Annals of Anatomy 254 (2024) 152266

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

Fasting duration impacts ribosome protein 6 phosphorylation in zebrafish brain: New insights in aquatic organisms' welfare

Maria Raggio [a], Daniela Giaquinto [a], Chiara Attanasio [a], Antonio Palladino [b], Vincenzo Esposito [a], Giuseppe Radaelli [c], Elena De Felice [d], Paolo de Girolamo [a], Livia D'Angelo [a,*]

[a] Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Via F. Delpino, Naples 1 I-80137, Italy
[b] Department of Agricultural Science, University of Naples Federico II, Viale dell'Università, Portici, Napoli I-80055, Italy
[c] Department of Comparative Biomedicine and Food Science (BCA), University of Padova, Viale dell'Università 16, Legnaro, Padova I-35020, Italy
[d] School of Biosciences and Veterinary Medicine, University of Camerino, Camerino I-62032, Italy

ARTICLE INFO

Keywords:
S6
PS6
Central nervous system
Neuronal activation
Food withdrawal metabolism

ABSTRACT

Background: Short- or mid-term fasting, full or partial, triggers metabolic response known to have in turn health effects in an organism. At central level, the metabolic stimulus triggered by fasting is known to be perceived firstly by hypothalamic neurons.
In the field of neuroscience, ribosomal protein S6 (S6) phosphorylation is commonly used as a readout of the mammalian target of rapamycin complex 1 signalling activation or as a marker for neuronal activity. The aim of this study is addressed to evaluate whether the phosphorylation of S6 occurs in the central neurons of zebrafish exposed to four (short-term) and seven (mid-term) days of complete fasting.
Methods: Group-housed adult zebrafish were exposed to four and seven days of complete food withdrawal. At the end of the experimental period, Western blotting analyses were carried out to measure the expression levels of the phosphorylated S6 (pS6) by comparing the two experimental conditions versus the control group. The same antibody was then used to identify the distribution pattern of pS6 immunoreactive neurons in the whole brain and in the taste buds.
Results: We did not observe increased pS6 levels expression in the brain of animals exposed to short-term fasting compared to the control, whereas the expression increased in brain homogenates of animals exposed to mid-term fasting. pS6 immunoreactivity was reported in some hypothalamic neurons, as well as in the dorsal area of telencephalon and preoptic area, a neurosecretory region homolog to the mammalian paraventricular nucleus. Remarkably, we observed pS6 immunostaining in the sensory cells of taste buds lining the oral epithelium.
Conclusions: Taken together, our data show that in zebrafish, differently from other fish species, seven days of fasting triggers neuronal activity. Furthermore, the immunostaining on sensory cells of taste buds suggests that metabolic changes may modulate also peripheral sensory cells. This event may have valuable implications when using zebrafish to design metabolic studies involving fasting as well as practical consequences on the animal welfare, in particularly stressful conditions, such as transportation.

1. Introduction

Fasting is increasingly used to evaluate the metabolic response of an organism and its implication in key cellular pathways (*i.e.* protein synthesis, mitochondrial activity, etc.) (Di Francesco et al., 2018). Despite the evidence that each feeding regimen may activate a different pathway, the health effects globally showed encouraging results in mammals (Di Francesco et al., 2018). Based on this, there is a growing number of experimental designs addressed to expose living organisms to the fasting condition. The fasting-induced metabolic stimulus is generally measured in terms of neuronal response in key brain regions sensitive to perceive metabolic changes. For instance, hypothalamic neurons promptly respond to metabolic cues either by phosphorylation of certain protein or by synthesizing appetite-regulating neuropeptides (Knight et al., 2012). Immediate early genes such as *c-fos* (Lau et al., 2011) or phosphorylation of erk (Miningou and Blackwell, 2020) have

* Corresponding author at: Department of Veterinary medicine and Animal Production, University of Naples Federico II, Via F. Delpino, Naples 1 I-80137, Italy.
E-mail address: livia.dangelo@unina.it (L. D'Angelo).

https://doi.org/10.1016/j.aanat.2024.152266
Received 29 December 2023; Received in revised form 3 April 2024; Accepted 11 April 2024
Available online 18 April 2024

been widely used to visualize the neurons that respond to numerous stimuli, including fasting.

Another commonly used marker in the neuroscientific studies is represented by the phosphorylation of the ribosomal protein S6 (S6), a structural component of the ribosome (Biever et al., 2015; Knight et al., 2012). S6 is an evolutionary conserved protein that spans 236–253 residues in species as distant as yeast, plants, invertebrates, and vertebrates (Meyuhas, 2008). Numerous reports have demonstrated that S6 is subject to phosphorylation in response to multiple physiological, pathological, and pharmacological stimuli (Meyuhas, 2015). Notably, this modification can be detected in both the cytosol and the nucleus (Pende et al., 2004). Phosphorylation of S6 is commonly used as a readout of the mammalian target of rapamycin complex 1 signalling activation or as a marker for neuronal activity (Biever et al., 2015).

The research goal of this study is to determine when S6 becomes phosphorylated in the central neurons in response to short- and mid-term fasting in zebrafish, and the different brain areas where immunoreactivity to pS6 is mainly detected. To this aim, adult zebrafish will be exposed to four and seven days of food deprivation, corresponding to short- and mid- term fasting respectively, and compared to control animals, which are fed twice per day. The majority of studies report that the time of fasting frequently used in zebrafish feeding assays, known to induce transcriptional and hormonal changes, is based on seven-days or 14-days fasting, complete withholding of food for seven or 14 days, respectively (Temple et al., 2003; Lawrence, 2007; Xia et al., 2014). Here we decided to consider shorter periods of time, equal to four days of fasting, and compared with the seven days, whose metabolic effects are well-known in literature in terms of metabolic and/or hormonal effects, such as overexpression of central anorexic neuropeptides (Volkoff, 2016).

Our group has previously evaluated pS6 in the hypothalamic neurons of another fish species, *Nothobranchius furzeri*, also known as the African turquoise killifish. Interestingly, we documented immunoreactivity to pS6 only in hypothalamic neurons synthesizing NPY mRNA, a well-established orexigenic neuropeptide (Montesano et al., 2019) in the adult but not old specimens, thus hypothesizing that four days of complete food withholding enables the activation of the orexigenic pathway during adulthood.

Interestingly, in this study we report that zebrafish displays a different metabolic response compared to the African turquoise killifish, reinforcing the concept that each fish species has its specific adaptation pattern. Furthermore, our findings may serve for future studies addressed to investigate the regulation of feeding in zebrafish as well as in close-related species. Food intake is indeed among the most well-studied mechanisms in fish for several reasons, among which the development of different strategies evolved depending on the ecological habitats, as well as morphological organizations of the digestive tract (Volkoff, 2019).

