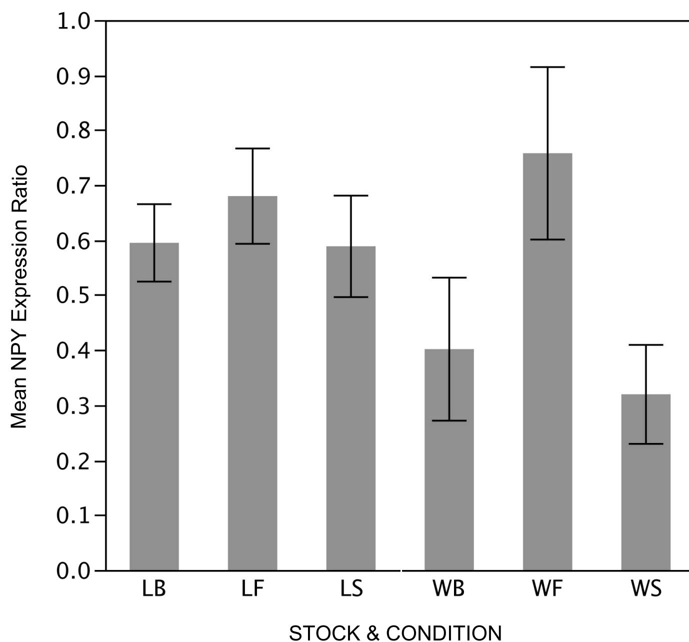


Figure 17. NPY expression ratios (NPY/β-actin) for all experimental animals. Letters **L** and **W** indicate lab and wild stock, respectively; letters **S, F,** and **B** indicate starved, fed, and brooding conditions, respectively.

Expression ratios for POMC were largely unreliable, as indicated by the standard error bars in Figure 18. While there appears to be differential expression, failed reactions resulted in a reduced sample size (N=17) for this test, and POMC ratios did not differ between stocks or conditions.

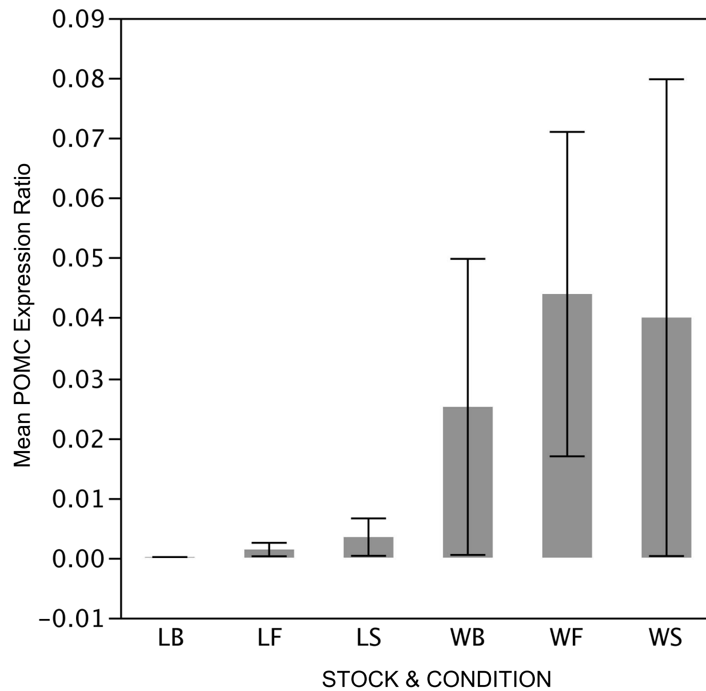


Figure 18. POMC expression ratios (POMC/β-actin) for 17 experimental animals. Letters **L** and **W** indicate lab and wild stock, respectively; letters **S, F,** and **B** indicate starved, fed, and brooding conditions, respectively.

