

Characterization of appetite-regulating factors in platyfish, *Xiphophorus maculatus* (Cyprinodontiformes Poeciliidae) and cunner, *Tautoglabrus adspersus*

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Abstract:

The regulation of energy in fish, like most vertebrates, is a complex process that involves a number of chemical signals originating and networking in different parts of the brain and throughout the body. These signals include anorexigenic hormones that suppress feeding and hunger [e.g. cholecystokinin (CCK) and cocaine-and amphetamine-regulated transcript (CART)] as well as orexigenic peptides that stimulate feeding behaviour and food intake [e.g. orexin and neuropeptide Y (NPY)]. Platyfish, *Xiphophorus maculatus*, are freshwater viviparous fish found in tropical waters from South America to northern Mexico. Although these fish have been the object of numerous physiology and behavioural studies, very little is known about the endocrine mechanisms regulating their feeding. In order to elucidate the role of these peptides in the regulation of feeding, we examined the effects of peripheral injections of CCK and orexin on feeding behaviour and food intake. Injections of CCK decreased both food intake and searching behaviour, while injections of orexin increased searching behaviour but did not seem to have an effect on food consumption. In order to better characterize these peptides, we performed tissue distribution and gene expression studies. Tissue distribution studies show that CCK, CART, NPY and orexin all show a widespread distribution in brain and several peripheral tissues, including intestine. In addition, we compared the expression of these peptides in brain and gut between fed and 10-day fasted platyfish using qPCR. Fasting caused decreases in both CCK and CART mRNA expressions in the brain and a decrease in CCK expression in the intestine. There was also a significant increase in orexin mRNA expression in the brain as a result of fasting. Cunnners, *Tautoglabrus adspersus*, are cold-water oviparous (egg laying) marine fish that can be found from Northern Newfoundland to all along the coast of the western North Atlantic. In cunner, fasting for 10 days caused a decrease in CCK in the brain, and a significant increase in orexin expression in the brain. Fasting had no significant effect on either NPY or CART expression. Furthermore, fasting had no effect on the expression of the peptides studied in the intestine. We also compared the expression of these peptides as a result of fasting in males and females of both species. In platyfish, there was no significant gender specific differences found in the expression of the peptides. In cunner, females showed a significantly higher NPY expression in the brain than males, although this was unique to

NPY. The widespread distribution and the fasting-induced changes in expression of these peptides suggest that they might have several physiological roles in platyfish, including the regulation of feeding.

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Table of contents:

| | |
|--|----|
| Introduction | 1 |
| Differences in reproductive strategies and energy allocation in fish | 2 |
| Differences in energy allocation with gender | 4 |
| Central and peripheral control of appetite regulation | 6 |
| Neurological control | 6 |
| Peripheral control | 7 |
| Cholecystokinin | 7 |
| Neuropeptide Y | 9 |
| Orexin | 10 |
| CART | 12 |
| Species used in this study | 14 |
| Platyfish | 14 |
| Cunners | 15 |
| Project Objectives | 17 |
| Materials and Methods | 18 |
| Study Animals | 18 |
| Platyfish | 18 |
| Tissue collection | 19 |
| RNA Extraction | 19 |
| cDNA synthesis | 20 |
| Tissue Distribution | 20 |

| | |
|--|----|
| IP study animals | 21 |
| Fasting study animals | 22 |
| Cunners | 23 |
| Fasting study animals | 23 |
| Tissue collection | 24 |
| Real-time quantitative PCR | 24 |
| Real-time quantitative PCR data analysis | 25 |
| Statistical analysis | 25 |
| Results | 29 |
| Characterization of Appetite Regulators in Platyfish | 29 |
| Intraperitoneal Injections – Food Intake/Search Behaviour | 29 |
| Intraperitoneal injections – CCK and orexin | 29 |
| Tissue Distribution | 33 |
| Tissue distribution studies – orexin, NPY, CART, CCK | 33 |
| Orexin | 34 |
| NPY | 34 |
| CART | 34 |
| CCK | 35 |
| Effects of fasting and gender on gene expression in platyfish & cunner | 37 |
| Effects of fasting on transcript expression in brain & intestine – platyfish (males & females) | 37 |
| Effects of fasting on mRNA expression in brain & intestine between sexes – platyfish | 39 |
| Effects of fasting on transcript expression in brain & | |

| | |
|--|----|
| Intestine – cunner (males & females) | 41 |
| Effects of fasting on mRNA expression in brain & gut between sexes – cunner | 43 |
| Discussion | 45 |
| Intraperitoneal (IP) injection studies | 45 |
| Tissue Distribution | 46 |
| Effects of fasting on expression of appetite regulators | 48 |
| CCK | 48 |
| NPY | 49 |
| Orexin | 50 |
| CART | 51 |
| Influence of gender on feeding and feeding responses | 54 |
| Conclusions | 56 |

Introduction:

Energy regulation in fish is a complex process that involves a number of chemical signals originating and networking in different parts of the brain and throughout the body (Hélène Volkoff, Unniappan, & Kelly, 2009). Some of these chemical signals are classified as orexigenic, as they act to stimulate hunger and feeding behaviours, whereas others act as anorexigenic compounds, which halt food intake and bestow feelings of satiety. This concept of energy balance is also intrinsically linked with reproduction. Many of the hormones that regulate feeding behaviours also influence reproduction and sexual motivation in vertebrates (J. E. Schneider, Wise, Benton, Brozek, & Keen-Rhinehart, 2013)

As feeding and reproductive processes are both energetically taxing and depend on the availability of resources, animals often have to make a choice between the two (J. E. Schneider et al., 2013). Plentiful food supplies favour reproduction whereas lack of available food resources restricts reproductive processes in favour of other energy demands (Jill E. Schneider, 2004). Furthermore, different reproductive strategies (e.g. egg-laying or internal egg development) lead to different reproductive efforts and different allocations of energy during the life history (Gunderson, 1997). Sexually dimorphic differences in energy expenditure may also occur, as shown in rodents, ranging from differences in the stress response, exploratory and emotional behaviours, as well as feeding behaviours (Øverli, Sørensen, & Nilsson, 2006; Ray & Hansen, 2004; P. A. Russell, 1977; Shors & Wood, 1995).

Feeding and reproduction are both controlled in part by central neural chemical signals that act to regulate energy homeostasis (Hoskins, Xu, & Volkoff, 2008; Mircea, Lujan, & Pierson, 2007). Among these chemical signals are hormones such as cholecystokinin (CCK), neuropeptide Y (NPY), orexin, and cocaine-and amphetamine-regulated transcript (CART).

Differences in reproductive strategies and energy allocation in fish

Few vertebrate groups display the wide diversity in reproductive strategies that fishes do. This diversity involves alternate methods regarding breeding opportunity, fecundity type, oocyte recruitment, spawning pattern, and parental care (Saborido-Rey, Murua, Tomkiewicz, & Lowerre-Barbieri, 2009). In fish, there are two major reproductive modes, oviparity and viviparity.

Oviparity is defined as the spawning of unfertilized oocytes or fertilized eggs and is generally considered to be the ancestral form of reproduction (Lodé, 2012). Oviparity occurs in the majority of fishes, including cunner. It is typically considered to be lecithotrophic reproduction, where laid eggs are provided with an abundant yolk and a chorion and are protected by a robust and complex eggshell (Dumont & Brummett, 1985; Hamlett & Koob, 1999). Although many fish are considered oviparous, the term ovuliparity is generally used where females release ova in the environment, which are then fertilized externally by the male (Lodé, 2012). Fertilization occurs when a male sprays milt on the ova and then development occurs outside the parental body (Lodé, 2012). Oviparity in fish exists as one of two adaptive extremes for supplying the maximum number of offspring into the next

generation, by having high reproductive rates coupled with low survival rates of offspring (Thibault & Schultz, 1978).

Viviparity is defined as live-bearing or maintenance of development by either parent in or on any part of the body and is considered to be a more recent adaptation brought about through an increase in parental investment (Campbell, 1972; Clutton-Brock, 1991; Wake, 1992). All viviparous animals provide protection to the developing embryo, and the young are born at a relatively advanced state of development, avoiding the high mortality rates found by eggs and larvae from oviparous species (Gunderson, 1997). In viviparous fish species, the young develop *in utero* and nutrients are provided by specialized placenta-like structures (e.g. yolk-sac placentas in sharks or trophotaenial placentas in godeids) (Wourms, 1981; Wourms & Lombardi, 1992).

In ovoviviparous (a specific type of viviparity) fish species, such as platyfish, embryonic development and hatching occur within the maternal body, however there is no direct exchange of nutrients between the mother and embryo as the eggs develop using yolk reserves (Thibault & Schultz, 1978).

These two strategies differ with regards to reproductive effort and maternal investment in offspring (Gunderson, 1997). Whereas paternal investment in offspring ends with insemination/spawning, maternal investment ends at spawning for oviparous species and at birth for viviparous fish (Basolo & Wagner Jr, 2006; Schartl, Walter, Shen, Garcia, Catchen, Amores, Braasch, Chalopin, Volff, & Lesch, 2013).

Prior to sexual maturity, fish allocate all energy resources into survival and growth, but once the process of maturation begins, they apportion energy into the production of gametes and other reproductive behaviours. The amount of energy allocated for the reproductive processes depends on the type of reproductive strategy (Saborido-Rey et al., 2009). Viviparity is associated with reduced reproductive effort when compared to oviparity, as they mature later in life and place more energy into growth processes than reproductive processes (Gunderson, 1997) As such, differences in the regulation of energy should be expected between species that display a specific type of parental investment.

Differences in energy allocation with gender

Since males and females have different roles in regards to reproduction and parental care, they may allocate energy resources differently. Except in some species where males show a greater degree of parental care, females generally invest much more energy into offspring than males (Andersson, 1994; Lodé, 2012). The amounts of energy invested in gametes, by males and females is often assumed to differ as females aim to maximize offspring survival while males focus to inseminate as many females as possible (Parker, 1970; Robert, 1972). Specifically, females tend to have a higher rate of gamete biomass production as compared to males (Hayward & Gillooly, 2011). Although some males may not invest energy into the harbouring of young, they invest energy into reproduction in a multitude of other ways – those behavioural, morphological, and physiological: including sperm

production, competition with other males, and reproductive behaviours (Taborsky, 1998).

With regards to appetite regulating hormones, differences in the expression levels of these peptides between genders may reveal differences in the regulation of energy. For example, in rats, food deprived females have an increase in food intake during the re-feeding phase when compared to males (Gayle, Desai, Casillas, Beloosesky, & Ross, 2006). Furthermore, female pregnant rats and humans have higher serum levels of orexin A accompanied by greater daily food intake when compared to males (Kanenishi et al., 2004; Sun, Tian, Yao, Li, & Higuchi, 2006). Female rats also have a higher expression of preproorexin in the hypothalamus when compared to males (Jöhren, Neidert, Kummer, & Dominiak, 2002).

Sexually dimorphic differences in appetite-regulating hormone expression are also found in fish, as can be seen with ghrelin. Ghrelin is a hormone-releasing peptide found mainly in the stomach, as well as the brain and other peripheral tissues (Horvath, Diano, Sotonyi, Heiman, & Tschöp, 2001; Kojima et al., 1999; Parhar, Sato, & Sakuma, 2003). Ghrelin has a number of regulatory actions including energy balance, gastric motility, and feeding behaviour, particularly as an appetite stimulator (Date et al., 2001; Horvath et al., 2001; Unniappan et al., 2002). In the cichlid fish Nile tilapia (*Oreochromis niloticus*), ghrelin mRNA expression is significantly higher in food-deprived sexually mature females compared to males (Parhar et al., 2003). Furthermore, female rainbow trout (*Oncorhynchus mykiss*) have a higher density of ghrelin cells in the stomach than males, suggesting gender-related differences in peptide expression (Sakata et al., 2004).

Central and peripheral control of appetite regulation

Neurological control

Among vertebrates, feeding centres within the brain ultimately control appetite regulation. The brain receives and processes information gathered from metabolic, neural and endocrine signals from the body concerning nutritional status or the presence of food in the gut (Jason, Gillian, & Terence, 2004). Feeding-related signals from the brain consist of neurohormones, which are primarily secreted by the hypothalamus and other regions of the brain, which act directly on feeding centres to stimulate or inhibit feeding. These neurohormones include peptides such as NPY, orexins, and CART.