2. Material and methods

2.1. Experimental animals

The experimental protocols involving animals were approved by the Italian Ministry of Health (n° 291/2022-PR). All experiments were performed on group-housed males *Danio rerio* belonging to the AB strain, at six months of age. Animal maintenance was performed as described (Aleström et al., 2020).

Fish (n=10/group) were divided in the following experimental groups: control, four and seven days of fasting. The two time-points were selected based on the literature ((Xia et al., 2014; Montesano et al., 2019)). Control group was fed twice/day with SDS Diets 400 (https://www.sds-diets.com/sds_en/), a specific aquatic diet for regular maintenance.

2.2. Tissue sampling

Fish were euthanized, around 10 a.m. to avoid the effects of circadian rhythms, with an overdose of anaesthetics. Fish were placed for approximately ten minutes in a methanesulfonate solution (MS-222, Tricane-S®, Western Chemical Inc., Gujarat, India) at a concentration of 1 mg/mL in buffered ethyl 3-aminobenzoate, until no vital signs (body and operculum movement, righting reflex) were observed. For morphological staining, whole heads of three animals were fixed in Bouin's solution overnight (ON). Then, samples were dehydrated in a graded ethanol series, embedded in paraffin, and 7 μm thick serial sagittal sections were cut.

For western blot analysis, whole brains from seven animals were dissected on ice and stored at −80° until the use.

2.3. Western blotting

Brains were homogenised in 200 μl of lysing buffer (# FNN0021, Invitrogen, CA, USA) and centrifuged at 13,628gfor ten minutes at 4 °C to remove nuclei and cell debris. Supernatants were then collected and extracted protein concentration was measured by Bradford assay (# 5000006 Bio-Rad, CA, USA). Lysates containing 30 μg of proteins were resuspended in Laemmli buffer (# 1610747 Bio-Rad, CA, USA) and loaded on a 4–15% Mini-PROTEAN TGX Stain-Free precast electrophoresis gel (# 4568083 Bio-Rad, CA, USA). Electrophoresis was then performed followed by protein transfer to a nitrocellulose membrane (# 1704158 Bio-Rad, CA, USA) via a Mini Trans-Blot apparatus (Bio-Rad, CA, USA). Membranes were blocked with 5% bovine serum albumin solution (# 1196.02 SERVA Electrophoresis Gmbh, Heidelberg, Germany) dissolved in Tris-buffered saline (TBS # 1706435 Bio-Rad, Ca, USA) with Tween buffer (# 1706531 Bio-Rad, Ca, USA) (TBST) for 1 hour (h) at room temperature (RT) on a blot shaker. The blot was incubated first with primary polyclonal anti-rabbit phospho-S6 ribosomal protein antibody (Ser235/236 antibody # 2211, Cell Signaling Technology, USA) diluted 1:1500 in 5% bovine serum albumin solution and incubated ON at 4 C° on a shaking platform. After washing in TBST, the membrane was incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (# 1706515 Bio-Rad, CA, USA) diluted 1:3000 in protease free 5% bovine serum albumin fraction V solution for one hour RT. After washing in TBST the target protein was detected by chemiluminescence incubating the membrane with clarity western ECL substrate (# 1705060 Bio-Rad, CA, USA) for five minutes. For reprobing the membranes, restore western blot stripping buffer (# 21059 Thermo Fisher Scientific, Waltham, MA, USA) was used for 45 minutes at RT. The same blot was then incubated with primary monoclonal anti-rabbit antibody against S6 (S6 ribosomal protein 5G10 rabbit mAB # 2217, Cell Signaling Technology, USA) diluted 1:1500 in 5% bovine serum albumin solution and incubated ON at 4 C° on a shaking platform. After washing in TBST, the membrane was incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase diluted 1:3000 in protease free 5% bovine serum albumin fraction V solution for 1 h RT. After washing in TBST the target protein was detected by chemiluminescence incubating the membrane with clarity western ECL substrate for five minutes. Finally, the same blot was stripped again to visualized β-actin as housekeeping protein. Restore western blot stripping buffer was used for 45 min at RT. The same blot was incubated with primary anti-rabbit antibody against β-actin (A5060, Sigma, Sant Louis, MO, United States) diluted 1:4000 in 5% bovine serum albumin solution and incubated ON at 4 C° on a shaking platform. After washing in TBST, the membrane was incubated with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase diluted 1:3000 in protease free 5% bovine serum albumin fraction V solution for 1 h RT. After washing in TBST the target protein was detected by chemiluminescence incubating the membrane with clarity western ECL substrate for 5 min. The bands were visualized by ChemiDoc molecular imager (Bio-Rad, CA, USA). Archival samples of

mouse brains stored in the Department of Veterinary Medicine and Animal Production of University of Naples Federico II were used as further antibodies control.

A further control was done performing separate western blot for the detection of one side of phospho-S6 ribosomal protein and on the other side of S6 ribosomal protein. In both cases, β-actin was used as housekeeping protein (Supplementary Appendix A). Antibodies are listed in Table 1.

2.4. Protein expression measurement and analysis

Protein band relative quantification was performed using 2.9.0 version of Image J software after conversion of the original raw image into a JPEG file format. The measurement reflects the relative amount of pS6 protein expressed as pS6/S6 ratio. As further control we quantified the ratio pS6/S6/β-actin. Differences between groups were analyzed by ANOVA followed by Tukey's HSD post-hoc test using GraphPad Prism 9 software. The graph in Fig. 1 and S1 report mean values ± standard deviation (* indicates a p value <0.05, *** indicate a p value <0.005, **** indicate a p value <0.001).

2.5. Immunohistochemistry

The immunohistochemical experiments were concomitantly performed on sections belonging to the specimens of each of the experimental groups. All experiments were repeated three times. After deparaffinization, antigen unmasking was carried out by dipping the sections in 0.01 M sodium citrate buffer pH 6.0 and heating them in a microwave oven for ten minutes at 750 W. Then, to block endogenous peroxidase activity, sections were incubated with 3% hydrogen peroxide for 30 min at RT. After washing with 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 15 minutes, sections were pre-incubated for one hour at RT with the blocking solution (cat# n191356, MP biomedical LLC, Solon, OH, USA) (1:5 in 0.01 M PBS). The sections were then incubated with primary rabbit antibodies against S6 (1:100, S6 ribosomal protein 5G10 rabbit mAB # 2217, Cell Signaling Technology, USA) and with its phosphorylated form (1:200, phospho-S6 ribosomal protein Ser235/236 antibody # 2211, Cell Signaling Technology, USA), at 4°C in humid chamber ON. The day after, the sections were rinsed in PBS for 15 minutes and incubated for 30 minutes at RT with ultrapolymer cocktail (cat# UNIHRP-015, ImmunoReagents, Inc., Raleigh, NC, USA). Immunoreactive sites were visualized using a fresh solution of 10 mg of 3–3' diaminobenzidine tetrahydrocloride (DAB, cat# D5905, Sigma-Aldrich, Darmstadt, Germany) in 15 mL of 0.5 M Tris buffer, pH 7.6, containing 0.03% hydrogen peroxide. Antibodies are listed in Table 1.