The melting curves for each reaction were examined to assess whether or not any contaminating products were formed, the fluorescence of which could lead to artificial results. β-actin and NPY amplicons both melted consistently at 84 °C; the melting curve for POMC PCR amplicons was not as clean, though still with a single peak at ~88.5°C (Appendix 2). The differences in the melting peaks are consistent with amplicon size and %GC content (Table 1).

Discussion

The intended end of this research trajectory was a characterization of the metabolic and feeding-regulatory changes brought about by brooding-entailed starvation between two genetically distinct lineages of a single Haplochromine cichlid species. This was motivated by a larger purpose—the improved understanding of metabolism and feeding regulation in fishes generally using a superlatively diverse and evolutionarily interesting model organism. Members of family Cichlidae, including *Astatotilapia burtoni*, offer themselves up as a promising model for many research programs; given the incomplete understanding of feeding regulation—in vertebrates generally and fish specifically, discussed at length in the introduction—*Astatotilapia burtoni* and related species are relatively untapped resources for scientific discovery. Seen in this light, behavioral polymorphisms of a single species arising accidentally from laboratory living conditions serve as an artificial 'mutant screen' within the larger natural 'mutant screen' afforded by the recent and rapid radiations of these fishes in the Great Lakes of East Africa. Working backward from studies in *A. burtoni* in this way is sure to offer many novel insights into the control of feeding and metabolism in fishes at large and vertebrates in general.

To these ends, the study has produced results that support its initial hypotheses:

- During conditions of starvation (brooding-induced or experimentally controlled), the lab stock *A. burtoni* do not appear able to appropriately reduce their metabolic rate and so expend more energy during starvation.

- Feelings of hunger—the downstream manifestations of melanocortin signaling—likely influence poor broodcare motivation observed in lab stock *A. burtoni*.

In the following sections, specific results will be adduced to justify these conclusions and shortcomings will be citing that might prevent our full claim to them.

Differences in metabolism

Female fishes of the laboratory stock responded markedly to feeding cessation when compared to females of the wild stock. These laboratory stock fishes were found to lose more weight in the absence of food than their wild counterparts. This suggests that homeostatic mechanisms underlying metabolism are better regulated in wild stock females than in lab stock females, causing the former to maintain closer-to-constant body weight and energy expenditure across conditions. Maintaining a balance in energy expenditure is important for the ability to cope with environmental stressors, such as the deprivation of food, in order to ensure survival in the wild. The trait of more labile body weight on the part of the lab stock may result from the relative domestication they have experienced through years of inbreeding in the laboratory. This is to say, labile body weight could result from captivity-specific selection pressures put on the stock over many generations in a laboratory setting. As fishes in the lab are fed daily, having a food source that is both regular and certain could decrease the selective importance of traits that promote survival in wild conditions where food is scarce.

Both starved and brooding females lost weight in the absence of feeding, but only in the former was this weight loss significantly different between the stocks. Why did the brooding females not differ in percentage weight change wile the starved fishes did? As described in the Materials and Methods section, the brooding fishes were fed ad libitum each day. Perhaps, when the fry reached full development, they would spit them out and resume feeding, only to take them back up afterward. This was never observed to have happened, but it is known to occur and has support in the literature—recall findings by Mrowka [1986] that satiation restores brood care motivation. An alternative explanation is that something related to the physiology of reproduction is affecting the body weight of the organism—several reproductive hormones have been proposed to play a role in fish feeding; if so, these could counteract potential irregularities in other hunger or satiety signaling.

If the loss of proper metabolic control is a factor contributing to observed differences in maternal behavior between the two stocks, then metabolic rates for brooding lab stock females will be higher than those of brooding wild stock females. Trends revealed by respriometry are consistent with hypotheses that laboratory stock females have higher metabolic rates than wild stock females under starvation conditions; as observed in the percentage weight-change data, brooding females did not differ substantially in metabolic rate between stocks. At least, though, there is consistency

here—as detailed in the introduction, the two chief determinants of body weight changes are energy input and energy expenditure. There would be cause for concern If, in absence of energy input, we observed significant weight loss under conditions of low metabolic rate. The significantly greater decrease in metabolic rate seen in starved lab stock females relative to starved wild stock females in just one week's time is compelling evidence that the wild stock is better able to control their metabolic rate in physically stressful conditions.

