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Growth hormone regulates intestinal gene expression of nutrient transporters in tilapia (*Oreochromis mossambicus*)

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ABSTRACT

Among the various ways that growth hormone (GH) underlies the growth physiology of teleost fishes, GH stimulates transport pathways that facilitate the absorption of nutrients across intestinal epithelia. The current study investigated the effects of GH on the gene expression of nutrient transporters in an omnivorous teleost, the Mozambique tilapia (*Oreochromis mossambicus*). We employed pituitary gland removal (hypophysectomy) and hormone replacement to assess whether GH directs the gene expression of the *GH receptor* (*ghr2*), the peptide transporters, *pept1a, pept1b* and *pept2*, the amino acid transporter, *slc7a9*, the Na^+/glucose cotransporter, *sglt1*, the glucose transporter, *glut2*, and the myo-inositol transporter, *smit2*, in anterior, middle, and posterior intestine. *ghr2* was predominantly expressed in posterior intestine, while *pept1a*, *pept1b*, *slc7a9*, *sglt1*, *glut2*, and *smit2* exhibited the highest mRNA levels in anterior and/or middle intestine. While hypophysectomized tilapia exhibited diminished expression of *ghr2*, *pept1a*, *pept1b*, *slc7a9*, and *glut2* compared with intact and sham-operated controls, only *ghr2*, *pept1a*, *pept1b* and *glut2* levels were restored by GH replacement. Our findings indicate that GH supports growth, at least in part, by stimulating the gene expression of its cognate receptor and key nutrient transporters in the intestine.

1. Introduction

Growth in vertebrates, including teleost fishes, is principally controlled by the growth hormone (GH)/insulin-like growth-factor (IGF) system (Reinecke et al., 2005; Duan et al., 2010; Pérez-Sánchez et al., 2018). Under favorable environmental conditions such as when food is available, factors within the GH/IGF system regulate the absorption of nutrients from the diet (Collie and Ferraris, 1995; Pérez-Sánchez and Le Bail 1999; Mommsen, 2001) and the allocation of acquired nutrients toward anabolic processes (Reindl and Sheridan, 2012). Thus, plasma GH and IGF1 levels are sensitive to both short-term (periprandial) and long-term (prolonged fasting) nutritional conditions (Uchida et al., 2003; Bertucci et al., 2019). With respect to their periprandial dynamics, plasma GH and IGF1 typically rise within several hours after a meal (Fox et al., 2009; Canosa et al., 2005). Alternatively, coho salmon (*Oncorhynchus kisutch*), masu salmon (*O. masou*), and Mozambique tilapia (*Oreochromis mossambicus*) subjected to prolonged nutrient restriction exhibited lower plasma IGF1 and hepatic *igf1* mRNA levels compared with fed controls (Duan and Plisetskaya, 1993; Uchida et al., 2003; Pierce et al., 2007; Fox et al., 2010; Kawaguchi et al., 2013). During prolonged fasts, however, plasma GH generally rises to mobilize energy reserves (Fox et al., 2009; Breves et al., 2014; Shimizu et al., 2009; Small and Peterson, 2005). Collectively, these patterns indicate that GH signaling responds to nutrient availability in fashions that match adaptive metabolic strategies with environmental conditions. While one consequence of the postprandial rise in plasma GH is to promote a rise in plasma IGF1 that will stimulate growth (Shimizu et al., 2009), how the GH/IGF system directs nutrient absorptive processes in the gastrointestinal tract remains unresolved (Collie and Ferraris, 1995; Reshkin et al., 1989; Farmanfarmaian and Sun, 1999).

In fishes, the proximal region of the midgut (intestine), is generally the primary site of nutrient absorption (Diaz et al., 1997; Olsen et al., 1999; Nordrum et al., 2000). Apically located transmembrane proteins mediate the active and passive transport of nutrients into the interior of enterocytes. Then, nutrients are transported across the basolateral membrane and distributed via blood to organs and tissues (Grosell et al., 2010). Small peptides are absorbed more efficiently by fishes than free amino acids (Terjesen et al., 2006; Zhang et al., 2006). The