Negative controls were carried out by substituting each primary antibody with normal serum in the specific step.

Table 1
Antibodies list used for western blot, immunohistochemistry and immunofluorescence methods.

Antibody	Dilution WB	Dilution IHC-IF	Catalogue code
Anti-rabbit phospho-S6 ribosomal protein polyclonal, polyclonal antibody produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Ser235 and Ser236 of human ribosomal protein S6	1:1500	1:200 (IHC) 1:100 (IF)	Cell Signaling Technology Cat#2211
Anti-rabbit S6 ribosomal protein monoclonal, monoclonal antibody produced by immunizing animals with a synthetic peptide corresponding to residues of human S6 ribosomal protein	1:1500	1:100 (IHC)	Cell Signaling Technology Cat# 2217
Anti-rabbit Actin polyclonacl antibody	1:4000	-	Sigma-Aldrich Cat# A5060
Anti-Rabbit Calbindin D28K Polyclonal Antibody	-	1:100 (IF)	Invitrogen Cat# PA1–931
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	1:3000	-	Bio-Rad Cat# 1706515
Normal Goat Serum		1:5 (IHC) 1:5 (IF)	MP Biomedicals, LLC Cat# 191356
UltraPolymer Goat Anti-Rabbit/Mouse IgG (H&L) conjugated to HRP			ImmunoReagents Cat# UNIHRP-015
Rhodamine Red-X AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) conjugated to tetramethylrhodamine-5-(and 6) isothiocyanate fluorochrome		1:200 (IF)	Jackson ImmunoResearch Cat# 111–297–003
Fluorescein (FITC) AffiniPure Goat Anti-Rabbit IgG, F(ab')$_2$ fragment specific conjugated to fluoroscein isothiocyanate fluorochrome		1:50 (IF)	Jackson ImmunoResearch Cat# 111–095–006

2.6. Double immunofluorescence

After dewaxing, the sections were rinsed in 0.1 M PBS every five minutes for three times and pre-incubated for one hour at RT with the blocking solution (1:5 goat serum and PBS), and then incubated with the first primary antibody Calbindin D28k (1:100, cat# PA1–931, Invitrogen) at 4 °C in humid chamber ON. Then the sections were washed in PBS every 5 minutes for three times and incubated with Rhodamine Red-X AffiniPure Fab Fragment Goat Anti-Rabbit IgG (H+L) conjugated to tetramethylrhodamine-5-(and 6) isothiocyanate fluorochrome (1:200, cat# 111–297–003, Jackson ImmunoResearch, Cambridge, UK) at 37 °C for two hours. Thereafter, the sections were rinsed in PBS and incubated with pS6 (1:100, cat# BK2211S, Cell Signaling) at 4 °C in humid chamber ON. After rinsing in PBS, the sections were treated with Fluorescein (FITC) AffiniPure Goat Anti-Rabbit IgG, F(ab')$_2$ fragment specific conjugated to fluoroscein isothiocyanate fluorochrome (1:50, cat# 111–095–006, Jackson ImmunoResearch, Cambridge, UK) for 2 h at 37 °C. Finally, the sections were washed with PBS and mounted with fluoroshield mounting medium with DAPI (cat #ab104139 Abcam). Antibodies are listed in Table 1.

2.7. Image acquisition

Images were observed and analysed with Leica—DM6B (Leica, Wetzlar, Germany) and processed with LasX software. The immunofluorescence images in fluorescence were acquired with a microscope ApoTome.2, Zeiss (Right Microscope). Digital raw images were optimized for image resolution, contrast, evenness of illumination, and background using Adobe Photoshop CC 2018 (Adobe Systems, San Jose, CA, USA). Anatomical structures were identified according to (Wullimann et al., 1996).

3. Results

3.1. pS6 brain expression under fasting conditions

The comparison of the expression level of phosphorylated S6, throughout western blotting analysis and calculated as pS6/S6 ratio, in control animals and in those exposed to fasting for four and seven days revealed a sharp activation of S6 protein in fish fasted for a longer time with a significant increased expression (0.48) after seven days compared to the control group (Fig. 1A, B). Conversely, the difference in pS6

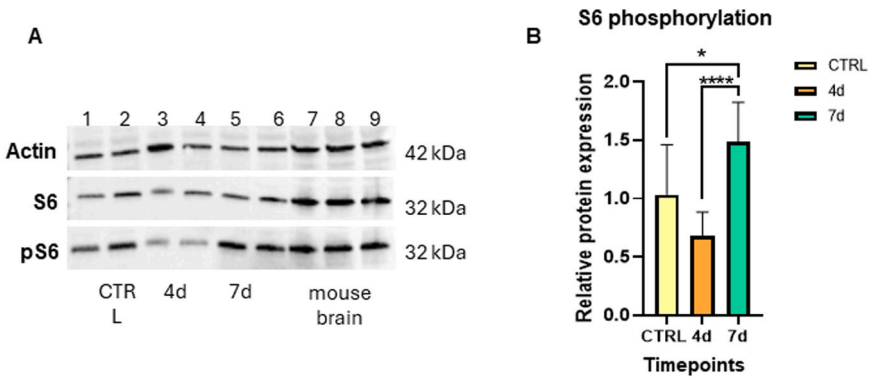

Fig. 1. A. Western blot images revealing the different expression of S6 and its activated form, pS6, in zebrafish brain exposed to the different experimental conditions. The figure report only two representative samples (out of 7 used for each experimental condition) for zebrafish, and one for each of the three different mouse brain areas (7- hippocampus, 8 - striatum, and 9 - frontal lobe), used as antibody control. B. Quantitative analysis of the expression levels of pS6, calculated as pS6/S6 ratio. The comparative analysis discloses a clear increase of the protein activated form (pS6) in fish fasted for seven days compared to control fish, normally fed, and, even more, to fish fasted for a shorter period of time (four days). Also, a decrease in pS6 expression levels can be noticed in the four days fasting group in comparison to the control group. The graph reports mean values ± standard deviation, * indicates a p value <0.05, **** indicate a p value <0.001.

expression between animals fasted for four days and those normally fed is slighter, accounting for 0.32 discrepancy. However, in this latter case we reported a decreased expression of pS6 in fasted animals compared to control ones rather than an increase. This observation highlights that the increase in pS6 relative expression levels in fish undergoing long-term fasting in comparison to those fasted for a shorter period of time is extremely significant, being resulting the relative protein expression increased of a 0.8 value (Fig. 1A, B). The expression levels of S6 and pS6 in the brain of fish exposed to the different experimental conditions normalized on β-actin amount are reported in Figure S1.