One potential shortcoming of the determination of metabolic rate from respirometry in brooding fishes is that the respiration of the offspring certainly contributes to the measured O_2 consumption; perhaps it does so to a significant degree [Steffensen, 1989]. If this is the case, however, the oxygen usage of brooding mothers is actually lower across the board. Barring substantial differences in brood size, this is unlikely to be a considerable confound for the conclusions drawn herein.

Differences in melanocortin signaling

Relative expression levels of two neuropeptides known to have integral and opposing effects in the tangle of neural circuitry implicated in the regulation of feeding—the orexigenic melanocortin signal neuropeptide Y (NPY) and its anorexigenic melanocortin counterpart pro-opiomelanocortin (POMC)—were quantified using real time qPCR, where $ACTB$ (β-actin) provided the reference expression. Primer efficiencies were not determined directly by the author; they were, however, determined in the same laboratory and using cDNA from organisms of the same species and generation. Reduced NPY expression in wild stock females from the starved and brooding conditions narrowly escaped significance at the 5% level, but the strong trend is still telling. More telling still, NPY expression did not differ between brooding, starved, or fed lab stock fishes, suggesting that during brooding and starvation, NPY expression is the same in the laboratory stock as it is during fed conditions. It is easily deduced from these findings that NPY, a potent appetite stimulating neurotransmitter in fishes, is not regulated in the same way between stocks and may be contributing to the laboratory stock phenotype of overly-hungry motherhood.

There is, as always, a caveat emptor for these seemingly acceptable results: in addition to its orexigenic properties, NPY also is known to have effects that work to suppress metabolism (see pg. 23). While this makes good sense—released in times of nutritional want, NPY should encourage energy intake while discouraging its expenditure—it must be reconciled with observations that metabolic rate remained

high in starved laboratory stock females and was found to be lower in starved wild stock females.

It is important to recognize that, despite its central role in the control of feeding, NPY is only a small piece of a much larger puzzle (Figure 7); perhaps the metabolism-increasing effects of decreased NPY expression observed in starved wild stock females are offset by the smooth functioning of other metabo-suppressive mechanisms. If this is the case, a new hypothesis emerges: since laboratory stock females maintained higher metabolic rates in the face of increased NPY expression, something involved in NPY's downstream metabolic signaling has gone awry. This account, leaving room for the proper function of NPY's downstream orexigenic pathway, is in light with the present findings and suggests a future direction for this line of research.

That the reaction to determine the differential expression of POMC between stocks largely failed may not be particularly important—fasting experiments in other fish species have failed to show alterations in POMC mRNA levels, and suggest that it may not have direct actions at all; instead its actions may be regulated at the posttranscriptional level [Cerda-Reverter et al., 2003]. It is actually α-MSH that is processed from POMC and which binds to MC3R and MC4R to inhibit food consumption. Since POMC is processed into other products that have other functions unrelated to the control of feeding, such as ACTH and β-endorphin, perhaps its expression levels reflect the regulation of other body systems. In fishes at least, quantifying POMC mRNA may not be equivalent to quantifying the levels of satiety induced by α-MSH.

Setbacks; looking forward

Originally, a larger sample size was desired for each feeding condition; several stumbling-blocks along the pathway of experimentation kept them smaller than would be ideal.