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transport of di- and tripeptides is mediated by apically located members of the peptide transporter (PepT/SLC15A) family (Daniel and Kottra 2004; Verri et al., 2010; Con et al., 2017; Wang et al., 2017). There are at least three PepT variants expressed in teleost intestine, PepT1a/SLC15A1a, PepT1b/SLC15A1b, and PepT2/SLC15A2 (Romano et al., 2006; Bucking and Schulte, 2012; Ronkin et al., 2015). PepT1 is a high capacity, low affinity, H^+-dependent cotransporter of protons and oligopeptides with three or fewer amino acids (Daniel, 2004; Benner et al., 2011). By contrast, PepT2 is a high affinity, low capacity, H^+-dependent cotransporter more selective for substrate binding than PepT1 (Romano et al., 2006). The transport of free amino acids is largely mediated by heterotrimeric transporters formed by light and heavy-chain proteins, such as the neutral and dibasic amino acid transporter complex ($B^{0,+}AT$) encoded by *slc3a1* and *slc7a9* genes (Wagner et al., 2001; Nitzan et al., 2017). The chemical digestion of carbohydrates yields glucose that is available for intestinal uptake by Na^+/glucose cotransporter 1 (SGLT1/SLC5A1) and glucose transporter 2 (GLUT2/SLC2A2) across the apical and basolateral membranes of enterocytes, respectively (Polakof et al., 2012; Chen et al., 2017). Inositol is a vitamin-like nutrient that is often included in formulated fish diets (Waagbo et al., 1998; Shiau and Su, 2005). Myo-inositol, the most abundant isomer of inositol, is transported by Na^+/myo-inositol transporter 2 (SMIT2/SLC5A11) (Aouameur et al., 2007). In Nile tilapia (*O. niloticus*), *sglt1* and *smit2* were more highly expressed in the intestine than any other identified *slc5* gene transcripts (Subramaniam et al., 2019).

The teleost gastrointestinal tract is an established target of GH given the varied physiological responses by intestine to GH administration (Farmanfarmaian and Sun, 1999). Individual teleost fishes contain two putative GH receptors (GHRs) that group into distinct clades, GHR1 and GHR2 (Saera-Vila et al., 2005; Jiao et al., 2006). Accordingly, Mozambique tilapia express two *ghr* gene transcripts denoted *ghr1* and *ghr2* (Kajimura et al., 2004; Pierce et al., 2007). *ghr1* encodes the putative receptor for somatolactin in tilapia (Pierce et al., 2007; Uchida et al., 2009). In this study, we targeted *ghr2* which encodes the primary receptor for GH and is expressed in the intestine (Pierce et al., 2007, 2012). Given the coincident increases in plasma GH and intestinal absorptive capacities that occur following a meal (Collie and Ferraris, 1995), we hypothesized that a regulatory link connects GH with particular nutrient transporters. In turn, the current study assessed whether GH directs the expression of *ghr2* and nutrient transporters in intestinal segments of hypophysectomized tilapia injected with GH.

2. Materials and methods

2.1. Animals

Male Mozambique tilapia (70–150 g) were selected from a population reared in outdoor tanks supplied with a continuous flow of municipal freshwater (FW) at the Hawai'i Institute of Marine Biology. The fish were maintained at 24–26 °C under natural photoperiod and fed a commercial diet (Skretting, Tooele, UT). During the experimental period and recovery from hypophysectomy (4 days), however, fish were not fed to avoid confounding the effects of between-subject variance in feed intake and GH treatment (Con et al, 2017). The Institutional Animal Care and Use Committee of the University of Hawai'i approved all housing, surgical, and experimental protocols.

2.2. Hypophysectomy and GH replacement

Hypophysectomy was performed by the transorbital technique described by Nishioka (1994). Prior to surgery, fish were anesthetized in buffered tricaine methanesulfonate (100 mg/L, Argent Chemical Laboratories, Redmond, WA) and 2-phenoxyethanol (2-PE; 0.3 ml/L, Sigma, St. Louis, MO) in FW. After the procedure, fish recovered in experimental aquaria containing recirculating brackish water (BW; 12 ppt) composed of seawater (Kaneohe Bay, Hawaii) diluted with municipal FW. Fish were maintained in BW for 3 days. Experimental aquaria were maintained at 24–26 °C. Fish were treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ) and not fed following surgery.