In early lesion studies in rats, removal of specific areas of the hypothalamus caused the inhibition of feeding (Anand & Brobeck, 1951), suggesting that major feeding centres are located in the hypothalamus. Similarly, in goldfish (*Carassius auratus*), changes in feeding responses are elicited when the inferior lobes of the hypothalamus are either electrically stimulated or lesioned suggesting that the hypothalamus plays a major role in appetite regulation in fish (Demski, 1973; Roberts & Savage, 1978). The hypothalamus produces many neuropeptides that act to inhibit or stimulate feeding.

Peripheral control

Chemical signals from the periphery consist of factors secreted into the bloodstream that bind to hypothalamic receptors via the hypothalamus. These factors, which include nutrients and gastrointestinal (GI) hormones, can either cross the blood brain barrier and act directly on hypothalamic feeding centres or have an indirect effect. The brainstem acts as a relay centre for sensory signals originating from the GI tract to innervate with the hypothalamus (Hélène Volkoff, Unniappan, et al., 2009). Peripheral signals can also be mechanical, e.g. mechanoreceptors sending signals related to distension of the gut to the brain via the vagus nerve. Similar to central hormones, peripheral hormones, which are widely conserved amongst vertebrates, (Hélène Volkoff, Unniappan, et al., 2009) can be classified as orexigenic, which work to stimulate appetite or anorexigenic, which cause appetite suppression. Many gastrointestinal peptides are also synthesized in the brain and are thus often referred to as “brain/gut” peptides (Hélène Volkoff, 2006). Examples of these peripheral hormones are CCK, which is an anorexigenic peptide and NPY, which is orexigenic.

Cholecystokinin

Cholecystokinin (CCK) is a highly conserved anorexigenic peptide found within the gastrointestinal tract (GIT) and brain of vertebrates (Moran & Kinzig, 2004; H. Volkoff, 2006). CCK was first discovered in the digestive tract of dogs, as a

previously unknown peptide that caused gallbladder contractions (Ivy & Oldberg, 1928). Eventually CCK was established as a hormone involved in appetite regulation that stimulates release of pancreatic enzymes. CCK binds to receptors on the pancreas and gallbladder, causing their contractions and aiding in digestion. The presence of fats and proteins in the gut stimulates the release of CCK into the blood stream (Liddle, Goldfine, Rosen, Taplitz, & Williams, 1985; Moran & Kinzig, 2004). CCK also decreases gastric emptying (Raybould & Tache, 1988) and has been shown to have a primary role in suppressing appetite in salmonids injected with CCK (Olsson, Aldman, Larsson, & Holmgren, 1999).

Although produced by both the brain and gut, its primary site of secretion is the enteroendocrine cells of the GIT (Gibbs, Young, & Smith, 1973). CCK is one of many gut hormones that work to influence food intake by signalling satiety (H. Volkoff, 2006). In rats and goldfish, intraperitoneal (IP) injections established the role of CCK as a satiety signal by decreasing feeding (Gibbs et al., 1973; Himick & Peter, 1994). There are many regions of the brain that also contain CCK, including the hypothalamus (Moran & Kinzig, 2004). In goldfish, central injections of CCK have been shown to suppress food intake (H. Volkoff, 2006; H. Volkoff, Eykelbosh, & Ector Peter, 2003). Alternatively, in several species of mammals and fish (Murashita, Fukada, Hosokawa, & Masumoto, 2006), including cunner (Babichuk & Volkoff, 2013; Hayes & Volkoff, 2014), food deprivation rapidly decreases CCK levels within days or weeks. In rodents, greater numbers of CCK-immunopositive cells are found in the hypothalamic periventricular nucleus of females than of males suggesting sexually dimorphic neural CCK pathways (De Vries, 1990). Similarly, in goldfish,

there is evidence that there exists a link between the expression of CCK and gender, as females have higher CCK transcript levels than males at certain times of the year in specific brain regions (Peyon, Saied, Lin, & Peter, 1999).

Neuropeptide Y

Neuropeptide Y (NPY) is a peptide produced mainly by the brain that stimulates feeding in vertebrates (Cerdá-Reverter & Larhammar, 2000; López-Patiño et al., 1999; H. Volkoff, 2006). It is a member of the NPY family of peptides that also includes peptide YY (PYY) and pancreatic polypeptide (PP) (Cerdá-Reverter & Larhammar, 2000). It acts as one of the most powerful appetite stimulators in both mammals and fish (Narnaware, Peyon, Lin, & Peter, 2000; J. T. Silverstein & Plisetskaya, 2000; Valassi, Scacchi, & Cavagnini, 2008). The NPY peptide has been shown to be present in the brain of several fish, including platyfish (Cepriano & Schreibman, 1993).

NPY operates by binding to G-protein coupled receptors that are distributed throughout the brain and peripheral tissues of vertebrates (Fredriksson, Larson, Yan, Postlethwait, & Larhammar, 2004; Larhammar, 1996; Larsson et al., 2005). Both central and peripheral injections of mammalian or fish NPY increase feeding behaviour in goldfish (de Pedro et al., 2000; López-Patiño et al., 1999; Narnaware et al., 2000) and catfish (J. T. Silverstein & Plisetskaya, 2000). NPY expression increases after 2 weeks of fasting in the winter skate (*Raja ocellata*) (Hélène Volkoff, Xu, MacDonald, & Hoskins, 2009) and after 2-3 weeks of fasting in both the chinook salmon (*Onchorhynchus tshawytscha*) and coho salmon (*Onchorhynchus kisutch*) (J.

T. Silverstein, Breininger, Baskin, & Plisetskaya, 1998) reinforcing the idea of NPY as an appetite regulator in fish.

Injection studies in rats show that NPY reduces sexual behaviours in both males and females and may play a key role in decreasing sexual motivation while increasing food intake (Clark, Kalra, & Kalra, 1985). In addition, NPY influences growth hormone secretions in both mammals and teleost fishes including the platyfish which might suggest its involvement in the timing of the sexual maturation process (Cepriano & Schreibman, 1993). Furthermore, in the cichlid fish *Cichlasoma dimerus* NPY has been shown to influence gonadotropin secretions in both males and females (Di Yorio, Delgadin, Sirkin, & Vissio, 2015).

Orexin

Orexins, which consist of two forms, orexin-A and orexin-B, derived from the same precursor molecule preproorexin, (Li, Hu, & de Lecea, 2013), are brain peptides that stimulate feeding behaviours in both mammals and fish (Matsuda, Azuma, & Kang, 2012; Panula, 2010; Wong, Ng, Lee, Ng, & Chow, 2011). Orexins were originally discovered simultaneously by two separate research groups, one of which isolated the ligand from rat brain tissue and named it orexin (Sakurai et al., 1998), while the other isolated it from the hypothalamus and named it hypocretin (de Lecea et al., 1998). Of the two forms, orexin-A has been shown to be more potent than orexin-B in stimulating feeding in fish (Helene Volkoff, Bjorklund, & Peter, 1999). In the fish brain, based on goldfish and zebrafish (*Danio rerio*) studies, orexin is mainly produced in the hypothalamus and the telencephalon (Gema Huesa,

Anthony N. van den Pol, & Thomas E. Finger, 2005; Kaslin, Nystedt, Östergård, Peitsaro, & Panula, 2004). Although the principal site of orexin release is in the brain, the discovery of orexin-like immunoreactivity in neurons in the gut of rats (Kirchgessner & Liu, 1999) coupled with their potential role as stimulating intestinal fluid secretion in guinea pigs (Kirchgessner, 2002) suggests that orexins may prepare the gut for digestion. In addition to its role as regulators of feeding, orexins have important roles in regards to sleep, movement, and arousal (Nakamachi et al., 2006; H. Volkoff, 2006). In both rats (Sakurai et al., 1998) and goldfish, (Helene Volkoff et al., 1999) ICV injections of orexin cause increased locomotion, while in zebrafish, orexin brain fibres interact with cholinergic and aminergic neurons pointing to orexins involvement in wakefulness (Kaslin et al., 2004).

In both mammals (Sakurai, 2006; Sakurai et al., 1998) and goldfish (Miura et al., 2007; Nakamachi et al., 2006; Helene Volkoff et al., 1999) injections of orexins stimulate hunger. Prolonged periods of fasting increase hypothalamic preproorexin mRNA expression in both mammals (Sakurai, 2002; Zhao, Guo, Du, & Liu, 2005) and zebrafish (Novak et al., 2005) further solidifying its role in appetite modulation.

Recent evidence in both fish and mammals points to a role orexins may play in the control of reproduction. In rats, orexin fibers have been localized in brain areas involved in the control of the hypothalamo-gonadotropic axis (Martyńska et al., 2006), and orexins stimulate gonadotropin-releasing hormone secretion *in vitro* (Sasson, Dearth, White, Chappell, & Mellon, 2006) and inhibit luteinizing hormone (LH) secretion (S. H. Russell et al., 2001). In goldfish, treatment with orexin-A

induces an inhibition of spawning behaviour and a decrease in the expression of chicken GnRH-II in the hypothalamus and optic-tectum-thalamus (Hoskins et al., 2008). Chicken GnRH-II is a brain peptide that stimulates growth hormone release and is often linked to reproductive processes such as spawning, as seen in female goldfish (H. Volkoff & Peter, 1999).

CART

Cocaine-and amphetamine-regulated transcript (CART) was first identified in rats as a transcript produced after administration of psychomotor stimulants such as cocaine and amphetamine (Douglass, McKinzie, & Couceyro, 1995; Hunter et al., 2004). In mammals, CART mRNA is widely expressed in the brain, particularly the hypothalamus (Hunter et al., 2004) but has also been localized in other peripheral tissues including the GI tract (Murphy et al., 2000). In goldfish, CART mRNA expression is more widespread including the brain, pituitary, gonads, and kidneys (H. Volkoff & R. E. Peter, 2001). In several fish species, including goldfish (H. Volkoff & R. E. Peter, 2001) and medaka (Murashita & Kurokawa, 2011), several forms of CART peptide exist. CART acts as an appetite-regulating hormone in vertebrates and also plays roles in body weight regulation, stress response, and other physiological functions (Rogge, Jones, Hubert, Lin, & Kuhar, 2008).

In both goldfish (H. Volkoff & Peter, 2000) and rats (Lambert et al., 1998), central injections of CART fragments decrease food consumption, indicating its role as an anorexigenic hormone. In goldfish, CART mRNA expression in the olfactory bulbs and hypothalamus (H. Volkoff & R. E. Peter, 2001) increases shortly following

a meal, while decreasing in the telencephalon-preoptic region, hypothalamus, and olfactory bulb following periods of fasting (H. Volkoff & R. E. Peter, 2001). Food deprivation studies in other species, including cod (*Gadus morhua*) (Kehoe & Volkoff, 2007), catfish (Kobayashi, Peterson, & Waldbieser, 2008), common carp (*Cyprinus carpio*) (Wan et al., 2012), and rat (Savontaus, Conwell, & Wardlaw, 2002), have shown a fasting-induced decrease in CART transcript expression further establishing its role as an appetite inhibitor.

Studies in mice have shown that ventral premammillary nucleus CART neurons interact with brain areas involved in reproduction, indicating that they may modulate leptin's effects on reproduction (Rondini, Baddini, Sousa, Bittencourt, & Elias, 2004). Leptin is a hormone secreted by white adipose tissue that is involved in a number of endocrine related processes (Ahima, Saper, Flier, & Elmquist, 2000; Casanueva & Dieguez, 1999), including the facilitation of GnRH secretion (Rondini et al., 2004). The involvement of CART in the neuroendocrine control of reproduction has also been shown in catfish, where CART may play a role in prompting the preparatory phase in the annual sexual cycle (Barsagade et al., 2010). In male rats, fasting for two days caused an increased CART mRNA expression that was not present in females (L. Xu, Bloem, Gaszner, Roubos, & Kozicz, 2009). This lead to a conclusion, that CART-containing neurons that contain CART may be involved in leptin-mediated feeding control in male rats only, thus showing a sex-specific control of energy balance (L. Xu et al., 2009).