3.2. pS6 immunolocalization in the adult zebrafish brain under fasting conditions

pS6 immunolabeling was carried out on sagittal sections of the whole head of control animals (adult zebrafish) and compared to the signal observed in the brain of animals exposed to the two fasting conditions. In the brain of control animals (Fig. 2A), the labelling was appreciated in a few neurons of the dorsal telencephalon (Fig. 2A,B), in neurons located in the preoptic area (Fig. 2A,C) and along the subventricular region of the hypothalamic recess (Fig. 2A,D). Weak immunostaining was seen in the cerebellar corpus and valvula (Fig. 2A).

Faintly immunostained neurons were detected in the brain of animals exposed to four days of fasting (Fig. 3A). Very weak

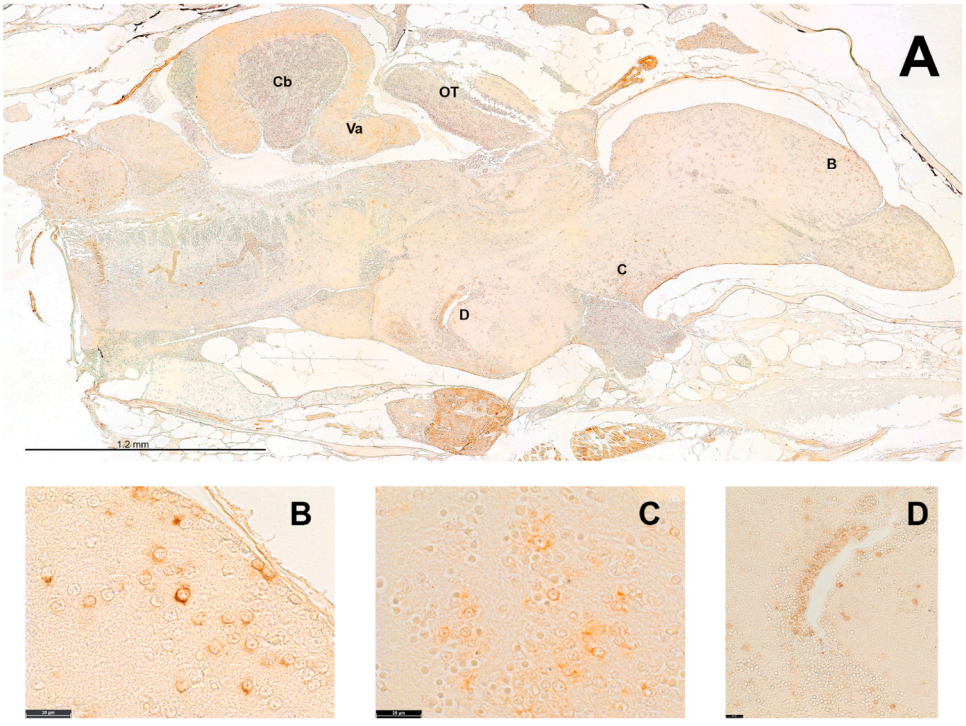

Fig. 2. pS6 immunoreactivity in the brain of adult zebrafish used as control animals. A. Overview of the whole encephalon showing immunopositivity to pS6 in few neurons in the dorsal telencephalon (B), preoptic area (C) and along the hypothalamic recess (D). B. Higher magnification of scattered immunoreactive neurons in the dorsal telencephalon. C. Higher magnification of immunopositive neurons in the preoptic area. D. Higher magnification of positive neurons along the hypothalamic recess. Scale bars: A = 1.2 mm, B,C,D = 20 μm. Abbreviations: OT: optic tectum, Va: Cerebellar valvula, Cb: Cerebellar corpus.

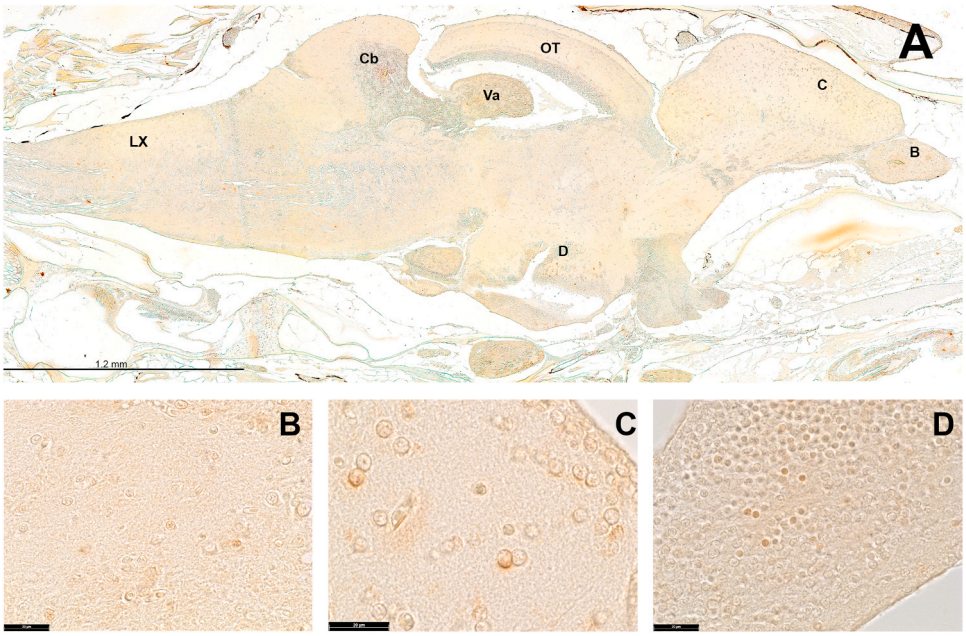

Fig. 3. pS6 immunoreactivity in the brain of adult zebrafish exposed to 4 days of fasting. A. Overview of the whole encephalon showing faint immunopositivity to pS6 in few neurons in the olfactory bulbs (B), dorsal telencephalon (C), and along the hypothalamic recess (C). B. Higher magnification of very weakly stained immunoreactive neurons in the dorsal telencephalon. C. Higher magnification of immunopositive neurons in the dorsal telencephalon. D. Higher magnification of faintly positive neurons along the hypothalamic recess. Scale bars: A = 1.2 mm, B,C,D = 20 μm. Abbreviations: OT: optic tectum, Va: Cerebellar valvula, Cb: Cerebellar corpus., LX: Lobe of vagal nerve.

immunopositivity was detected in the olfactory bulbs (Fig. 3A,B), in the dorsal telencephalon (Fig. 3A,C) and in proximity of the hypothalamic recess (Fig. 3A,D). Weak immunopositivity was observed in the cerebellar corpus and valvula (Fig. 3A).