Three days after hypophysectomy, fish (n = 6–9) were anesthetized with 2-PE (0.3 ml/l) and administered ovine GH (oGH; 5 μg/g body weight) or saline vehicle (0.9% NaCl) by a single intraperitoneal injection (1.0 μL/g body weight). All animals were treated in the same fashion prior to injections. The concentration of oGH administered in the current study was based on previous studies in which oGH was shown to regulate components of the GH/IGF/IGFBP system in Mozambique tilapia (Pierce et al., 2012; Breves et al., 2014; Douros et al., 2017). Intact and sham-operated groups were included as controls. oGH was obtained from the National Hormone and Peptide Program (NIDDK-oGH-15). After injection, fish were returned to the experimental aquaria and sampled after 12 h. Our previous study showed that GH elicits clear effects on the GH/IGF/IGFBP system within 12 h (Breves et al., 2014). At the time of sampling, fish were lethally anesthetized and three intestinal segments (anterior, middle, and posterior) were collected. The anterior, middle, and posterior segments corresponded to the hepatic loop, gastric loop, and terminal segment, respectively (Seale et al., 2014). Tissue samples were washed with 0.9% NaCl, snap frozen in liquid nitrogen, and stored at −80 °C. The completeness of all hypophysectomies was confirmed by postmortem inspection of the cranial cavity.

2.3. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from homogenized tissue samples using TRI Reagent (MRC, Cincinnati, OH) according to the manufacturer's protocols. Reverse transcription negative control reactions confirmed the absence of contaminating DNA. RNA quality and quantity were determined by spectrophotometry using a NanoDrop One (Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 500 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Reference and target genes were assayed by the relative quantification method (Pfaffl, 2001). Primer pairs and their amplification efficiencies and amplicon sizes are provided in Table 1. The qRT-PCR reaction volume (15 μL) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of forward and reverse primers, and 1–3 μL of undiluted cDNA (equivalent to 25–75 ng of total RNA). PCR cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Gene expression is reported as the ratio of target to reference RNA levels; 18 s ribosomal RNA (18 s) levels were used to normalize the levels of target genes.

2.4. Statistical analyses

Data were analyzed by two-way ANOVA with treatment and intestinal segment as main effects. Data that did not meet the assumptions of variance (Brown-Forsythe and Bartlett's tests) and normality (Kolmogorov-Smirnov test) were log-transformed prior to further analysis. Significant main effects of treatment and intestinal segment ($P < 0.05$) were followed up by Fisher's protected LSD test. All statistical tests were performed using GraphPad Prism 8.0 (San Diego, CA).

3. Results

Significant effects of treatment, intestinal segment, and an interaction were observed for intestinal *ghr2* expression (Fig. 1A). *ghr2* levels in the anterior and middle intestine were diminished following hypophysectomy compared with intact and sham-saline controls; oGH restored *ghr2* expression in both segments.

Table 1
Gene specific primers used for qRT-PCR.

Gene	Primer Sequence (5′-3′)	R^2	Eff.%	Amplicon size (bp)	Accession no.	Reference
18s	F: GCTACCACATCCAAGGAAGGC R: TTCGTCACTACCTCCCCGAGT	0.99	97	69	AF497908	Magdeldin et al. (2007)
ghr2	F: CACACCTCGATCTGGACATATTACA R: CGGTTGGACAATGTCATTAACAA	0.89	106	102	EF452496	Pierce et al. (2007)
pept1a	F: TAAAACCCTGCCTGACTTCC R: AATCCTCATTAGCCCCAAAA	0.98	102	131	XM_003459630	Con et al. (2017)
pept1b	F: CCAAGCCAGAACAAGGTAACA R: GGCTCAATTAGTCCCAAGTCC	0.99	97	100	XM_003447363	Con et al. (2017)
pept2	F: CCAGTTTGGCGAGGAGCATA R: CACTGCACGTCACCTCTCAA	0.99	101	123	XM_005475385.4	Newly-designed primers
slc7a9	F: ATACGACGGCTGGAACAATC R: AGATAGCTCACATTCACCAGCA	0.96	93	132	XM_003445502	Nitzan et al. (2017)
sglt1	F: CCCGAGTACTTGAAGAAGAG R: GCAATAACAGCGAGGTAGA	0.94	92	164	XM_019361133.1	Subramaniam et al. (2019)
smit2	F: GAGACGGAAGAAGGAAGATG R: GCCCAGTAACCAATGATAAAG	0.99	96	150	XM_005461087.3	Subramaniam et al. (2019)
glut2	F: GGCACTCTAGCTCTGGCTGTGT R: GGGTGGTGACCTGGGTCTTCTT	0.99	99	185	XM_003442884.5	Chen et al. (2017)

