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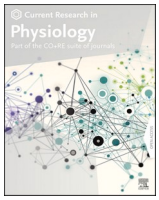

Cell-based homologous expression system for *in-vitro* characterization of environmental effects on transmembrane peptide transport in fish

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A B S T R A C T

All organisms encounter environmental changes that lead to physiological adjustments that could drive evolutionary adaptations. The ability to adjust performance in order to cope with environmental changes depends on the organism's physiological plasticity. These adjustments can be reflected in behavioral, physiological, and molecular changes, which interact and affect each other. Deciphering the role of molecular adjustments in physiological changes will help to understand how multiple levels of biological organization are synchronized during adaptations. Transmembrane transporters, which facilitate a cell's interaction with its surroundings, are prime targets for molecular studies of the environmental effects on an organism's physiology. Fish are subjected to environmental fluctuations and exhibit different coping mechanisms. To study the molecular adjustments of fish transporters to their external surrounding, suitable experimental systems must be established. The Mozambique tilapia (*Oreochromis mossambicus*) is an excellent model for environmental stress studies, due to its extreme salinity tolerance. We established a homologous cellular-based expression system and uptake assay that allowed us to study the effects of environmental conditions on transmembrane transport. We applied our expression system to investigate the effects of environmental conditions on the activity of PepT2, a transmembrane transporter critical in the absorption of dietary peptides and drugs. We created a stable, modified fish cell-line, in which we exogenously expressed the tilapia PepT2, and tested the effects of water temperature and salinity on the uptake of a fluorescent di-peptide, β-Ala-Lys-AMCA. While temperature affected only Vmax, medium salinity had a bi-directional effect, with significantly reduced Vmax in hyposaline conditions and significantly increased Km in hypersaline conditions. These assays demonstrate the importance of suitable experimental systems for fish ecophysiology studies. Furthermore, our *in-vitro* results show how the effect of hypersaline conditions on the transporter activity can explain expression shifts seen in the intestine of saltwater-acclimated fish, emphasizing the importance of complimentary studies in better understanding environmental physiology. This research highlights the advantages of using homologous expression systems to study environmental effects encountered by fish, in a relevant cellular context. The presented tools and methods can be adapted to study other transporters *in-vitro*.

1. Introduction

Organisms routinely experience changes in environmental conditions that can be natural or anthropogenic in origin. The change can be acute or chronic, and result from traversing between habitats, cyclical events (diurnal or season), or climatic episodes. Environmental changes can perturb an organism's homeostasis and induce physiological adjustments, aimed to adapt to the new conditions and restore physiological stability (Hoffmann and Hercus, 2000). These adjustments can be reflected in behavioral changes (Øverli et al., 2006), tissue and cell function and metabolism (Kültz et al., 2013; Nitzan et al., 2019; Root et al., 2021; Vargas-Chacoff et al., 2009), and gene and protein expression and function (Bodinier et al., 2009; Chew et al., 2014; Daniel et al., 1991; Hiroi et al., 2008; Sardella and Brauner, 2008). The plasticity of physiological traits, which varies between and within species, is the key element that allows organisms to respond to environmental stress and maintains homeostasis (Hoffmann and Hercus, 2000). Deciphering the underlying molecular adjustments that occur during environmental changes will contribute to a broader understanding of physiological mechanisms.

Various aquatic environments exhibit unique fluctuations of environmental factors such as water salinity, pH levels and temperature, which have been extensively studied for their effects on fish physiological traits. Habitat conditions affect fish cells and tissues in a more direct manner than in mammals, due to the special nature of the aquatic environment and fish physiology. For example, as poikilothermic organisms that generally cannot regulate their body temperature, fish tissues and organs are exposed to changing environmental temperatures

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and respond by activating various mechanisms that affect their function (Nitzan et al., 2019). In addition, the direct exposure of some fish tissues to the external aquatic or similar environment, such as the gills and intestine, affect their morphology, cell populations, and molecular-expression patterns, as part of osmoregulatory mechanisms (Con et al., 2017; Inokuchi and Kaneko, 2012; Kokou et al., 2019; Kültz et al., 2013; McCormick et al., 2003; Nitzan et al., 2017; Root et al., 2021). These tissues are responsible for continuously, yet dynamic interchanging fluxes of different molecules with the surrounding environment (e.g., ions, water, salts, etc.). For these specific responses to environmental conditions, it is important to establish tools and experimental systems that address specific questions related to fish ecophysiology.