Species used in this study

Platyfish

The platyfish (*Xiphophorus maculatus*), of the family Poeciliidae (order: Cyprinodontiformes) are viviparous (livebearing) teleost fish that can be found from northern Mexico, through central and South America (Boswell et al., 2009; Ponce de León, Rodríguez, & León, 2012). Platyfish and other species of *Xiphophorus* are neotropical, open water fish that live in a variety of habitats including streams, lakes, and ponds with muddy beds as well as areas with dense growths of aquatic and semi-aquatic vegetation (Zaret, 2013). Platyfish are omnivorous, with a diet that usually consists of terrestrial and aquatic insects, aquatic crustaceans, and plant matter (Arthington, 1989). Poeciliids are popular aquarium fish subject to important ornamental fisheries or aquaculture (Abasali & Mohamad, 2011). *Xiphophorus* fishes have been used as a research model as early as the 1930's, and their importance in research has even lead to the development of a collective database of the *Xiphophorus* genome, the Xiphophorus Genetic Stock Centre (D. K. Kallman, 2001; K. Kallman, 1965). Platyfish display several "typical mammalian" features, including complex behaviours, live birth, and melanoma formation (Schartl, Walter, Shen, Garcia, Catchen, Amores, Braasch, Chalopin, Volff, Lesch, et al., 2013). These attributes make them unique models to better understand the molecular and evolutionary biology of such traits.

Due to the specialization of the male anal fin into an intromittent organ, the gonopodium, gender determination of the platyfish can be visually ascertained on

live fish (K. D. Kallman & Schreibman, 1973), a trait that makes them very popular in studies that require sex determination and selective breeding (McKenzie Jr, Crews, Kallman, Policansky, & Sohn, 1983). In females, sexual maturity can only be determined via autopsy, since unlike males there is no specialized fin to indicate the ability to reproduce (Schreibman & Kallman, 1978).

Platyfish are ovoviviparous, as eggs are said to be “well-provisioned” with nutrients, and embryonic development and hatching occur within the maternal body (Kawaguchi, Tomita, Sano, & Kaneko, 2015; Nelson, 2006; Wourms, 1981).

Although these fish have been widely studied in fields ranging from ecology, evolution, genetics, and genomics to systematics (Kang, Schartl, Walter, & Meyer, 2013) as well as used as models in cancer research (Boswell et al., 2009), very little is known about the endocrine regulation of feeding and reproduction in these fish. Investigating the differences in levels of appetite-regulating hormones in both sexes may contribute to the understanding of how these hormones may operate in relation to different reproductive strategies.

Cunners

Cunners, *Tautoglabrus adspersus*, are members of the family Labridae (order: Perciformes), which consists of oviparous (egg laying) marine fish. Cunners are cold-water marine fish and can be found from Northern Newfoundland to all along the coast of the western North Atlantic (J. M. Green, Martel, & Martin, 1984). Typically, cunners are found in shallow, coastal waters and in a variety of different substrates ranging from sandy bottoms during the summer spawning months to

rocky crevices during the winter (John M Green & Farwell, 1971). In Newfoundland, the spawning season for cunners occurs over a 4-to 6-week period in July and August (Martel & Green, 1987; Pottle & Green, 1979). Because mature female cunners are able to spawn daily, even in the absence of a male, they are of particular scientific interest to research involving reproduction (Mills et al., 2003). During the spawning season, female cunners have been shown to exhibit differences in feeding strategies (J. M. Green et al., 1984).

Cunners have proven to be an invaluable tool in food deprivation research. When water temperatures drop below 5°C, cunners enter a state of torpor in which they do not feed and become metabolically depressed (Costa, Driedzic, & Gamperl, 2013; John M Green & Farwell, 1971). Food deprivation studies in cunner have shown altered hormone mRNA expression levels during short-term summer fasting and during natural winter torpor (Babichuk & Volkoff, 2013).

Cunners are an important fish to study with regards to the aquaculture of Newfoundland. This is primarily because of their potential usefulness in preying upon sea lice, *Lepeophtheirus salmonis*, in and around farmed Atlantic Salmon, *Salmo salar*, cages (Groner, Cox, Gettinby, & Revie, 2013). There has been much discussion over their potential role as a “cleaner” fish and tank trials have indicated that cunners feed upon sea lice. This is important as developing chemical-free control measures to sea lice eliminates potential risks to the environment and is a main priority of the salmon farming industry. Their abundance in northern coastal waters coupled with their proliferative spawning make cunners an excellent study

organism to investigate the links between feeding and reproduction in an oviparous teleost.

Project Objectives

The objectives of this study were to examine and compare the role of appetite-regulating hormones in the regulation of feeding and reproduction in fish with two different reproductive strategies, viviparity and oviparity, the platyfish and the cunner, respectively. It is possible that differences in reproductive strategies might reflect differences in feeding between sexes and between reproductive stages, potentially translating into different expressions levels of appetite-regulating hormones.

Since there have been minimal studies outlining the regulation of energy in platyfish, we needed to understand the role appetite regulating hormones played in that species. In order to elucidate how feeding is regulated in platyfish, we examined the effects of injections of appetite-regulators on feeding, in particular CCK and orexin. Fish were injected and observed for behavioural changes in response to feeding. This was measured as 1) numbers of pellets consumed and 2) number of food search attempts, and compared to saline injections. In addition, the expressions of CCK, CART, NPY, and orexin were measured using quantitative real time PCR (qPCR) and compared between fed and fasted platyfish in order to better understand their roles in energy regulation.

Gene expression levels of CCK, NPY, CART, and orexin were also compared between fed and 10-day fasted male and female cunners. Although studies have

been completed with cunner detailing the effects of feeding on gene expression, none of these compared levels of expression between males and females. An attempt to perform IP injections was made using cunner in order to help characterize the effects of appetite-regulating hormones but injections were not successful. Therefore we compared the effects of fasting on the expression of appetite-regulating hormones between these two species.

Performing these studies in platyfish and cunner will add a greater understanding of the role that these hormones have on the regulation of energy balance in vertebrates, which is still not a completely understood process. With a greater understanding of how expression levels of appetite-regulating hormones differ between genders and different reproductive strategies it may be possible to optimize aquaculture-feeding practices resulting in lower costs and higher yields of fish.

Materials and Methods:

Study Animals

Platyfish – Tissue Distribution, IP, and fasting study animals

Platyfish used for these studies were obtained from ABCee's Aquatic Imports (Lasalle, QC, Canada). A mixture of male and female platyfish (weighing from 1.5 - 3 grams and measuring between 3.75 and 5.25 cm in length) were kept in 60L glass aquaria under a simulated photoperiod of 16H light: 8H dark, with constantly aerated and filtered water at 21°C (15 fish per tank) and fed to satiety once a day

(13:00), with small floating fish pellets for tropical fish (42% protein, 11% fat, 2% fibre, 8.5% moisture, 8% ash, Omega Sea, Sitka, Alaska, USA). Fish were acclimated under these standard conditions for several weeks before the start of an experiment. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

Tissue collection - platyfish

Platyfish sacrificed for tissue collection were anesthetized using 0.05% tricaine methanesulfonate (MS 222) (Syndel Laboratories, Vancouver, BC, Canada) followed by spinal section. The length and weight of all fish was recorded and tissue samples were collected. Tissues were preserved in *RNAlater* (Qiagen, Mississauga, ON, Canada) and stored at -20°C.

RNA Extraction

RNA extractions were performed using a trizol-chloroform and Tri-reagent extraction (BioShop, Burlington, Ontario, Canada). RNA concentrations were quantified at a wavelength of 260-nm using a Nanodrop spectrophotometer (ThermoScientific, Wilmington, North Carolina, USA). All samples used had absorbance ratios between 1.7 and 2.1 at wavelengths of 260 and 280 nm. If this was not achieved, samples were submitted to purification protocols using a GeneJET™ RNA Purification Kit (Fermentas, Burlington, Ontario, Canada). Isolated

RNA samples were stored at -80°C in 1.5mL nuclease-free Eppendorf tubes until further use.

cDNA synthesis

A total of 1 µg of RNA was transcribed from each tissue using a Verso cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's protocol. In some samples where 260/280 readings were not between 1.7-2.1, a genomic DNA clean-up step was completed before reverse transcription using the supplied DNase. cDNA products were stored at -20°C until further use. Following this, cDNA was used in a polymerase chain reaction (PCR) with GoTaq master mix 2 x (Promega, Madison, WI, USA) for a total reaction volume of 25 µl.

Tissue Distribution - platyfish

Several male and female fed platyfish were used for the tissue distribution study. RNA was extracted from brain, intestine, liver, spleen, gills, muscle tissue, testes, and ovaries as described above. These were reverse transcribed to cDNA using the methods described above. Primers specific for tissue distribution (Table 1) were designed for CCK, NPY, CART, and orexin using Primer3 software (<http://frodo.wi.mit.edu/>). Platyfish CCK, NPY, CART, and orexin gene-specific primers were designed using Primer3 software (<http://frodo.wi.mit.edu/>) based on previously cloned sequences (Table 1). Each PCR reaction protocol consisted of the following steps: 1) Denaturation, 5 minutes, 94°C, 30 cycles of 2) Denaturation, 30 seconds, 94°C, 3) Primer-specific annealing temperature, 30 seconds, 4) Elongation,

30 seconds, 72°C, and a final step of 5) Extension, 5 minutes, 72°C. In order to verify gene identity size, PCR products were separated by gel electrophoresis on a 1.5% agarose gel for 30 minutes at 120 volts. Images were captured using an Epichemi Darkroom Bioimaging System (UVP, Upland, California, USA) and analyzed using LabWorks 4.0 software. Elongation factor -1 α was used as the control gene. A blank (no cDNA) control was used in order to verify that amplification was due to gene expression and not contamination.

IP study animals - platyfish

Following the 2-week acclimation period, fish were placed in tanks separated by perforated grids into 3 compartments with 1 fish in each compartment (Figure 1). Fish were acclimated to these conditions for 72 h. Preliminary studies were conducted on several fish by performing sham injections using empty syringes in order to assess possible effects caused by perforating the abdominal cavity. Fish were allowed to recuperate for several days before being subjected to further studies. Experimental fish were lightly anesthetized in 0.05% MS 222 (Syndel Laboratories) and 10 μ l of fish physiological saline (Burnstock, 1958), sulfated CCK-8 (50 ng/g)(American Peptide Company, Sunnyvale, CA, USA) or mouse orexin A (100 ng/g) (American Peptide Company) was injected into the peritoneal cavity, caudal to the pelvic fins, using a 33-gauge needle attached to a 10 μ l Hamilton syringe.

For each experiment, three fish from a single tank were injected at a time and observed for feeding behaviour and food consumption. Compartments allowed the fish to see the presence of other fish in the tank (and thus avoid stress) and to

accurately quantify the number of pellets eaten by each fish. Fish were offered 10 pellets 15 minutes post-injection, the time at which observations began for 30 minutes. Observations were recorded manually into a laboratory notebook and well as a video recording using a Sony Handycam – DCR-SR42 (Sony Canada). Experiments were carried out at the regular feeding time the fish had been adapted to eat (13:00). Feeding/food-seeking behaviour was monitored and food consumption measured by counting the number of pellets eaten by each individual fish. Food consumption was converted to milligrams of food consumed/wet body weight/time feeding based on the mean pellet weight fed to fish (approximately 3.0mg/pellet).

Food intake was assessed for control fish submitted to sham injections as well as saline-treated animals, in order to verify that the injection procedures themselves did not influence feeding. Fish were tested in random order in terms of treatment and days.

Fasting study animals – platyfish

48 platyfish were randomly distributed amongst 4 tanks (12 fish per tank) and acclimated for 2 weeks (Table 2). In order to determine sex effects, at least 6 fish of each gender were placed in each tank. Gender was determined based on the presence or absence of the male gonopodium. After the 2 weeks acclimation period, two tanks were continually fed once a day (13:00) to satiety, acting as an experimental control and two tanks were subjected to fasting conditions. Fish were

then sampled 10 days after the beginning of the experiment. Brain and gut tissues were collected and preserved in *RNAlater* and stored at -20°C until RNA extractions were performed.