There were significant treatment, intestinal segment, and interaction effects on *pept1a* (Fig. 1B). Intestinal expression of *pept1a* was robust in the anterior and middle intestine, and nearly undetectable in the posterior intestine. *pepta1* levels were diminished in the middle intestine by > 100-fold following hypophysectomy and were restored by oGH. Significant effects of treatment, intestinal segment, and an interaction were observed for *pept1b* (Fig. 1C). Hypophysectomy diminished *pept1b* expression in the middle intestine by ~1000-fold and was restored by oGH. Resembling *pept1a*, *pept1b* levels were highest in the anterior and middle intestine. While expression levels were higher in the middle and posterior intestine, there were no significant treatment or interaction effects on *pept2* expression (data not shown).

There were significant treatment and intestinal segment main effects on *slc7a9* expression (Fig. 1D). *slc7a9* expression was highest in the middle intestine where levels were diminished following hypophysectomy; however, oGH did not rescue the marked fall in expression. There was only a significant main effect of intestinal segment on *sglt1*; *sglt1* levels were higher in the anterior and middle intestine compared with the posterior intestine (Fig. 1E). There were significant treatment and intestinal segment effects on *glut2* levels (Fig. 1F). *glut2* expression in the anterior intestine was diminished following hypophysectomy and restored by oGH . *glut2* levels were higher in the anterior and middle intestine compared with the posterior intestine. Lastly, there was no significant treatment effect on *smit2* levels; *smit2* expression was higher in the anterior and middle intestine compared with the posterior intestine (Fig. 1G).

4. Discussion

To our knowledge, the current study is the first to report links between GH and specific molecular targets in the intestine of Mozambique tilapia that underlie nutrient absorption. We specifically identified *ghr2*, *pept1a*, *pept1b*, and *glut2* as GH-stimulated gene transcripts in the anterior and/or middle intestine of tilapia. GH exerts its varied actions by binding to its cognate receptor in target tissues or, indirectly, through stimulating the release of IGFs from the liver. In previous investigations, intact and hypophysectomized tilapia, as well as cultured hepatocytes, were employed to characterize how plasma GH directs hepatic *ghr2/igf1* and plasma IGF1 patterns (Pierce et al., 2011, 2012; Breves et al., 2014). In the current study, we employed hypophysectomy and hormone replacement to reveal that *ghr2* expression in the anterior and middle intestine was similarly modulated by GH (Fig. 1A). Collectively, these results indicate that GH promotes the gene expression of its receptor at multiple sites to enhance tissue responsiveness to circulating GH under particular circumstances.

Intestinal *pept1* and *−2* expression is highly plastic in fishes (Terova et al., 2009; Koven and Schulte, 2012; Bucking and Schulte, 2012), including tilapia (Nitzan et al., 2017; Chourasia et al., 2018). For instance, tilapia *pept1a* is diminished during prolonged fasting and restored within 3 days of refeeding (Orozco et al., 2017). In Mozambique tilapia, plasma GH is elevated as early as 0.5 h following a meal, but is also elevated in fish fasted for up to 8 days compared with fed animals (Fox et al., 2009). In the present study, *pept1a* and *−1b* were dramatically stimulated in hypophysectomized fish injected with GH (Fig. 1B, C), indicating that di- and tripeptide absorption capacities are regulated (directly and/or indirectly) by GH. Importantly, this apparent link between GH and *pept1a/b* gene expression is consistent with the enhanced transport of peptides and amino acids by teleost intestine following GH administration (Collie and Ferraris, 1995; Sun and Farmanfarmaian, 1992; Farmanfarmaian and Sun, 1999; Walker et al., 2004). *pept1a*, and *−1b* levels were markedly greater in the anterior and middle intestine compared with the posterior intestine, consistent with the functional observation that di- and tripeptide uptake predominantly occurs in the proximal intestine of tilapia (Orozco et al., 2017). We thus propose that conditions that favor growth via the activation of the GH/IGF system in tilapia may stimulate GH-mediated nutrient uptake through transporters such as PepT1. Moreover, we found differences in *pept1* gene expression response patterns among the different intestinal segments; expression of both *pept1a* and *−1b* were > 10,000-fold higher in anterior and middle segments compared with the posterior intestine. These regions of high *pept1* gene expression correspond to regions previously found to be responsive to fasting and refeeding in Mozambique tilapia (Orozco et al., 2017).