More numerous immunoreactive neurons were observed in the brain of animals exposed to seven days of fasting (Fig. 4A). While weak immunolabeling was seen in the olfactory bulbs (Fig. 4A), intense labelling was seen in the neurons of the dorsal telencephalic zone (Fig. 4A,B) and in the preoptic area (Fig. 4A,C). Less numerous and weakly stained neurons were seen in the hypothalamic area (Fig. 4A,D).

As further control, we performed immunohistochemical experiments on the alternate sections of the same animals by using the antibody against S6. The labelling was almost widespread over telencephalon, diencephalon, midbrain and, to a less extent, in the rhombencephalon

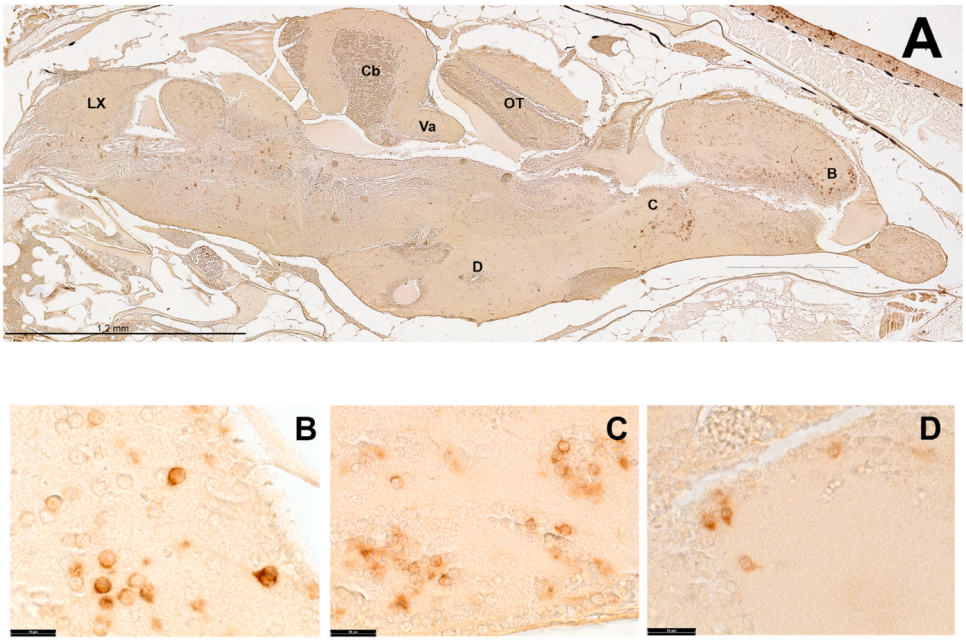

Fig. 4. pS6 immunoreactivity in the brain of adult zebrafish exposed to 7 days of fasting. A. Overview of the whole encephalon showing immunopositivity to pS6 in neurons in the dorsal and ventral telencephalon (B), preoptic area (C), and scarcely along the hypothalamic recess (D). B. Higher magnification of intensely stained immunoreactive neurons in the dorsal telencephalon. C. Higher magnification of immunopositive neurons in the preoptic area. D. Higher magnification of very few immunopositive neurons along the hypothalamic recess. Scale bars: A = 1.2 mm, B,C,D = 20 μm. Abbreviations: OT: optic tectum, Va: Cerebellar valvula, Cb: Cerebellar corpus, LX: Lobe of vagal nerve.

independently of the experimental condition [Supplementary appendix A]. Remarkably, we observed immunoreactivity to pS6 in the cells of the taste buds of control animals (Fig. 5A), undergoing four days of fasting (Fig. 5B) and in animals undergoing seven days of fasting (Fig. 5C) To verify which cellular phenotype (sensory or supporting cells) was immunoreactive to pS6, we conducted a double staining by using Calbindin D28k, a marker used to label sensory cells ((Yamamoto et al., 2000)). We detected co-localization of pS6 in all Calbindin immunostained cells in all control and experimental groups (Fig. 6).