Five of the six respirometers in the laboratory of Suzy Renn were brand new in the box. While this seems like a good thing, it was quickly discovered that they were not recognized by the computers—the power light on the respirometery hardware would light up, but the computer gave no indication that the device was plugged into it. After speaking at length to a LoligoSystems representative at odd hours (they are headquartered in Denmark), it was determined that the devices were not properly activated upon leaving the factory. Though no one at Loligo had ever encountered this problem before, after some troubleshooting in a program called myPCLab (Novus) under the guidance of Chris Wellstood, everything was operational. Unfortunately, this delay set respirometric measurements back almost two months.

During the first respirometry session, another unforeseen setback befell the design—a flush pump quit working, reducing the number of fishes per cohort from 6 to 5. A few times during the earlier respirometry sessions, experimenter error led to air-bubbles being trapped near the O_2 probe, resulting in bad data and prompting the exclusion of entire individuals from the respirometry analysis. These and other factors surely contributed to the outcome of this experiment.

In the future, research along these lines could be simply and beneficially emended in several ways. Sacrifices could be conducted after the first week as well as the second to better chart the gene expression changes that are occurring between the conditions. Other tissue types could be collected, such as the liver—where leptin is known to be synthesized in fishes—and the ovaries, to examine potential influences on these fronts. Localization of gene expression within the brain would allow for an examination of differences between the brainstem and the hypothalamic melanocortin systems, and even intra-hypothalamic differences between the various nuclei. Finally, a consideration of more feeding-related peptides, particularly those just upstream or downstream of the central melanocortin system, will be crucial.

In fine

An examination of maternal mouthbrooding in the East African cichlid fish *Pseudocrenilabrus multicolor* was carried out by Wolfgang Mrowka during the late 1980s; his findings revealed that this reproductive-care strategy occasions two extremely different periods in the life-history of these females. Between brooding periods, these fishes exhibit intense food intake and high energy turnover,reflected in the present experiment by the high oxygen consumption of non-reproductive females in the *fed* condition. Part of the food they consume during this "investment phase" contributes to maintenance, part of it to growth, part of it to the production of new eggs within the ovary, and part serves as energy storage for use in subsequent brooding cycles. The "consumption phase" (mouthbrooding itself), is characterized by the cessation of feeding, low energy turnover, and the utilization of stored energy investments made earlier on [Mrowka and Schierwater, 1988]. Support for this phase distinction was found in *A. burtoni* females of the *brooding* condition in the present study. There is evidence that mouthbrooding in cichlid fishes evolved from substrate-brooding, where cichlids lay their eggs in the open, on rocks, leaves, or logs. [Keenlyside, 1991]. Synchronous changes in the allocation of energy expenditure to the different stages of the reproductive cycle might have played a significant selective role during the

evolution of this important behavior.

In humans, the central melanocortin system is the best-characterized neuronal pathway involved in the regulation of energy homeostasis. This system, outlined in no small detail in the Introduction, is a collection of circuits unique in its capacity signals from a wide array of hormones, nutrients, and afferent neural inputs related to feeding. It is likely to be involved in integrating long-term hunger adipostatic signals such as those from leptin and insulin, primarily received by the hypothalamus, with more acute signals regulating to hunger and satiety, which are primarily received by the brainstem. The central melanocortin system is also unique from a regulatory viewpoint—it is composed of fibers expressing *both agonists and antagonists* of melanocortin receptors, and the positioning of the POMC and NPY/AgRP cell bodies adjacent to the median eminence, and of brainstem POMC cell bodies adjacent to the area postrema, imply that this system may be actively sampling blood-borne hormone and nutrient concentrations. Notably, there are reciprocal projections from the hypothalamic and brainstem sites of melanocortin cell bodies to integrative and motor centers with high densities of MC4R expression, such as the PVN and DMV (Figures 5, 6). All of this supports the idea that this system is ultimately important in the integration of many afferent inputs with behavioral and autonomic responses that adjust energy intake and expenditure in order to maintain energy homeostasis.