The systemic and local action of GH-dependent growth factors such as Igfs and Igfbps may further modulate the actions of GH on intestinal nutrient transport (Collie and Ferraris 1995; Sun and Farmanfarmaian, 1992; Farmanfarmaian and Sun, 1999; Walker et al., 2004). We found that while *slc7a9* levels were diminished following hypophysectomy, GH injection did not impact *slc7a9* levels (Fig. 1D). One explanation for this pattern is that one or more pituitary factors, beyond GH, underlie the regulation of *slc7a9* in tilapia. Moreover, the specific transporter(s) underlying GH-stimulated amino acid capacities may not include SLC7a9. The segment-specific expression of *sglt1* and *glut2* observed in the current study is consistent with higher transport of simple carbohydrates in the anterior intestine compared with the posterior intestine (Chen et al., 2017; Subramaniam et al., 2019). Interestingly, hypophysectomized fish exhibited reduced *glut2*, but not *sglt1*, in the anterior intestine (Fig. 1E, F). *glut2* levels in the anterior intestine were recovered by GH replacement. These results suggest that *glut2*, at least in part, may underlie the GH-stimulated uptake of glucose in teleosts

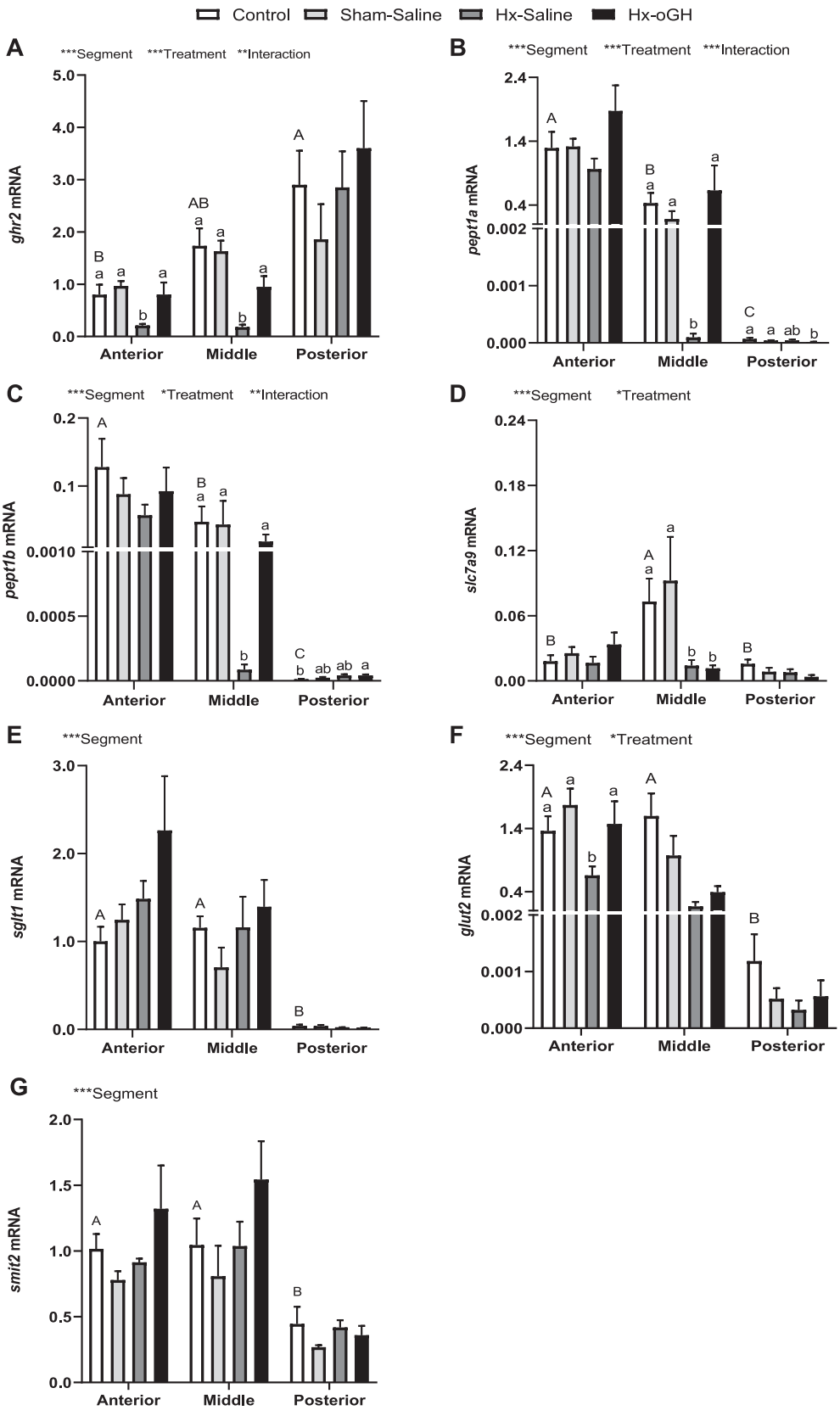

Fig. 1. Effects of hypophysectomy (Hx) and replacement with oGH on *ghr2* (A), *pept1a* (B), *pept1b* (C), *slc7a9* (D), *sglt1* (E), *glut2* (F), and *smit2* (G) levels in anterior, middle, and posterior intestine. mRNA levels are presented as relative expression of the target gene normalized to 18 s (means ± SEM; n = 6–9). Differences among groups were evaluated by two-way ANOVA. Significant effects of treatment, intestinal segment, or an interaction are indicated in respective panels (*P < 0.05, **P < 0.01, ***P < 0.001). When there was a significant treatment effect, *post hoc* comparisons (Fisher's protected LSD test) were made between groups within each intestinal segment. Within a given segment, means not sharing the same lower-case letter are significantly different (P < 0.05). When there was a significant intestinal segment effect, *post hoc* comparisons (Fisher's protected LSD test) were made between control groups. Control groups not sharing the same upper-case letter are significantly different (P < 0.05).