Cunners - Fasting study animals

Fasting study animals - cunner

Cunners (male and female; mature and immature) weighing between 7 and 29 grams and measuring between 7.8 and 13.3 cm in length, were collected off the coast of Norris Point (Norris Point, NL, Canada) in July 2014 and were acclimated for 2 weeks in 1m x 1m flow-through tanks under natural light and water temperature conditions at the Bonne Bay Marine Station (Norris Point, NL, Canada). The fish were fed 1-inch cubes of frozen chopped squid once daily at 13:00 to satiety. At the start of the experiment, 48 fish were randomly divided into two fed and two fasted tanks (Table 3). The control tanks were fed to satiety throughout the experiment using the same feeding regime that was used during acclimation, whereas the fasted groups were food deprived for a 10-day period. All fish were weighed, measured (total length), and sexed at the end of the experiments. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

Tissue collection - cunner

Only 10 cunners were sampled from each tank. Those sacrificed to use in the study were anesthetized using 0.05% tricaine methanesulfonate (MS 222) (Syndel Laboratories) followed by spinal section. Fish were measured, weighed and tissue samples (brain and intestine) were collected in *RNAlater* and stored at -20°C until RNA was extracted and reverse transcribed to cDNA as described above.

Real-time quantitative PCR

For gene characterization, whole brain and whole intestine (the entire length of the gastrointestinal tract) RNAs were isolated from both cunners and platyfish. qPCR primers were designed to span an intron sequence greater than 100 bp, in both platyfish (Table 1) and cunner (Table 2). Forward and reverse primers were designed to have approximately the same melting temperature. Multiple sets of primers for each gene of interest were tested using four serially diluted samples of cDNA and those with the highest efficiency and linearity were used in further studies. cDNA samples were diluted 1:3 in water for all qPCR reactions. Duplicate reactions were prepared using a mix containing 0.2 µl 10 µM forward primer, 0.2 µl µM reverse primer, 2.6 µl water, 5 µl SYBR FAST qPCR Master Mix (Kapa Biosystems, Boston, MA, USA), and 2 µl cDNA for a total reaction volume of 10 µl. 96-well plates were loaded with an epMotion® 5070 automated pipetting system (Eppendorf, Mississauga, ON, Canada). Real-time quantitative PCR was performed using a MasterCycler® Realplex 2S thermocycler (Eppendorf). Optimal primer annealing temperatures, efficiencies, and R² values were determined for all primer

pairs to ensure viability. The cycling conditions for the qPCR were as follows: 40 cycles; 1) Denaturation, 30 seconds, 94°C, 2) Primer-specific annealing temperature, 45 seconds, and 3) Elongation, 60 seconds, 72°C. A melting curve analysis was performed at the end of each qPCR to verify that only one PCR product was amplified. After testing several candidate genes (18s, elongation factor-1 α , and ubiquitin) for use as reference genes, elongation factor-1 α was found to have the lowest variability and most stable expression between fed and fasted treatment groups.

Real-time quantitative PCR data analysis

Gene expression levels were measured and quantified using Realplex 1.5 software (Eppendorf). Realplex compared all expression levels using relative quantification ($\Delta\Delta C_t$) to determine relative gene expression levels. Gene expression levels were normalized to the housekeeping gene elongation factor-1 α .

Statistical analysis

Statistical analysis was performed using Prism 6 GraphPad InStat program (Graphpad Software Inc., San Diego, California, USA). For the IP studies, both search behaviour and food intake were compared using one-way ANOVA. In the fasting experiments, a non-parametric Mann-Whitney t-test was performed to compare qPCR results from fed and fasted platyfish. For the platyfish gender comparison studies, a two-way ANOVA was used with factors being: sex and fed/fastened treatment groups measuring relative expression. Significance was set at $p < 0.05$

Table 1: Primers used in platyfish for qPCR analysis and tissue distribution with corresponding GenBank Accession ID

| Target Gene | Orientation | Primer Sequence (5'-3') | GenBank ID |
|------------------------------------|-------------|-------------------------------|--------------|
| qPCR Primers | | | |
| CCK | Forward | 5' CTGCCCTTCTCCCAAAG 3' | XM_005812187 |
| | Reverse | 5' TGTGTCCGAGGTGGGTCT 3' | |
| NPY | Forward | 5' CAGCCCTGAGACACTACATCA 3' | XM_005802707 |
| | Reverse | 5' GCAGCAGCTCTGAGACCAGT 3' | |
| Orexin | Forward | 5' TGGTACACCACCAACTTCCA3' | XM_005796773 |
| | Reverse | 5' ACACTCTGCGTGCTACATCC 3' | |
| CART* | Forward | 5' GCCCGAGTACGACTTCATCA 3' | XM_005808120 |
| | Reverse | 5'GACGGCAGCTGTTTGTTCCTT 3' | |
| EF | Forward | 5' CCGTTTTGAGGAGATCCAAA 3' | XM_005802839 |
| | Reverse | 5' AGGCATCCAGGAGAGTGGTT 3' | |
| Tissue Distribution Primers | | | |
| CCK | Forward | 5' TGAGGAGGACGCAGATTCC 3' | XM_005812187 |
| | Reverse | 5' TCAAACCTTTCTGCACTGCG 3' | |
| NPY | Forward | 5' GCCAAGTATTACTCAGCCCTG 3' | XM_005802707 |
| | Reverse | 5' CCACAATGATGGGTCGTATCTTG 3' | |
| Orexin | Forward | 5' TGTGTCTGAGTGCTGCAGA 3' | XM_005796773 |
| | Reverse | 5' AGTATCCCAGCTGCTTGGTT 3' | |
| CART* | Forward | 5' TGGTGAAGCTCTTGAGGTT 3' | XM_005808120 |
| | Reverse | 5' CAGTTGGCGCCTCTTCCG 3' | |
| EF | Forward | 5' CCGTTTTGAGGAGATCCAAA 3' | XM_005802839 |
| | Reverse | 5' AGGCATCCAGGAGAGTGGTT 3' | |

*Due to results obtained from agarose gel analysis, CART form 4 was used instead of CART form 1 as it could be consistently separated by gel electrophoresis.

Table 2: Primers used in cunner for qPCR analysis with corresponding GenBank

Accession ID

| Target Gene | Orientation | Primer Sequence (5'-3') | GenBank ID |
|-------------|-------------|-------------------------------|------------|
| CCK | Forward | 5' CTCCAGGAAAGGTTCTGTGC 3' | JX126917 |
| | Reverse | 5' CCATCCATCCCAAGTAGTCC 3' | |
| NPY | Forward | 5' AGACGGATACCCTGTGAAGC 3' | JX126916 |
| | Reverse | 5' TCTGTCTTGTGATGAGGTTGATG 3' | |
| Orexin | Forward | 5' GTCGCTCTGGCAGTAAGACC 3' | JX126914 |
| | Reverse | 5' TAAGCGGTCCACGTCTTTTT 3' | |
| CART | Forward | 5' AAAGGACCGAACCTGACCTC 3' | JX023541 |
| | Reverse | 5' GGGACTTGGCCAAACTTTTT 3' | |
| EF | Forward | 5' GGTACATCTCAGGCTGACTGCG 3' | JX126915 |
| | Reverse | 5' TCACACCGAGGGTGAAGG 3' | |

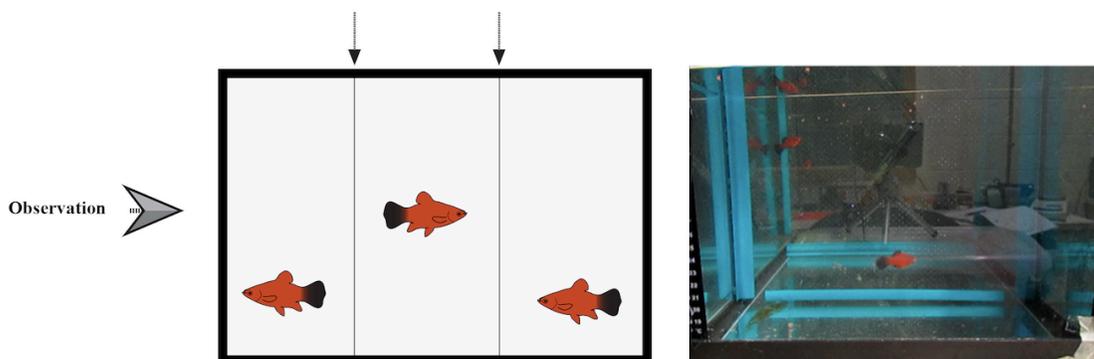


Figure 1: IP tank set-up, showing placement of fish during experiment as well as location of perforated dividers (diagram of side view and picture of front view).

Figure created by Dr. Helene Volkoff.

Table 3: Table showing fasting experiment tank set-up for platyfish, numbers and genders of fish in each experimental tank

| Treatment | # of females | # of males |
|------------------|---------------------|-------------------|
| Fed | 8 | 4 |
| Fast | 8 | 4 |
| Fed | 8 | 4 |
| Fast | 8 | 4 |

Table 4: Table showing fasting experiment tank set-up for cunners, showing numbers of fish in each experimental tank

| Treatment | # of fish* |
|------------------|-------------------|
| Fed | 12 |
| Fast | 12 |
| Fed | 12 |
| Fast | 12 |

* Not grouped by gender, since there is no external gender-specific characteristic to distinguish males from females

Results

Characterization of Appetite Regulators in Platyfish

Intraperitoneal Injections – Food Intake/Search Behaviour

Intraperitoneal (IP) injections – CCK and orexin

Injections of CCK significantly decreased both the number of search attempts (approaches to pellets) (Figure 2) and the number of pellets consumed when compared to the saline-injected group (Figure 3).

Injections of orexin significantly increased the number of search attempts (approaches to pellets) (Figure 2) but did not have an effect on the number of pellets consumed when compared to saline injections (Figure 3).

When comparing the effects of injections in males and females, there were no significant effects on either search behaviour (Figure 4) or the number of pellets consumed (Figure 5) due to the effects of sex.

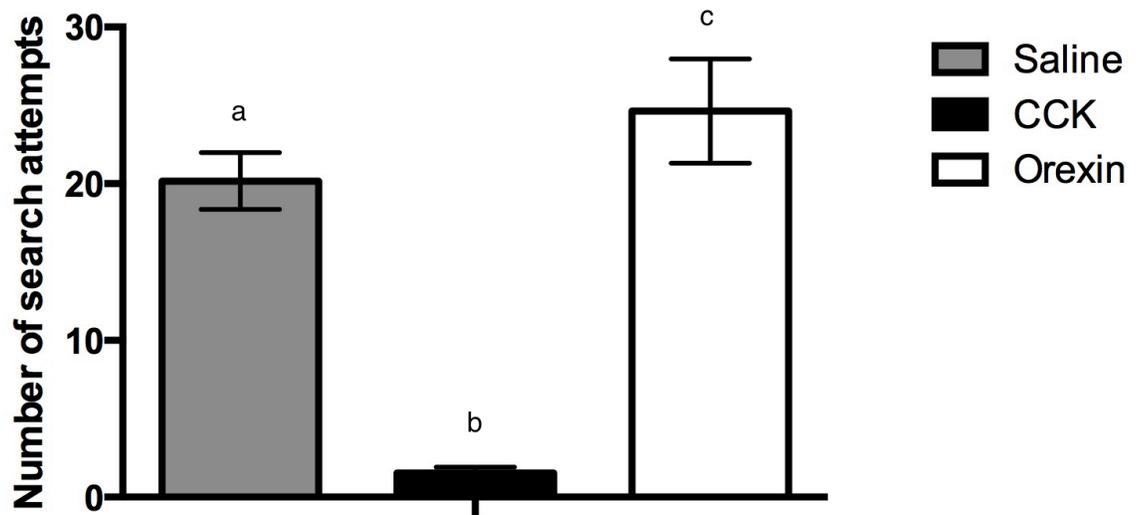


Figure 2: Number of search attempts in 30 minutes (approaches to pellets) displayed after injection with saline (n= 30), CCK at 50 ng/g (n=11) or orexin A at 100 ng/g (n=11). Data is expressed as mean \pm SEM. Different letters indicate significant difference (one-way ANOVA); significance considered at $p < 0.05$.