However, the efferent pathways downstream of the melanocortin system have only been partially characterized, and few details exist regarding the mechanisms by which the melanocortin system is able to store information about levels of energy stores and to integrate this information with output to affect food intake and energy expenditure. Given that the central melanocortin system is an active target area for the development of drugs to treat obesity, diabetes, and cachexia, it is important to understand the system in its full complexity, including the possibility that these circuits have roles in cardiovascular, natriuretic, and reproductive function [Cone, 2005]. Many neuropeptides implication in metabolism and feeding regulation are conserved throughout vertebrates, from fishes to mammals. Indeed, it has recently been demonstrated that NPY melanocortin signaling functions in tilapia as it does in mammals [Peddu et al., 2009], legitimizing the present study and bolstering evidence for its larger biological relevance.

This study set out to determine from the top-down whether there are whole-organism differences in metabolism and feeding regulation between laboratory stock and wild stock *A. burtoni* females undergoing a period of starvation, and whether brooding-induced starvation affects female *A. burtoni* differently than non-brooding

starvation. Gross weight changes, measurements of metabolic rate, and assays for differential gene expression provided three levels from which to zero-in on the mechanisms of metabolism during mouthbrooding. Alongside a control group of *A. burtoni* females who were fed as usual, this experimental tack allowed for the diagnosis of metabolic irregularity and separated the reproductive context of brooding from the act of starvation. Since the melanocortin system is the final destination of feeding signals broadcast bodily, a future objective of immediate interest is to home in on these more acute signals to try to determine the root of the regulatory impairment. This bottom- up aim was not addressed in the present study, but the results presented herein are foundational in kind; they represent a logical starting-place to begin to tease apart the intricacies of feeding and its relationship to behavior.