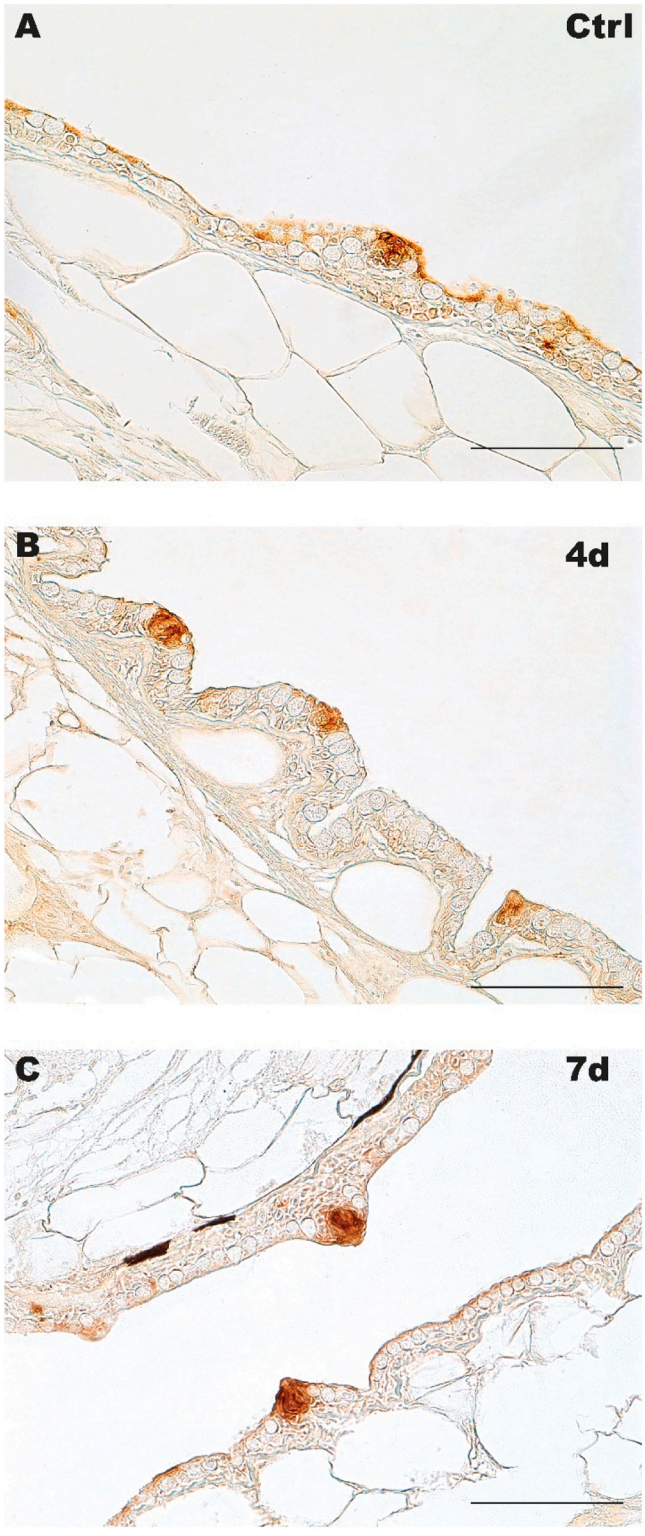

Fig. 5. pS6 immunoreactivity in the taste buds lining the oral epithelium of adult zebrafish. A. Sections of control animals displaying immunoreactivity to pS6 immunostaining, comparable to that observed in the B sections of animals fasted for four days. C. Sections of animals fasted for seven days showing marked immunoreactivity to pS6.

4. Discussion

This report is addressed to evaluate the neuronal response of sexually mature zebrafish upon four and seven days of fasting, maintained under standard husbandry conditions (Alestrom et al., 2020). Phosphorylation of S6 protein, known to be a marker of activated neurons in response to fasting in mouse ((Knight et al., 2012)) and fish ((Montesano et al., 2019)), was significantly increased in the brain homogenates of animals fasted for seven days, compared to four days of fasting and age-matched control. Despite the difference that in the African turquoise killifish the expression levels were measured only in the diencephalic region ((Montesano et al., 2019)) while our quantification is based on the whole brain homogenate, the immunostaining experiments confirm that the highest immunoreactivity occurs upon seven days of fasting in the zebrafish brain. Very interestingly, we observed a decrease of pS6 expression in the brain homogenates of animals exposed to short-term fasting when compared to control animals. pS6 levels are directly related to mTOR activity ((Alirezaei et al., 2010)). Our data allow us to speculate that the S6 phosphorylation may take place upon prolonged exposure to a determined stimulus (*i.e.* seven days). It is indeed reported that complete activation of S6 kinases is triggered by activated ERK ((Meyuhas, 2008); Romeo et al., 2012). This process may require time and thus could justify the low levels of pS6 upon 4 days of fasting. Future experiments may help us to compare the expression of posphoERK and pS6 upon fasting and confirm or reject this hypothesis.

Coherently with expression data, we observed stronger immunostaining in neuronal cells in the telencephalic hemispheres, preoptic and hypothalamic areas of animals exposed at mid-term fasting compared to the other two groups. Notably, despite differences in the immunostaining signal, the immunoreactive neurons were localised in the same brain regions in all experimental groups. In the telencephalon, immunopositive neurons were mainly located in the dorsal areas, which are considered the homologous pallial structure of mammals, although the exact subdivisions of the pallial territories and their homologies with mammalian brains are still matter of debate among neuroanatomists (Briscoe and Ragsdale, 2018). The telencephalon receives olfactory, visual, acoustic, lateral line, somatosensory, and in some species electrosensory input from relays in the preglomerular complex (Briscoe and Ragsdale, 2019; (Wullimann, 2020). Telencephalic neurons could have been activated likely due in response to the increased need of searching food. Most remarkably, it is likely that the telencephalic neurons may receive neuronal projections from the hypothalamus. In fish, neuroanatomical studies pointed out that neurons from secondary gustatory visceral nucleus send ascending fibres to hypothalamic nuclei and the tertiary gustatory nucleus (Rink and Wullimann, 1998; Yáñez et al., 2017) as well as to the telencephalon (for review, (Korsching, 2020).

pS6 immunostaining was remarkably observed in neurons of the preoptic area, a neurosecretory region in the zebrafish brain homolog to the mammalian paraventricular nucleus. The region is thus a functionally complex neuroendocrine centre controlling hormone release in the pituitary gland (Herget et al., 2014) and our findings agree with the pivotal roles of those neurons in the fish homeostasis. Unsurprisingly, we detected immunopositivity in the neurons of the hypothalamic area, although a weaker staining compared to the preoptic area. The basic mechanisms involved in regulation of food intake in fish appear to be similar to those of mammals, by means of expression of key orexigenic neuropeptides (Volkoff, 2016) despite some differences implying the existence of fish-specific mechanisms. However, we have not assessed the distribution pattern of neuropeptides involved in the food intake pathway which are beyond the scope of this manuscript. The fact that we