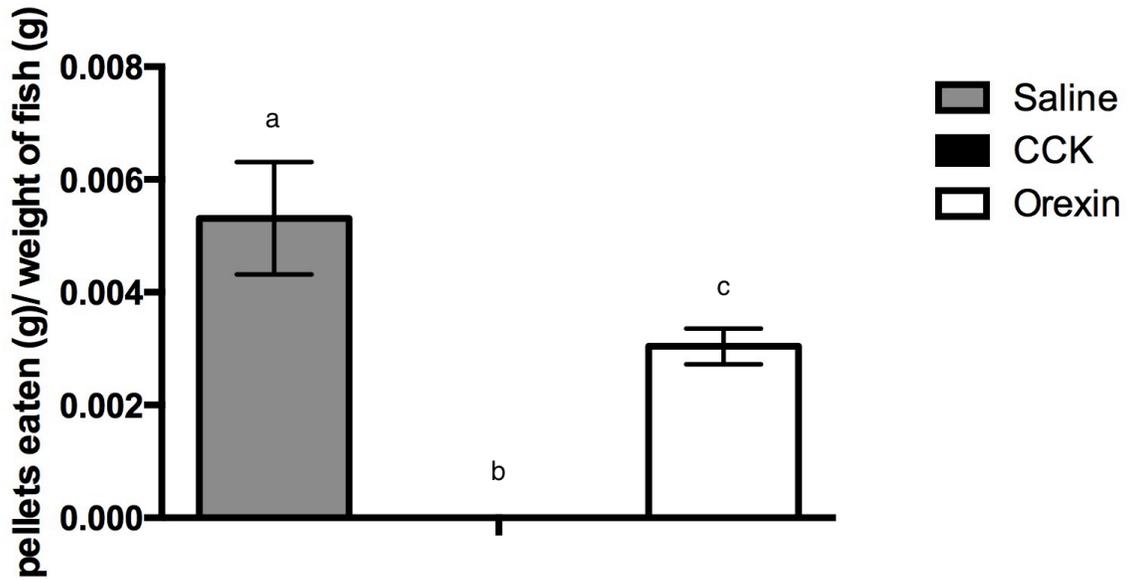


Figure 3: Food intake 15 min post-injection, quantified as weight of pellet(g) x number of pellets / weight of fish(g). Fish injected with saline (n= 30), CCK at 50 ng/g (n=11) or orexin A at 100 ng/g (n=11). Data is expressed as mean \pm SEM. Different letters indicate significant difference (One-way ANOVA); significance considered at $p < 0.05$.

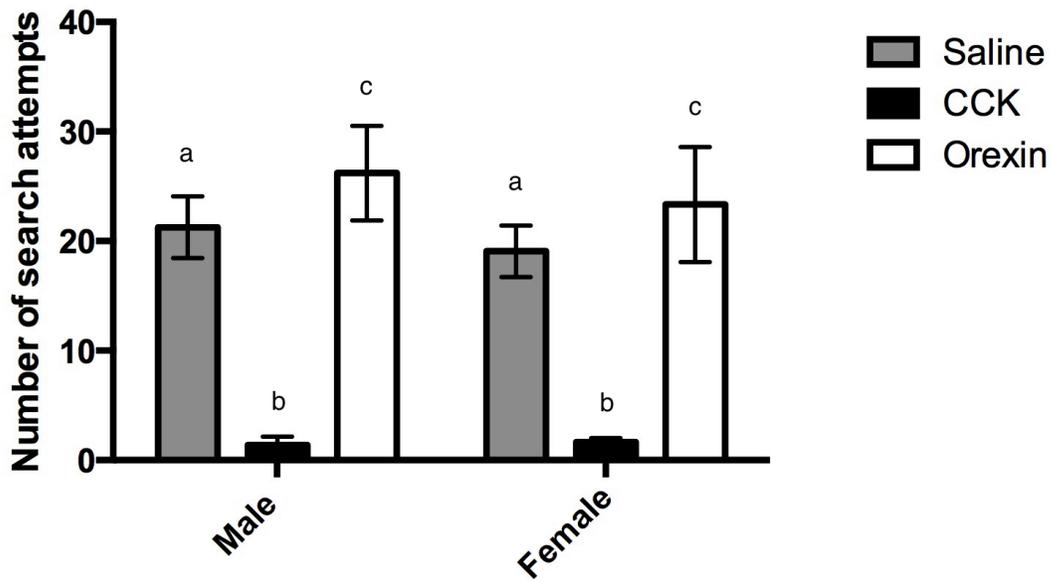


Figure 4: Number of search attempts in males and females in 30 minutes (approaches to pellets) displayed after injection with saline (male n=15, female n=15), CCK at 50 ng/g (male n=5, female n=6) or orexin A at 100 ng/g (male n=5, female n=6). Data is expressed as mean \pm SEM. Different letters indicate significant difference (one-way ANOVA); significance considered at $p < 0.05$.

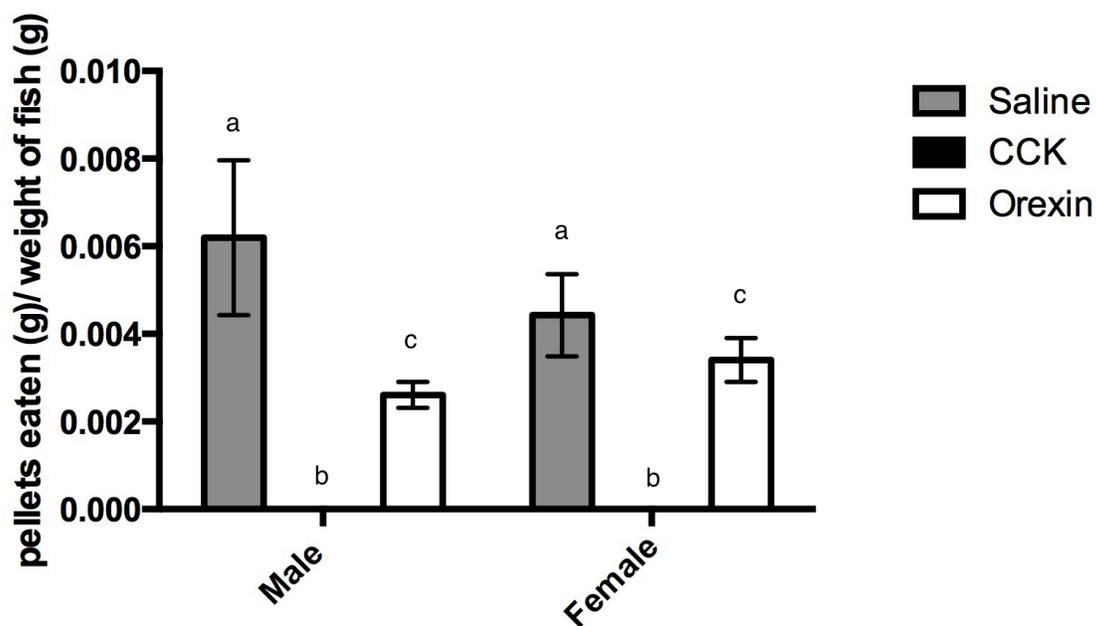


Figure 5: Food intake in male and female platyfish 15 min post-injection, quantified as weight of pellet(g) x number of pellets / weight of fish(g). Fish injected with saline (male n=15, female n=15), CCK at 50 ng/g (male n=5, female n=6) or orexin A at 100 ng/g (male n=5, female n=6). Data is expressed as mean \pm SEM. Different letters indicate significant difference (One-way ANOVA); significance considered at $p < 0.05$.

Tissue Distribution

Tissue distribution studies – orexin, NPY, CART, CCK

Tissues distribution studies were performed in the platyfish. Transcript fragments of CCK, NPY, orexin, and CART were amplified in brain, intestine, liver, spleen, gills, muscle tissue, testes, and ovaries using RT-PCR in conjunction with no-

template controls. Each tissue sample was also tested with the housekeeping gene EF1- α to ensure that cDNA was present in all the samples.

Orexin

For tissues distributions of orexin, a 210 bp region of the transcript was amplified. Orexin was present in all tissues examined (Figure 6) with apparent high expression in the gills, spleen, and testes.

NPY

The 162 bp NPY fragment was seen NPY in all the samples examined, except for muscle (Figure 6). NPY was apparently expressed to a higher degree in the brain than all of the other tissues, based on the strength of the bands.

CART

A 160 bp region of CART was amplified for the tissue distributions. CART was present in all tissues examined except for the muscle (Figure 6). The apparent highest expression of CART was found in the intestine based on the strength of the bands, followed by the testes. All of the other tissues expressed CART with equal intensity.

CCK

A 189 bp CCK fragment was amplified. CCK was present in the brain, intestine, spleen, gills, testes, and ovaries (Figure 6). The highest apparent expression, based on the brightness/strength of the bands was in the brain and the ovaries, followed by the intestine and testes. Bands were present in both the spleen and gill samples, but the strength was considerably weaker than the other tissues.

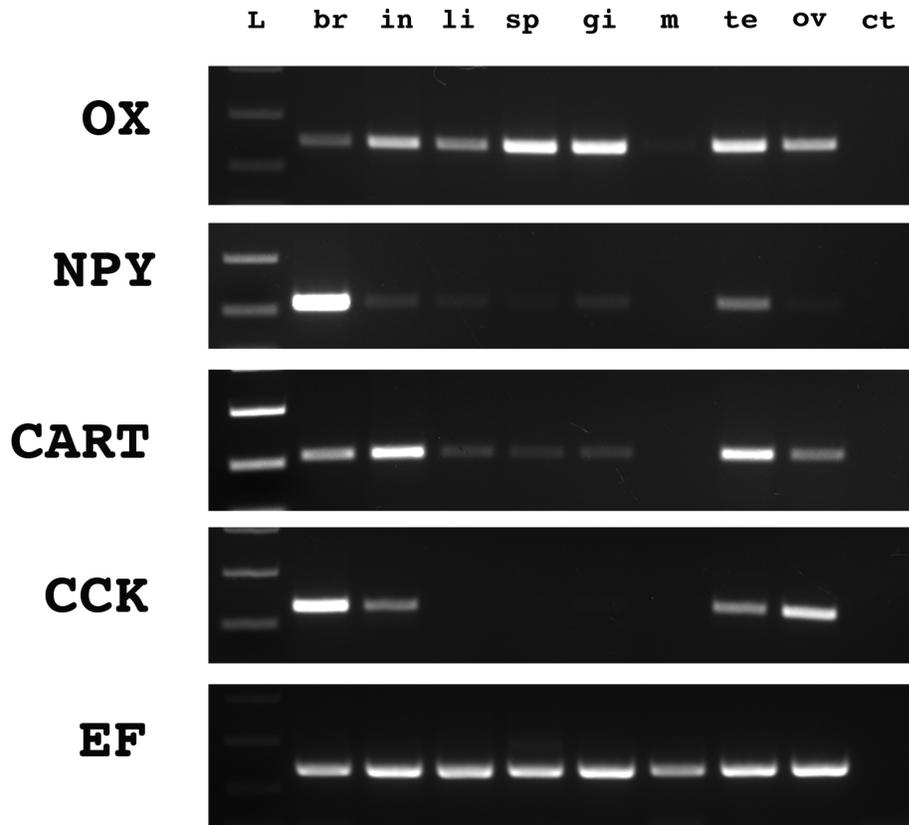


Figure 6: Tissue distribution of orexin, NPY, CART, CCK, and EF in platyfish. Transcript fragments were amplified using RT-PCR and visualized on an agarose gel with ethidium bromide. Samples from left to right are as follows: brain (br), intestine (in), liver (li), spleen (sp), gills (gi), muscle tissue (m), testes (te), and ovaries (ov).

Effects of fasting and sex differences on gene expression in platy and cunner

*Effects of fasting on transcript expression in brain and intestine – platyfish
(males and females)*

CCK expression was significantly lower in fasted than fed fish in both brain [Figure 7a; fed ($n=14$); fasted ($n=12$)] and intestine [Figure 7b; fed ($n=12$); fasted ($n=15$)].