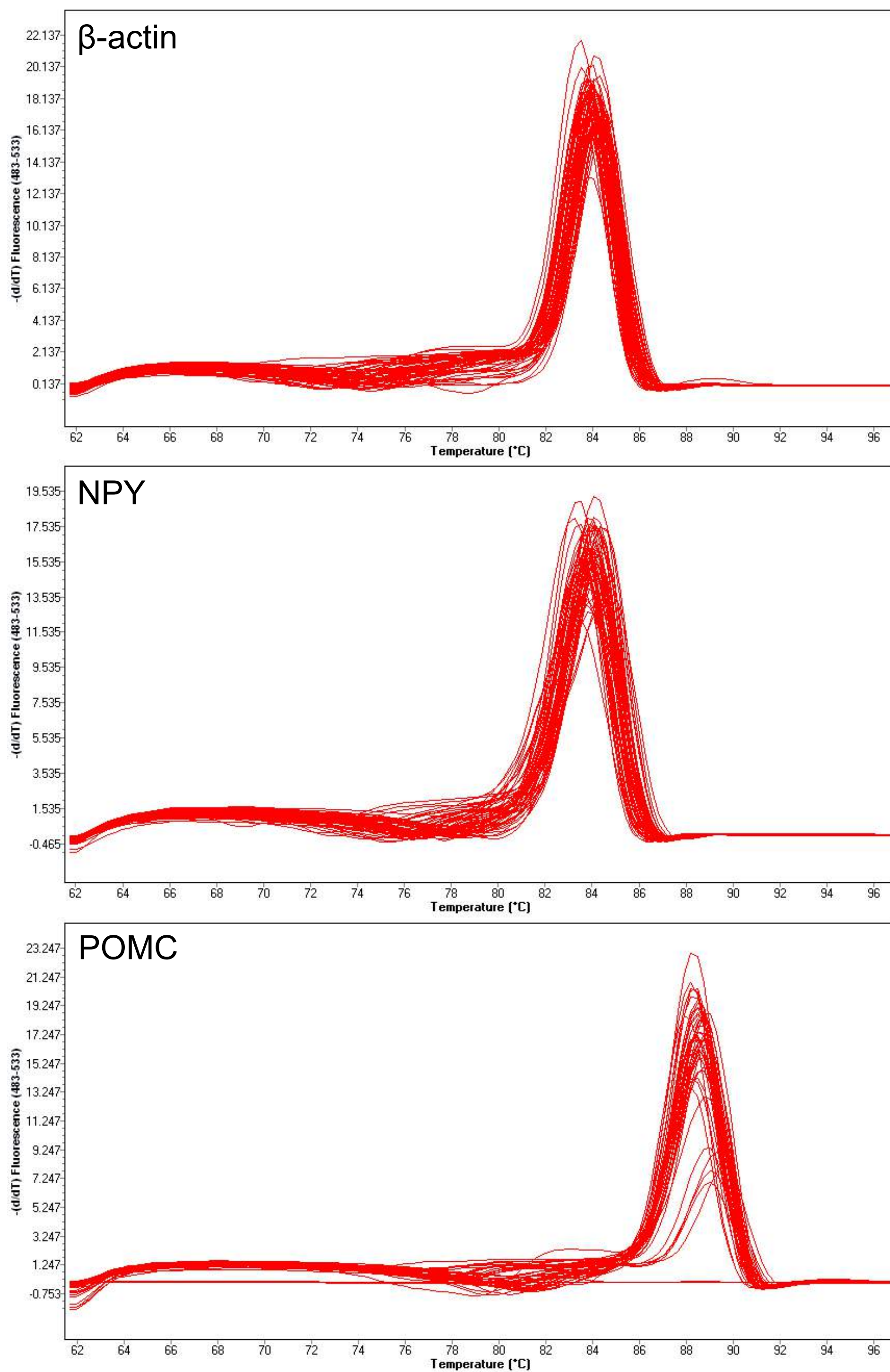
Appendicies

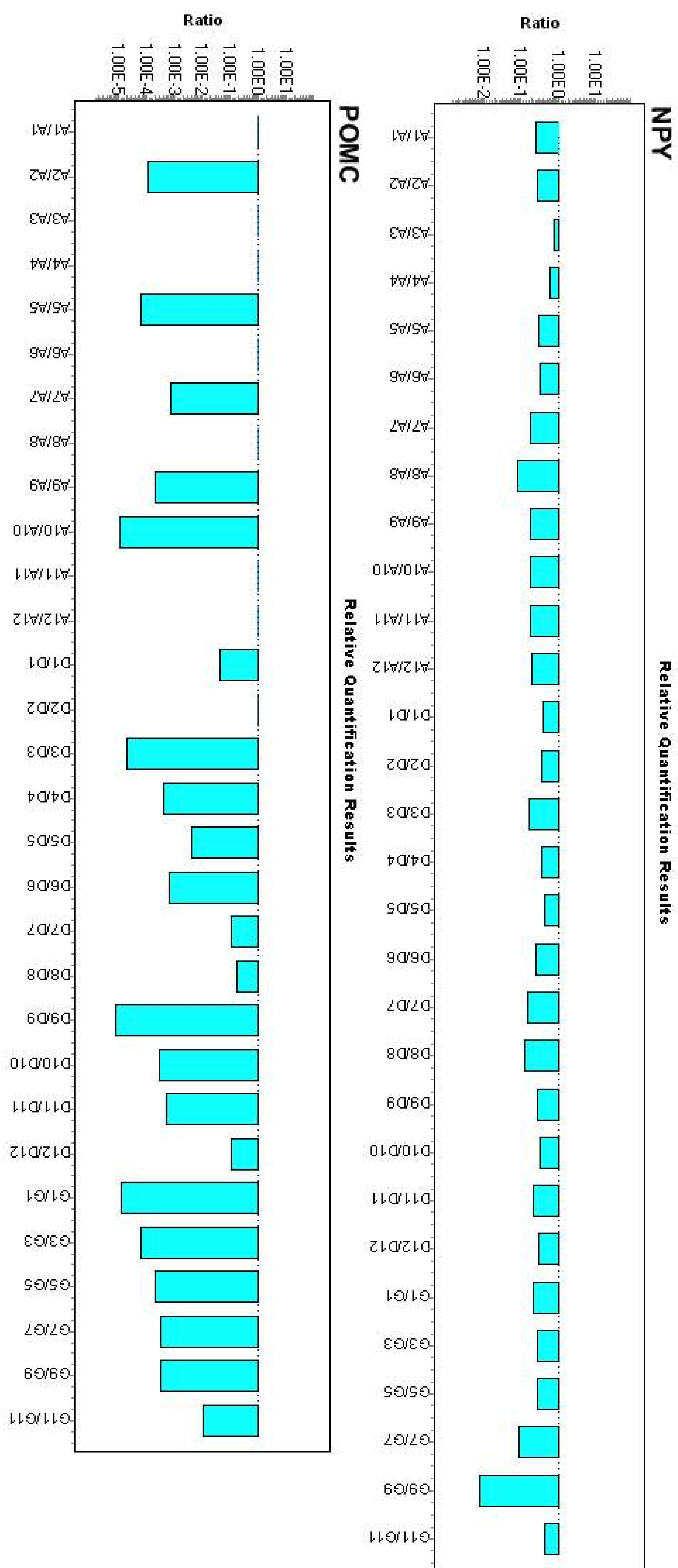
Appendix 1: RNA quantity and quality

Fish ID	Concentration (ng/μl)	260/280	260/230
LS1	267.3	1.74	0.53
LB1	383.3	1.83	0.69
WB1	179.8	1.68	0.45
WF1	279.4	1.75	0.51
WS1	297.5	1.76	0.57
LF2	197.4	1.89	0.78
WB2	268.3	1.98	1.07
WS2	121.4	1.98	0.88
LS2	89.6	1.90	0.90
WF2	192.8	1.88	0.85
LB3	92.5	1.75	0.49
LF3	305.4	1.98	1.04
WF3	146.6	1.95	0.57
WB3	117.1	1.85	0.60
WS3	410.1	1.96	0.82
LB4	356.1	2.03	0.85
LF4	4.83	2.03	1.73
LS4	309.5	1.93	1.00
WB4	612.5	1.83	1.71
WS4	171.5	1.95	0.85
LF5	242.9	1.99	1.31
LB5	184.2	1.94	1.25
LS5	165.3	1.79	0.57
WS5	75.1	1.86	1.15
WF5	164.3	1.93	0.93
LS6	95.8	2.00	1.43
WF6	84.2	1.99	1.22
LF6	75.2	1.97	0.82