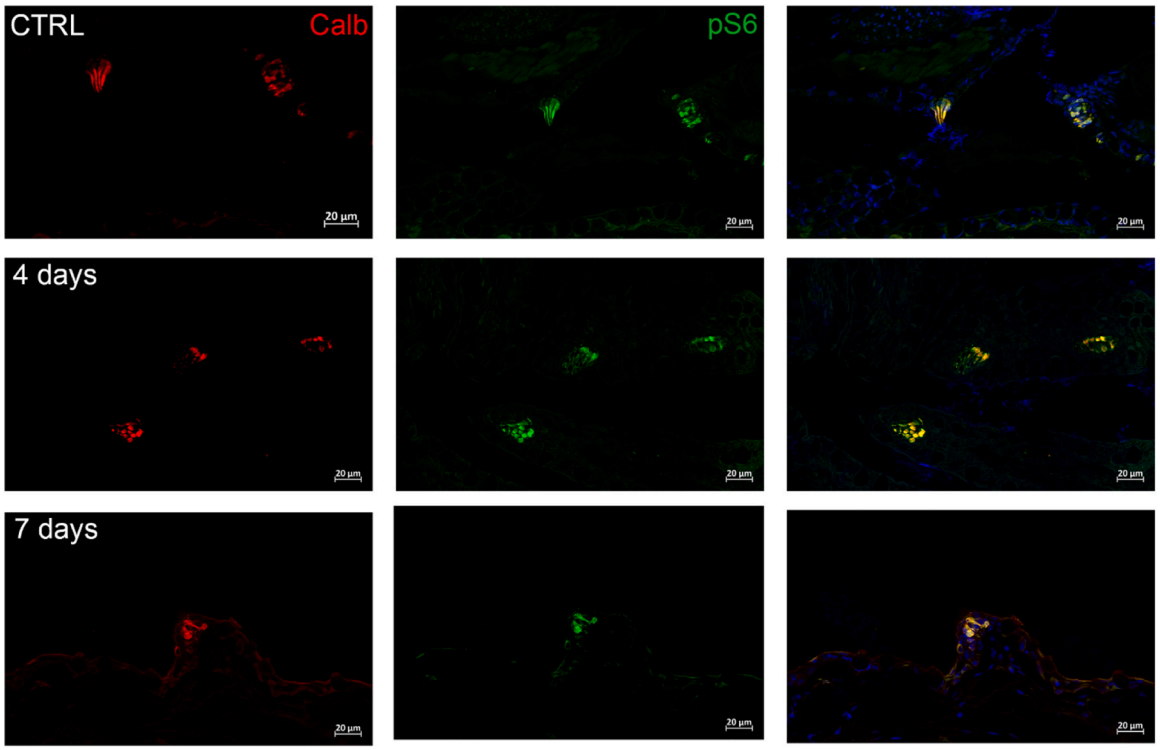

Fig. 6. pS6/Calb co-localization in the taste buds lining the oral epithelium of adult zebrafish in control and experimental animals. Sections of control (CTRL) animals and animals exposed to four- and seven days of fasting displaying co-staining to Calbindin D28k (red) and pS6 (green). Calbindin D28k was used as marker of sensory cells.

could not observe sharp differences in the immunoreactivity of pS6 in the hypothalamic area when comparing animals upon four and seven days of fasting suggests that the described mechanism might be considered mainly a stress response to fasting. Hypothalamus in fish is also known to induce adaptive changes in food intake and energy expenditure according to the exposure to stressful conditions (Delgado et al., 2017). This hypothesis is further corroborated by the behavioural observations we recorded during the daily management performed along the experimental period. The bottom occupancy of the tank, which is normally part of the so-called burst-and-coast swimming style (Kalueff et al., 2013), was reduced in the experimental tank housing animals fasted for four days and almost absent in the group of seven days of fasting (personal observations). The fasting-related stress, however, did not influence the normal shoaling behavior rather the swimming activity slowed down, likely due to save energies (Wright et al., 2006). Finally, animals lingered on the right side of the tank where food is usually administered.

Most remarkably and still focusing on the morphological observations, immunoreactivity to pS6 was detected in the taste buds lining the mouth epithelium in the three experimental groups. pS6 was localised in sensory cells type, as confirmed by immunofluorescence observation. The pS6 immunolabeling seen in the control animals is likely due to the overnight fasting that all animals underwent before being suppressed. This observation is not surprising if we consider that taste buds are at the forefront of food intake in fish (Kasumyan, 2019) and their cells are likely prone to respond to metabolic stimuli and become activated. This may likely be due to the first cellular response to metabolic status changes. Future studies are necessary to dissect the sensory cell perception and related modulation by homeostatic feedback.

5. Conclusions

Our data demonstrate that, in zebrafish, four days of fasting do not determine increased levels of pS6 indicative of neuronal activation, differently from other fish species, such as the African turquoise killifish. Differently, seven days of fasting triggers a neuronal response. Interestingly, also taste bud sensory cells appear activated in response to fasting. These findings may also lead to valuable and practical consideration when referring to zebrafish welfare in certain contexts, such as transportation. Notably, fasting is the recommended approach to minimise stress and protect health of adult animals during transport (Alestrom et al., 2020). Over long distances, for instance from America or Canada to Europe, transportation can last longer than 2–3 days impacting animal health and welfare. This aspect should be taken into debt consideration when adult fish transportation has to be performed (Varga, 2016). Further, this specific issue is even more relevant with the ever-increasing use of zebrafish as model organism for scientific purposes (Choi et al., 2021). Following this, in fact, a growing number of institutions and researchers are interested in sharing zebrafish lines and, therefore, shipping live zebrafish.

Funding

This manuscript was supported by fundings of University of Naples Federico II (000019-2019-UNINA grants)

Ethical statement

The animal study protocol was approved by the Italian Ministry of Health (n° 291/2022-PR).

CRediT authorship contribution statement

Daniela Giaquinto: Visualization, Data curation. **Maria Raggio:** Methodology, Investigation, Data curation. **Giuseppe Radaelli:** Writing – review & editing, Validation. **Vincenzo Esposito:** Writing – review & editing, Validation. **Antonio Palladino:** Validation, Formal analysis. **Chiara Attanasio:** Writing – review & editing, Formal analysis. **Livia D'**

Angelo: Writing – original draft, Supervision, Data curation, Conceptualization. **Paolo de Girolamo:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Elena De Felice:** Writing – review & editing, Visualization, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paolo de Girolamo reports financial support was provided by University of Naples Federico II Department of Veterinary Medicine and Animal Production. Maria Raggio, Daniela Giaquinto, Chiara Attanasio, Paolo de Girolamo, Livia D'Angelo reports a relationship with University of Naples Federico II Department of Veterinary Medicine and Animal Production that includes: employment. Livia D'Angelo has patent pending to Optional. Paolo de Girolamo, Giuseppe Radaelli and Livia D'Angelo acted as Guest Editor for a special issue If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

2020. Korsching, S.I., 2020. Taste and Smell in Zebrafish. In: Fritzsch, B. (Ed.) and Meyerhof, W. (Volume Editor), The Senses: A Comprehensive Reference, vol. 3. Elsevier, Academic Press 466–492. https://10.1016/B978-0-12-809324-5.24155-2.

Aleström, P., D'Angelo, L., Midtlyng, P.J., Schorderet, D.F., Schulte-Merker, S., Sohm, F., Warner, S., 2020. Zebrafish: housing and husbandry recommendations. Lab Anim. 54 (3), 213–224. https://doi.org/10.1177/0023677219869037.

Alirezaei, M., Kemball, C.,C., Flynn, C.,T., Wood, M.,R., Whitton, J.,L., Kiosses, W.,B., 2010. Short-term fasting induces profound neuronal autophagy. Autophagy 6 (6), 702–710. https://doi.org/10.4161/auto.6.6.12376.