NPY expression did not change significantly in either brain [Figure 7c; fed ($n=11$); fasted ($n=15$)] or intestine [Figure 7d; fed ($n=12$); fasted ($n=12$)] for 10 days.

Orexin mRNA expression in the brain significantly increased after the fasting period ($n=11$) as compared to the fed fish ($n=10$) sampled at the same time (Figure 7e). However, this was not seen in the gut, as fasting seemed to have no significant effect on orexin expression [Figure 7f; fed ($n=11$); fasted ($n=11$)].

CART transcript expression in the brain showed a significant decrease after the 10-days of fasting (fed: $n=11$; fasted $n=10$) (Figure 7g). In the intestine there was no significant change in the expression of CART due to fasting ($n=13$ for each treatment) (Figure 7h).

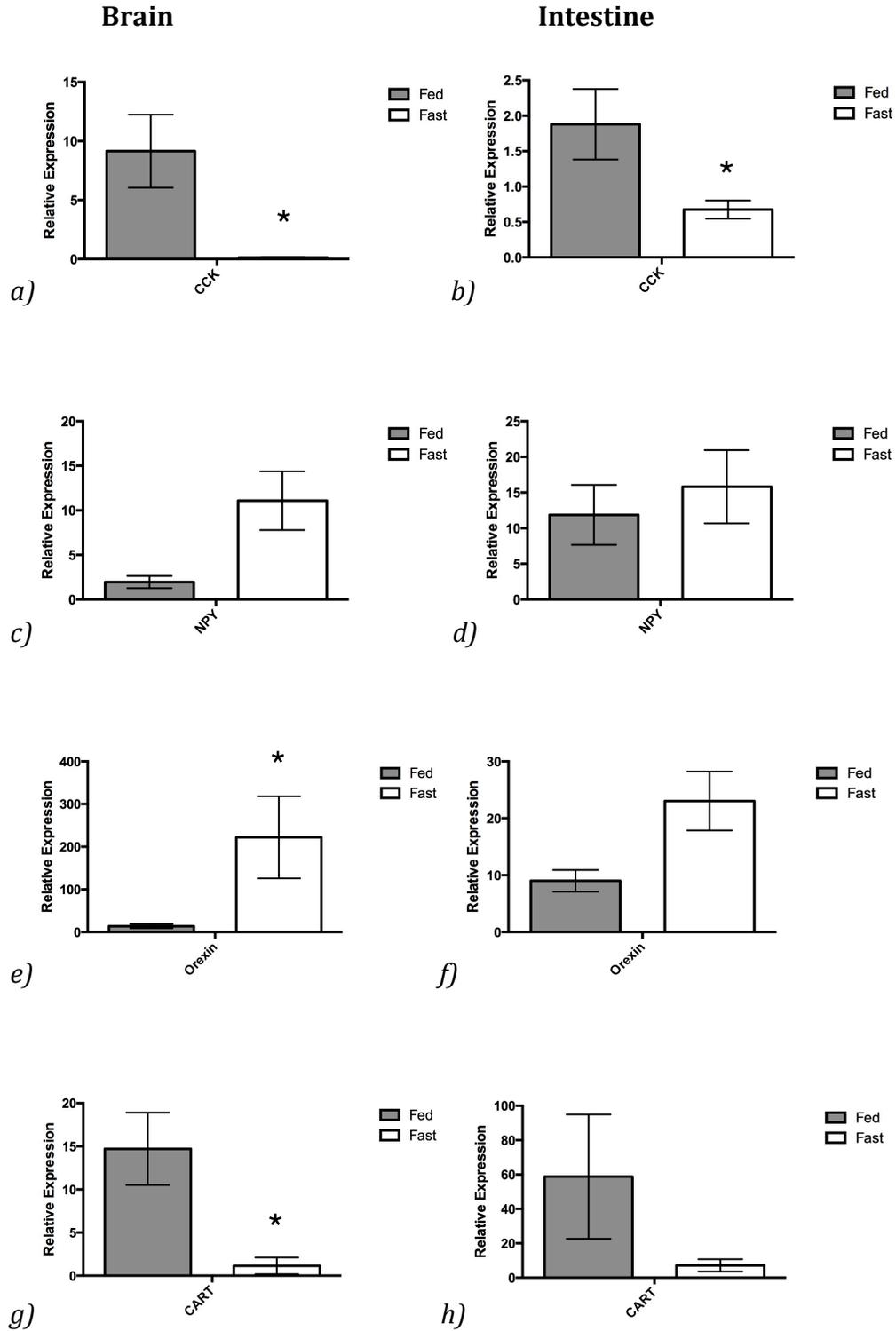


Figure 7: Relative expression of CCK (a,b), NPY (c,d), orexin (e,f) and CART (g,h) in fed and fasted platyfish brain and intestine. Stars indicate significant difference between fed and fasted states (Student's t test). Data are expressed as mean \pm SEM, with significance considered at $p < 0.05$.

Effects of fasting on mRNA expression in brain and intestine between sexes – platyfish

Platyfish can be sexed without surgery, so fish were divided into groups of 2 females for every 1 male in order to reduce stress on the female fish due to sexual competition between males. In order to assess any gender-specific differences in expression as a response to fasting, expression was further analyzed to compare males and females using a two-way ANOVA.

There were no significant differences in expression between fed males and females or fasted males and females in either of CCK, NPY, orexin, or CART transcripts ($n = 5-8$ per group) in either brain (Figure 8a, c, e, g) or intestine (Figure 8b, d, f, h) tissues.

The response to fasting was similar in males and females with a decrease in both CCK and CART expression and an increase in orexin expression in the brain and a decrease in CCK in the intestine.

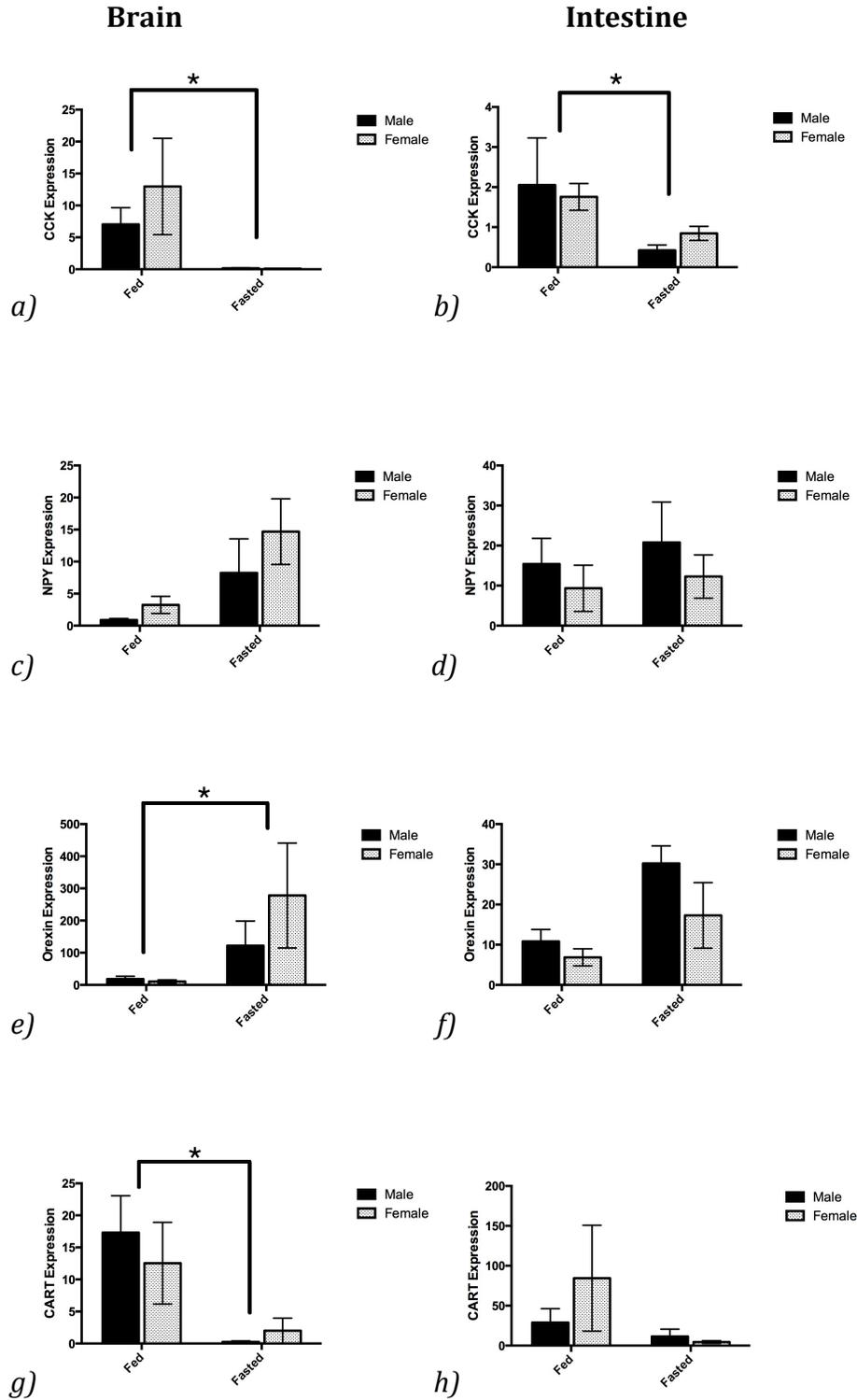


Figure 8: Relative expression of CCK (a,b), NPY (c,d), orexin (e,f) and CART (g,h) in fed vs. fasted platyfish brain and intestine in both males and females. Stars indicate significant difference between fed and fasted states, with letters dictating differences between genders (Two-way ANOVA). Data are expressed as mean \pm SEM, with significance considered at $p < 0.05$.

*Effects of fasting on transcript expression in brain and intestine – cunner
(males and females)*

In the brain, CCK expression was significantly lower in fasted fish than fed fish [Figure 9a; fed ($n=10$); fasted ($n=12$)]. In the intestine there was no significant change in CCK expression due to fasting [Figure 9b; fed ($n=11$); fasted ($n=14$)].

NPY expression in the brain showed no significant difference between fish sampled from the fed tanks ($n=10$) and those from fasted tanks ($n=10$) (Figure 9c). In the intestine, there were no significant increases found in the expression of NPY [Figure 9c; fed ($n=14$); fasted ($n=13$)].

Orexin transcript expression in the brain significantly increased after fasting [Figure 9e; fed ($n=10$); fasted ($n=13$)]. In the intestine, there was no significant increase in the expression of orexin as a result of the fasting [Figure 9f; fed ($n=11$); fasted ($n=13$)].

CART transcript expression showed no significant change in either the brain [Figure 9g; fed ($n=11$); fasted ($n=13$)] or the intestine [Figure 9h; fed ($n=14$); fasted ($n=11$)] as a result of the 10-days of fasting.