WB6	222.1	2.05	1.19
WS6	191.9	2.00	0.85

Appendix 2: Roche LightCycler 480 (qPCR) output

Melting curves; qPCR output (next page)




NPY
Relative Quantification Results
Ratio
1.00E-2
1.00E-1
1.00E0
1.00E1
A1/A1
A2/A2
A3/A3
A4/A4
A5/A5
A6/A6
A7/A7
A8/A8
A9/A9
A10/A10
A11/A11
A12/A12
D1/D1
D2/D2
D3/D3
D4/D4
D5/D5
D6/D6
D7/D7
D8/D8
D9/D9
D10/D10
D11/D11
D12/D12
G1/G1
G3/G3
G5/G5
G7/G7
G9/G9
G11/G11
POMC
Relative Quantification Results
Ratio
1.00E-5
1.00E-4
1.00E-3
1.00E-2
1.00E-1
1.00E0
1.00E1
A1/A1
A2/A2
A3/A3
A4/A4
A5/A5
A6/A6
A7/A7
A8/A8
A9/A9
A10/A10
A11/A11
A12/A12
D1/D1
D2/D2
D3/D3
D4/D4
D5/D5
D6/D6
D7/D7
D8/D8
D9/D9
D10/D10
D11/D11
D12/D12
G1/G1
G3/G3
G5/G5
G7/G7
G9/G9
G11/G11

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