Biever, A., Valjent, E., Puighermanal, E., 2015. Ribosomal Protein S6 Phosphorylation in the Nervous System: from regulation to function. Front Mol. Neurosci. 8, 75. https://doi.org/10.3389/fnmol.2015.00075.

Briscoe, S.D., Ragsdale, C.W., 2018. Homology, neocortex, and the evolution of developmental mechanisms. Science 362 (6411), 190–193. https://doi.org/10.1126/science.aau3711.

Choi, T.Y., Choi, T.I., Lee, Y.R., Choe, S.K., Kim, C.H., 2021. Zebrafish as an animal model for biomedical research. Exp. Mol. Med 53 (3), 310–317. https://doi.org/10.1038/s12276-021-00571-5.

Delgado, M.J., Cerdá-Reverter, J.M., Soengas, J.L., 2017. Hypothalamic Integration of Metabolic, Endocrine, and Circadian Signals in Fish: involvement in the Control of Food Intake. Front Neurosci. 11, 354. https://doi.org/10.3389/fnins.2017.00354.

Di Francesco, A., Di Germanio, C., Bernier, M., de Cabo, R., 2018. A time to fast. Science 362 (6416), 770–775. https://doi.org/10.1126/science.aau2095.

Herget, U., Wolf, A., Wullimann, M.F., Ryu, S., 2014. Molecular neuroanatomy and chemoarchitecture of the neurosecretory preoptic-hypothalamic area in zebrafish larvae. J. Comp. Neurol. 522 (7), 1542–1564. https://doi.org/10.1002/cne.23480.

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H., Zebrafish Neuroscience Research Consortium, 2013. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10 (1), 70–86. https://doi.org/10.1089/zeb.2012.0861.

Kasumyan, A.O., 2019. The taste system in fishes and the effects of environmental variables. J. Fish. Biol. 95 (1), 155–178. https://doi.org/10.1111/jfb.13940.

Knight, Z.A., Tan, K., Birsoy, K., Schmidt, S., Garrison, J.L., Wysocki, R.W., Emiliano, A., Ekstrand, M.I., Friedman, J.M., 2012. Molecular profiling of activated neurons by phosphorylated ribosome capture. Cell 151 (5), 1126–1137. https://doi.org/10.1016/j.cell.2012.10.039.

Lau, B.Y.B., Mathur, P., Gould, G.G., Guo, S., 2011. Identification of a brain center whose activity discriminates a choice behavior in zebrafish. Proc. Natl. Acad. Sci. USA 108 (6), 2581–2586. https://doi.org/10.1073/pnas.1018275108.

Lawrence, C., 2007. The husbandry of zebrafish (Danio rerio): a review. Aquacult 269, 1–20. https://doi.org/10.1016/j.aquaculture.2007.04.077.

Meyuhas, O., 2008. Physiological roles of ribosomal protein S6: one of its kind. Int Rev. Cell Mol. Biol. 268, 1–37. https://doi.org/10.1016/S1937-6448(08)00801-0.

Meyuhas, O., 2015. Ribosomal Protein S6 Phosphorylation: four decades of research. Int Rev. Cell Mol. Biol. 320, 41–73. https://doi.org/10.1016/bs.ircmb.2015.07.006.

Miningou, N., Blackwell, K.T., 2020. The road to ERK activation: do neurons take alternate routes? Cell Signal 68, 109541 (https://).

Montesano, A., Baumgart, M., Avallone, L., Castaldo, L., Lucini, C., Terzibasi Tozzini, E., Cellerino, A., D'Angelo, L., de Girolamo, P., 2019. Age-related central regulation of orexin and NPY in the short-lived African killifish Nothobranchius furzeri. J. Comp. Neurol. 527 (9), 1508–1526. https://doi.org/10.1002/cne.24638.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C., Thomas, G., 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol. Cell Biol. 24 (8), 3112–3124. https://doi.org/10.1128/MCB.24.8.3112-3124.2004.

Rink, E., Wullimann, M.F., 1998. Some forebrain connections of the gustatory system in the goldfish Carassius auratus visualized by separate DiI application to the hypothalamic inferior lobe and the torus lateralis. J. Comp. Neurol. 394, 152–170.

Temple, J.L., Millar, R.P., Rissman, E.F., 2003. An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior. Endocrinol 144, 13–19. https://doi.org/10.1210/en.2002-220883.

Varga, Z.M., 2016. Aquaculture, Husbandry, and Shipping at the Zebrafish International Resource Center. Methods Cell Biol. 135, 509–534. https://doi.org/10.1016/bs.mcb.2016.01.007.

Volkoff, H., 2016. The neuroendocrine regulation of food intake in fish: a review of current knowledge. Front Neurosci. 10, 540. https://doi.org/10.3389/fnins.2016.00540.

Volkoff, H., 2019. Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. Mol. Cell Endocrinol. 497, 110437 https://doi.org/10.1016/j.mce.2019.04.017.

Wright, D., Ward, A.J.W., Croft, D.P., Krause, J., 2006. Social organization, grouping, and domestication in fish. Zebrafish 3 (2), 141–155. https://doi.org/10.1089/zeb.2006.3.141.

Wullimann, M., F., 2020. Neural origins of basal diencephalon in teleost fishes: Radial versus tangential migration. J. Morphol. 281, 1133–1141. https://doi.org/10.1002/jmor.21237.

Wullimann, M., F., Rupp, B., Reichert, H., 1996. Neuroanatomy of the Zebrafish Brain. Springer Book Archive. eBook. https://doi.org/10.1007/978-3-0348-8979-7.

Xia, W., Smith, O., Zmora, N., Xu, S., Zohar, Y., 2014. Comprehensive analysis of GnRH2 neuronal projections in zebrafish. Sci. Rep. 4, 3676. https://doi.org/10.1038/srep03676.

Yamamoto, Y., Atoji, Y., Suzuki, Y., 2000. Calbindin D28k-immunoreactive afferent nerve endings in the laryngeal mucosa. Anat. Rec. 259 (3), 237–247. https://doi.org/10.1002/1097-0185(20000701)259:3<237::AID-AR20>3.0.CO;2-P.

Yáñez, J., Souto, Y., Piñeiro, L., Folgueira, M., Anadón, R., 2017. Gustatory and general visceral centers and their connections in the brain of adult zebrafish: a carbocyanine dye tract-tracing study. J. Comp. Neurol. 525 (2), 333–362. https://doi.org/10.1002/cne.24068. Epub 2016 Jul 10.