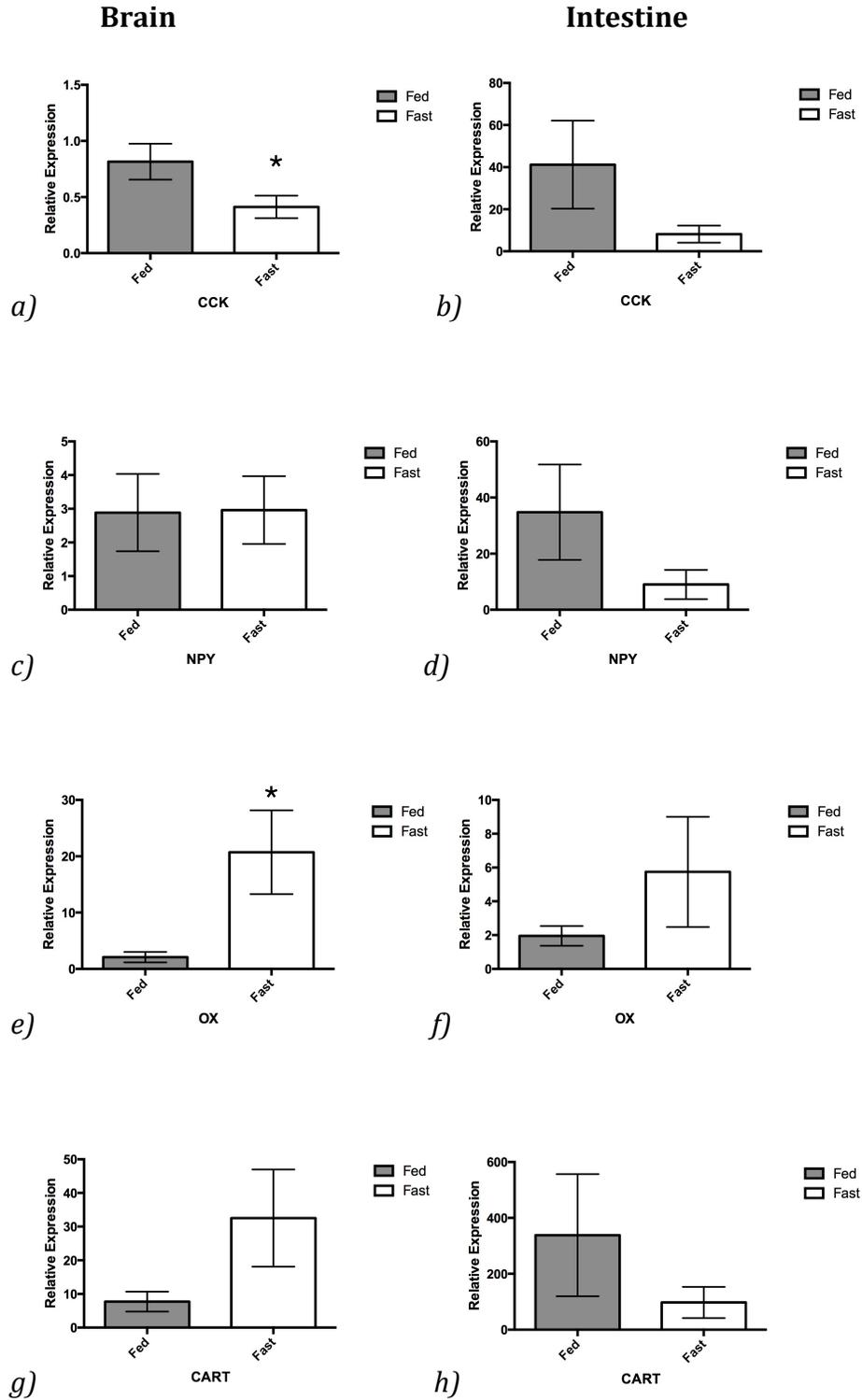


Figure 9: Relative expression of CCK (a,b), NPY (c,d), orexin (e,f) and CART (g,h) in fed and fasted cunner brain and intestine. Stars indicate significant difference between fed and fasted states (Student's t test). Data are expressed as mean \pm SEM, with significance considered at $p < 0.05$.

Effects of fasting on mRNA expression in brain and gut between sexes – cunner

Cunner gender determination requires dissection; therefore gender was determined based on the presence of ovaries or testes. Samples were grouped into fed and fasted groups and expression was re-analyzed using a two-way ANOVA.

NPY brain expression was higher in females than in males (Figure 10c). In the intestine, there was no significant change in the expression as a result of gender (Figure 10d).

There were no significant differences in expression between males and females fed and fasted fish in either of CCK, orexin, and CART transcripts. These results held true for the both brain (Figure 10a, e, g) and intestine (Figure 10b, f, h).

The response to fasting was similar in males and females with a decrease in CCK expression and an increase in orexin expression in the brain. In the intestine, the response to fasting was similar in males and females with no significant differences found in either CCK, NPY, orexin, or CART expression.

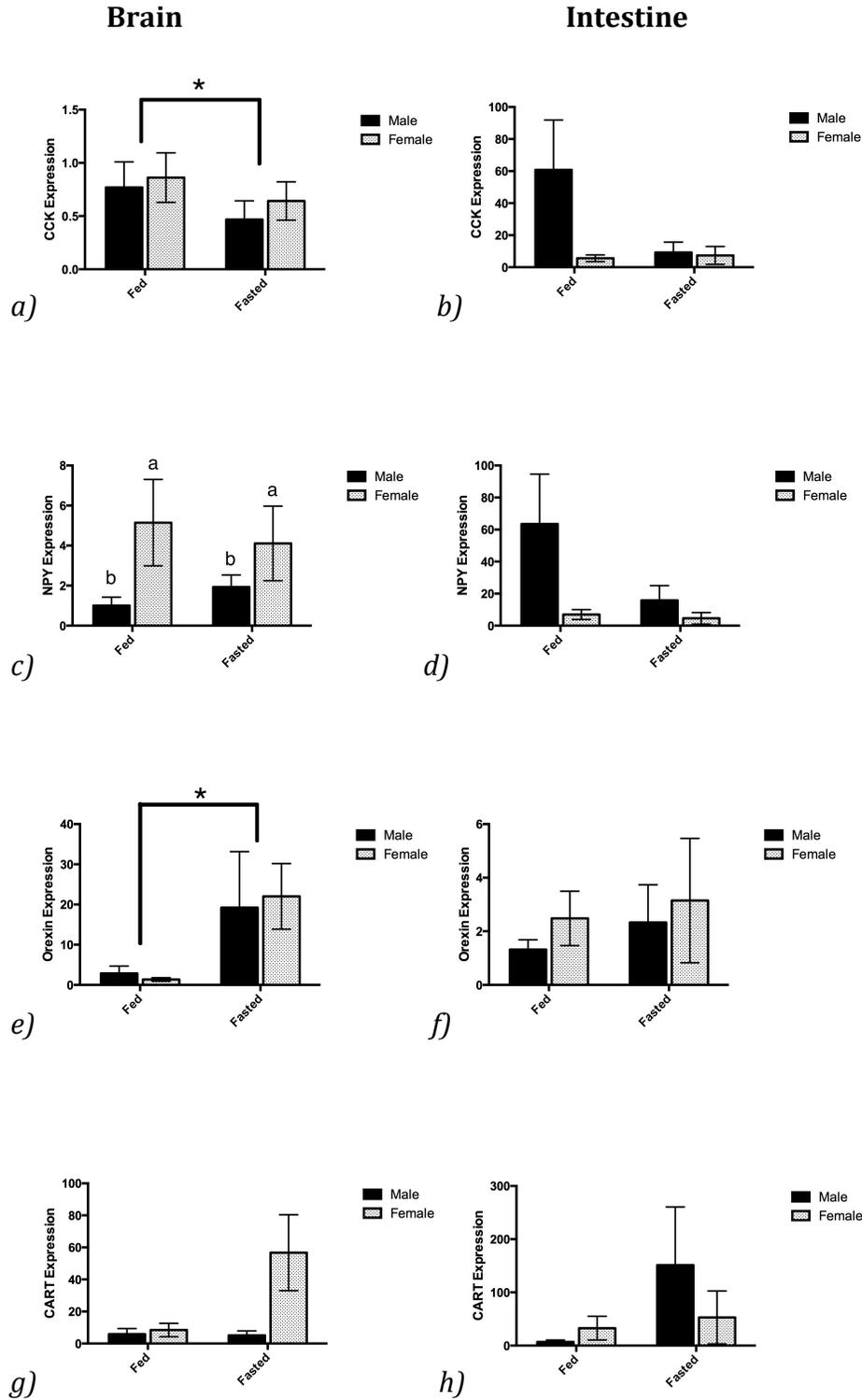


Figure 10: Relative expression of CCK (a,b), NPY (c,d), orexin (e,f) and CART (g,h) in fed vs. fasted cunner brain and intestine in both males and females. Stars indicate significant difference between fed and fasted states, with letters dictating differences between genders (Two-way ANOVA). Data are expressed as mean \pm SEM, with significance considered at $p < 0.05$.

Discussion

Intraperitoneal (IP) injection studies

Intraperitoneal injections of CCK or orexin caused a change in the number of search behaviours as well as a change in the number of pellets consumed in platyfish. These responses were not due to stress as both sham and saline injected fish were responsive to food and displayed active searching behaviours.

Injections of CCK caused a significant decrease in both the number of search attempts and the number of pellets eaten in the 30 minutes of recording after the injection period. This phenomenon has been documented in goldfish (Himick & Peter, 1994; H. Volkoff et al., 2003) and blind cavefish (Penney & Volkoff, 2014), as peripheral injections of sulfated CCK-8 cause suppression of food intake, supporting the role of CCK as an anorexigenic appetite-regulating hormone in fish. IP studies in rats have also shown that injections of CCK caused a decrease in feeding responses (Gibbs et al., 1973).

Injections of orexin resulted in an increase in search behaviours as compared to the saline-injected control fish. However, there was no change in the number of pellets consumed by the fish. IP orexin injections have previously been shown to increase both food intake and locomotion in ornate wrasse, *Thalassoma pavo* (Facciolo, Crudo, Giusi, Alò, & Canonaco, 2009) and blind cavefish, *Astyanax fasciatus mexicanus* (Penney & Volkoff, 2014). Similarly, ICV injections of orexin increase food intake and locomotor activity in goldfish (Nakamachi et al., 2006; Helene Volkoff et al., 1999).

It appears, from evidence in rodents (Gibbs et al., 1973), that orexin might be a major regulator for arousal/locomotion and that its role on the regulation of food intake is indirect, by increasing searching foraging behaviour. This may explain the results in the present studies, in which locomotion was greatly increased, with the fish moving around the tank and approaching the pellets, but not actively consuming them.

IP studies were attempted in cunner as well, but trials were unsuccessful, as cunners did not respond well to the injections. After injection with the saline, CCK, or orexin, cunners became metabolically depressed (a physiological adaptation for energy preservation) – which was evidenced by their vertical orientation in the water column (Personal communication – Sarah Tuziak, 2014).

Tissue Distribution

Expression studies were conducted in platyfish as although sequences are available, there are no studies characterizing these peptides in platyfish.

CCK was expressed in the brain and gut as well as several other peripheral tissues with apparent highest expression in the brain. Similar to these results, CCK has been localized in the brain and intestine of several other species of fish including goldfish (Peyon et al., 1999), rainbow trout (Jensen, Rourke, Møller, Jønson, & Johnsen, 2001) and flounder (Kurokawa, Suzuki, & Hashimoto, 2003). The presence of CCK expression in the gastrointestinal tract is not surprising given the role of CCK in the digestive process in fish, such as contraction of the gallbladder (Aldman & Holmgren, 1995; Einarsson, Davies, & Talbot, 1997) or stimulation of gastric

motility (Raybould & Tache, 1988). CCK was also expressed in gonads, similar to what is observed in other fish, such as winter skate (MacDonald & Volkoff, 2009).

NPY was detected in nearly all of the tissues studied. The highest apparent expression was found to be in the brain. High NPY brain expression has been reported in other fish species, such as winter skate (MacDonald & Volkoff, 2009) and Chinese perch (*Siniperca chuatsi*) (Liang, Li, Yao, Cheong, & Liao, 2007). NPY was also detected in a number of peripheral tissues in platyfish, including intestine and gonads. Similarly, NPY has been detected in the periphery in other species. For example, in Brazilian flounder (*Paralichthys orgignyanus*) (Campos et al., 2010) and Chinese perch (Liang et al., 2007), NPY was expressed in several peripheral tissues, including liver, spleen, and intestine.

Orexin was expressed in all tissues examined including brain, gills, spleen, and testes. Orexin detection in central tissues has been previously reported in other fish species, including goldfish (Gema Huesa, Anthony N van den Pol, & Thomas E Finger, 2005), zebrafish (Kaslin et al., 2004), cod (M. Xu & Volkoff, 2007), winter flounder (*Pleuronectes americanus*) (Buckley, MacDonald, Tuziak, & Volkoff, 2010), and orange grouper (Yan et al., 2011). Prepro-orexin mRNA was previously determined to be highly expressed in the gills, spleen, and testes (amongst other tissues) of the barfin flounder, *Verasper moseri* (Amiya et al., 2012) as well as in the liver of the orange grouper (Yan et al., 2011). The presence of orexin in peripheral tissues may help explain its role in appetite-regulation, locomotion, or arousal. Orexin was also expressed in the gonads, as previously reported in other fish species such as goldfish, where its role is not well understood, but there has been

some evidence that orexin may inhibit reproductive behaviour by inhibiting release of gonadotropin-releasing hormone (Hoskins et al., 2008).