(Deane and Woo, 2005; Sangiao-Alvarellos et al., 2005). In vertebrates, SMIT2 transports inositol phosphates that contribute to the signal transduction of neurotransmitters and growth factors (Aouameur et al., 2007). In the present study, expression of *smit2* along the entire length of the intestine was consistent with a recent study in Nile tilapia (Subramaniam et al., 2019) but we found no evidence that endocrine GH regulates intestinal *smit2*.

In the present study, the middle intestine was highly sensitive to GH treatment. In enterectomized rabbit, expression of the amino acid transporter B^0/ASC transporter 2 ($ATB^0/ASCT_2$) in the ileum was

stimulated by GH treatment (Avissar et al., 2004). The sensitivity of nutrient transporters to GH in rabbit ileum suggests that GH sensitivity is enhanced in the middle intestine of multiple vertebrate groups. Moreover, the region-specific responses by particular tilapia transporters (*pept1a, pept1b, slc7a9*, and *glut2*) to hypophysectomy are novel observations that warrant future investigation. We propose that differences in *ghr2*/GHR2 expression may account for these region-specific patterns. Indeed, receptors for GH/prolactin-family peptides are differentially expressed along the intestine of Mozambique tilapia and other vertebrates (Seale et al., 2014; Ran et al., 2016; Velayudhan et al., 2008). In conclusion, this study expands our understanding of how GH supports somatic growth through the identification of specific transporters that underlie nutrient absorptive capacities. The identification of such GH targets may contribute to the development of strategies for enhancing the growth of domesticated fishes (Daniel, 2004; Hediger et al., 2004; Verri et al., 2010).

CRediT authorship contribution statement

Cody Petro-Sakuma: Formal analysis, Investigation, Writing - original draft. **Fritzie T. Celino-Brady:** Validation, Formal analysis, Investigation, Writing - review & editing. **Jason P. Breves:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Andre P. Seale:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Project administration, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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