CART was expressed in nearly all of the tissues, but most strongly in the intestine followed by the gut and the gonads. CART plays a role in appetite regulation and has been localized in the GI tract of both mammals (Murphy et al., 2000) and fish (Babichuk & Volkoff, 2013). CART mRNA has been detected in forebrain of a number of other fish species, including goldfish (H. Volkoff & R. E. Peter, 2001), cod (Kehoe & Volkoff, 2007), salmon (Murashita, Kurokawa, Ebbesson, Stefansson, & Rønnestad, 2009), and winter flounder (MacDonald & Volkoff, 2009). CART has been implicated as having a role in the prompting of the preparatory phase in the annual sexual cycle of catfish, which may explain its expression in the gonads (Barsagade et al., 2010).

Tissue distribution studies were previously completed in cunner involving these peptides in a study by Hayes and Volkoff, 2014 (Hayes & Volkoff, 2014), so there was no need to perform them as part of this study.

Effects of fasting on expression of appetite regulators

CCK

In the platyfish fasting experiments, both sexes of platyfish were deprived of food for 10 days and both brain and gut tissues were collected after the fasting period was over. Changes in the transcript expression of CCK, NPY, orexin, and CART were measured to determine the effects of fasting.

In both platyfish and cunner, fasting induced significant decreases in CCK expression in brain. Decreases in CCK expression following fasting have previously been reported in the brain of fish including cunner (Babichuk & Volkoff, 2013), goldfish (Peyon et al., 1999), winter flounder (Hélène Volkoff, Xu, et al., 2009) and yellowtail (Murashita, Fukada, Hosokawa, & Masumoto, 2007).

Within the intestine, fasting induced decreased CCK expression in platyfish, but not in cunner. Similarly to the result in platyfish, CCK expression has been shown to decrease following fasting in the intestine in a number of other fish species, including winter flounder (Hélène Volkoff, Xu, et al., 2009) and yellowtail (Murashita et al., 2007).

The discrepancy between this study and the previous fasting study in cunner where one week of fasting caused a significant decrease in CCK transcript expression in the intestine (Babichuk & Volkoff, 2013; Hayes & Volkoff, 2014), might have been due to different experimental protocols, such as the length of fasting period (10 days vs. 7 days), area of intestine sampled, or size of the fish used.

NPY

In both platyfish and cunner, NPY expression was not significantly altered by the 10-day fasting, in either the brain or gut samples. Similar results have been shown for cunner in a previous study (Babichuk & Volkoff, 2013). Similarly, NPY expression in the brain is not altered after fasting in cod (Kehoe & Volkoff, 2007).

In other fish including goldfish (H. Volkoff & R. Peter, 2001), salmon (J. T. Silverstein et al., 1998), catfish (J. Silverstein, Wolters, & Holland, 1999), winter flounder (MacDonald & Volkoff, 2009), and brazilian flounder (Campos et al., 2010), fasting results in increased NPY transcript expression in brain.

Using whole brains may mask the effects that specific regions of the brain have on NPY expression (Hoskins & Volkoff, 2012). In a similar experiment involving tilapia, one week of fasting showed no NPY mRNA increase in whole brain tissue (Riley et al., 2008), mirroring the results found in our study. Different regions of the brain may respond differently to the effects of fasting. In a continuing study, it may be important to look at separate brain areas in order to better elucidate the effects of fasting on NPY expression in platyfish.

Orexin

In the brain, orexin transcript expression was significantly increased after fasting in both cunner and platyfish. Fasting induced decreases in orexin brain expression have previously been shown in several fish species including zebrafish (Novak et al., 2005), goldfish (Nakamachi et al., 2006), and in the hypothalamus in winter flounder (Buckley et al., 2010). In a previous study on cunner, fasting for 1 or 2 weeks had no significant effect on orexin expression in either the hypothalamus or telencephalon (Babichuk & Volkoff, 2013). The contrasting results may have been due to different sizes of the fish used since our study included smaller fish.

There were no significant differences in orexin expression between the fed and fasted groups in either platyfish or cunner intestine. These results are

consistent with those of a previous study on cunner showing no effects of fasting (4 weeks) on intestinal orexin expression (Hayes & Volkoff, 2014). Unfortunately to our knowledge, there are no other studies examining orexin expression in the intestine of fish which we can use to help draw conclusions.

In mammals, fasting increases orexin mRNA levels in the gastrointestinal tract (Korczyński, Ceregrzyn, Kato, Wolinski, & Zabielski, 2006) and orexins excite secretomotor neurons, modulate gastric and intestinal motility and secretion, and regulate hormone release from pancreatic endocrine cells (Kirchgessner, 2002). The differences found between mammals and fish might reflect differences in GIT morphology and digestive physiology.

CART

In platyfish, CART expression in the brain showed a significant decrease after fasting. These findings are consistently found in other fish species as well, including goldfish (Abbott & Volkoff, 2011), catfish (Kobayashi et al., 2008), and salmon (Murashita et al., 2009). In cunner, CART expression in the brain did not show any significant decrease as a result of fasting. This is similar to the result previously reported in cunner, where hypothalamic CART mRNA expression was not significantly altered due to fasting (Babichuk & Volkoff, 2013). In other species of fish including the common carp (Wan et al., 2012) and catfish (Kobayashi et al., 2008), forebrain or whole brain CART expression show a similar trend of down regulation as a result of periods of fasting.

In the intestine, there were no significant changes due to fasting in CART expression in either platyfish or cunner, perhaps suggesting that CART does not play a role in the balance of energy regulation in the intestine. In both platyfish and cunner, CART may play an entirely different role more related to its other functions such as body weight regulation or as a modulator of the stress response (Rogge et al., 2008). Therefore, the role of CART as an appetite-regulating hormone may be limited to its presence in the brain.

Platyfish and cunner display very different life histories, including different lifestyles, gut morphologies, and feeding habits. Due to these differences, expressions of appetite-regulators as a result of fasting could be very dissimilar. Platyfish live in a more temperate climate than cunner, this means that platyfish do not need to undergo torpor to avoid colder temperatures and are able to feed in every season. Cunner live in a much colder environment than platyfish do, and to avoid these colder temperatures undergo torpor where they down-regulate a number of appetite-regulating hormones (Babichuk & Volkoff, 2013). Cunner also lack a “true gut” (Hayes & Volkoff, 2014) and therefore may not release appetite-regulating peptides in the same way that platyfish are able to.

In platyfish there seems to be a trend where the action and effects of appetite regulating hormones are tissue-specific. In the brain, CCK and CART showed decreased expression as a result of fasting as has been demonstrated in previous

studies using other species. However, in gut tissue, expression was only decreased with regards to CCK. Since CCK has been mainly localized in the gut, this is not at all surprising. With CART, its decreased expression in response to feeding is only found in the brain and not the gut, suggesting a tissue specific mode of action. Orexin shows a similar trend as with CART in regards to tissue specificity, as fasting causes an increase in orexin mRNA expression in the brain only. With regards to NPY, there was no increase in brain expression due to fasting which was not expected. It is possible that NPY regulates energy balance in different contexts in platyfish. Because fish show a wide array of digestive tract morphologies amongst species, the release of hormones from these tissues may not be as well conserved as those released from the brain (Hoskins & Volkoff, 2012). Therefore, appetite-regulating hormone expression in the intestine may not mimic the effects found in the brain as a result of fasting.

A trend seen in fasting experiments in cunners seem to be tissue-specific modulation of metabolism in the body. In the brain, both CCK and orexin show an increase and a decrease in expression, respectively, relating to their known roles as appetite-regulating hormones. With regards to NPY and CART, fasting showed no significant effect on expression in the brain, suggesting other functions not related to energy balance. In the gut, there were no expected results for any of the four appetite-regulating hormones that were investigated. This may be due to the lack of a “defined stomach” in the family labridae (Hoskins & Volkoff, 2012). In this family, the anterior intestine therefore secretes corresponding gastric hormones instead. Therefore hormone expression in the gut may be localized to a specific region and

could potentially be masked by investigating the whole gut, which may dilute the effect of expression as a result of food deprivation.

Influence of gender on feeding and feeding responses

IP injections studies in platyfish were also analyzed by gender. We found no differences in the searching behaviours or the level of food intake between males and females, indicating that their peptide systems and response to exogenous peptides are probably the same.

In order to determine the possible effects of gender on expression of appetite-regulating hormones and their responses to feeding, the expression of CCK, NPY, orexin, and CART were compared between fed females and males, fasted females and males, and fed and fasted males and fed and fasted females.

Our results show that, in platyfish, there were no significant interactions between gender and fed/fast treatment in the platyfish gut or brain as a response to the 10-day fasting period. (i.e. there were no differences in expression between fed males and females and no differences in the response to fasting). This was seen in every appetite-regulating hormone that was studied in our experiment. In cunner, the only exception was the expression of NPY mRNA in the brain of cunner fasted for 10 days. Although fasting had no significant effect on the expression of NPY, females showed a significantly much higher expression than males of the same treatment groups. Interestingly, fasted female cunners had higher NPY levels than fasted males, suggesting that females may respond to fasting to a higher degree and are more susceptible to the effects of fasting

In mammals and some species of fish there seem to be sexually dimorphic differences in expression in certain appetite-regulating hormones. In goldfish, females have higher brain CCK expression than males at certain time of the year during different reproductive stages in specific brain regions (Peyon et al., 1999). In our studies, only one reproductive stage was analyzed in both platyfish and cunner, so examining multiple stages might reveal differences in expression of appetite-regulating hormones.

NPY, orexin, and CART have all been linked either to changes in sexual behaviours or as being key proponents in the sexual maturation process. In rats, no difference was detected in the mean levels of NPY gene expression in the arcuate nucleus between male and female rats (Urban, Bauer-Dantoin, & Levine, 1993). NPY has been linked to growth hormone secretion in the platyfish and has been implicated in the sexual maturation process (Cepriano & Schreibman, 1993). Also, NPY acts to influence aggression in fish and there are differences in expression in dominant and subordinate zebrafish males and females, which may be linked to reproductive behaviours (Filby, Paull, Hickmore, & Tyler, 2010).

In goldfish, orexin treatment has been shown to inhibit spawning behaviour and decrease expression of GnRH-II (Hoskins et al., 2008), but again this may not translate to sexually dimorphic responses in expression due to fasting. With CART, fasting for two days caused an increase in expression in males but this effect was absent in females (L. Xu et al., 2009). Perhaps gender has no bearing on the

expression and response of appetite-regulating hormones in certain species of fish and only differences in feeding strategies invoke changes in expression.

In further studies, investigating different stages of the reproductive cycle, when fish divert resources from feeding to reproductive processes, may elucidate the role that appetite-regulators have on reproduction. Furthermore, investigating the interaction between hormones involved in appetite and those involved in reproduction may provide more details into the regulation of energy balance in fish and other vertebrates.

Conclusions

Platyfish provide a useful model to study the effects of peripheral injections of appetite-regulating hormones because they respond well to treatments and show the effects fairly quickly. Platyfish are also useful to study the effect of fasting on expression of appetite-regulating hormones. In platyfish, fasting has a significant effect on a number of appetite-regulating hormones found in the brain, including CCK, orexin, and CART. However, in the gut no significant changes were seen in the expression of appetite-regulating hormones other than CCK. The fasting-induced changes in expression of these peptides suggest that they are involved in the control of feeding and metabolism and their action may be tissue specific. Cunnners also provide a useful model for studying the effects of appetite-regulating hormones in the control of energy balance. Cunnners go through a prolonged natural torpor, which results in a decrease in the expression of some appetite-regulating hormones

(Babichuk & Volkoff, 2013). However, short-term periods of fasting do not show these trends, as orexin expression in the brain has been shown to increase as a result of fasting for a period of 10 days.

Sexually dimorphic expression of appetite-regulators can be seen in a number of different species and peptides, but these may be specific to the individual species as well as the individual peptide. Although the links between energy balance and reproduction are unclear, there may be an interaction between appetite-regulating hormones and reproductive hormones in different stages of reproduction, which further studies may be able to verify.

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