The intestine is one of the fish organs exposed to the surrounding environment, after some processing. In addition to its role in nutrient and water absorption, the fish intestine is an osmoregulatory organ that has an important role in ion and acid-base regulation. The intestinal epithelial cells (also known as enterocytes) express many different transporters, ion channels, and pumps in the apical membrane, which faces the intestinal lumen, a dynamic environment that changes according to feeding regime, feed content, and water salinity (Genz et al., 2008; Taylor and Grosell, 2006, 2009). These changing conditions affect nutrient absorption and utilization and the expression of transmembrane proteins (Con et al., 2017; Hallali et al., 2018; Kokou et al., 2019; Nitzan et al., 2017). Transporters are an important group of membrane proteins, which facilitate the movement of solutes, e.g. nutrients, endogenous metabolites, and drugs, across the cell plasma and organellar membranes (Almén et al., 2009). Their importance in metabolism and homeostasis is mirrored by their involvement in many pathological states (Milne, 1964). In addition, they are responsible for the selective permeability of the plasma membrane, allowing cells to respond and affect their environments. In fish, the expression and function of transporters have been studied almost exclusively in *in-vivo* or *in-situ* experimental systems, mostly in relation to osmoregulation and nutrition (Bucking and Schulte, 2012; Con et al., 2017; Rojas-García et al., 2016; Weinrauch et al., 2019). However, *in-vitro* functional studies in fish are scarce and were mostly conducted in heterologous expression systems (Verri et al., 2012), which can be problematic when studying environmental effects on substrate transport, due to the large physiological difference between fish and other animal classes. For example, while temperature has a large influence on enzymatic reactions and protein folding, it also affects the membrane environment, which is crucial for the integrity of transmembrane protein activity (Lee, 2004; Saita and De Mendoza, 2015). As poikilothermic animals, fish have specific adaptations to temperature compared to mammals (Cossins and Macdonald, 1989; Robertson and Hazel, 1995). Therefore, homologous experimental systems will facilitate more accurate studies regarding the effects of fish-relevant environmental conditions on transport activity.

The peptide transporters (PepTs) have been extensively studied in many organisms, due to their importance in nutrient absorption, intestinal pathologies, drug transport, and tumor development (Ingersoll et al., 2012; Rubio-Aliaga and Daniel, 2008; Schniers et al., 2021; Spanier and Rohm, 2018; Tai et al., 2013). In addition, PepTs serve as well-established models for structural studies of solute carriers (Minhas and Newstead, 2020) and substrate recognition (Guettou et al., 2013; Killer et al., 2021). PepTs are secondary active transporters that use a proton gradient across the cell membrane as the driving force for di- and tri-peptide absorption. In mammals, two types of peptide transporters are known, PepT1, a high-capacity/low-affinity transporter, which is expressed mainly in the intestine, and PepT2, a low-capacity/high-affinity transporter, which is expressed mainly in the kidney. Due to a teleost specific genome duplication, bony fish PepT1 is present as two paralog genes, and all three transporters (PepT1a, PepT1b and PepT2) are expressed in the intestine (Con et al., 2017; Gonçalves et al., 2007). Their expression was found to change along the intestinal tract, with a correlation existing between their abundance, functional properties, and the substrate availability in the lumen (i.e., PepT1 variants are more abundant towards the anterior intestine, while PepT2 is more abundant towards the posterior intestine) (Con et al., 2017, 2019; Gomes et al., 2020; Vacca et al., 2022). Their expression pattern allows for highly efficient small-peptide absorption along the intestinal tract. Their expression was also found to be affected by water salinity in different species (Bucking and Schulte, 2012; Chourasia et al., 2018; Con et al., 2017; Kokou et al., 2019). Functional work has been conducted with these transporters, however, these studies used either *in-situ* systems, which do not allow characterization of a single specific transporter, or heterologous expression systems that are limited for studying environmental effects due to limited physiological relevance for the species of interest. In order to study these physiologically important transporters in a relevant environmental context, we established a homologous expression system, based on a fish cell line. This system enables studying the effect of different environmental conditions encountered by fish on the transport of small peptides. We decided to focus on PepT2, because it is a high-affinity transporter that, in fish, is expressed mainly in the intestine and the kidney, two important osmoregulatory organs. The expression of this transporter was shown to change dramatically along the intestine of the Mozambique tilapia after salinity change (Con et al., 2017). The significant salinity-dependent effect shown in intestinal PepT2 transcript levels, together with this transporter's major contribution to nutrient absorption and reabsorption, makes it a fitting candidate for molecular research aiming to elucidate the effect of environmental conditions at the protein level.

2. Materials and methods

2.1. Cell growth and maintenance

The cell line used in this study is an endothelial cell line originated from Mozambique tilapia bulbus arteriosus tissue (TmB; Lewis and Marks, 1985). TmB cells were maintained in L15 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1.25 U/ml Nystatin ("full L15") and were incubated at 25 °C with no supplemental CO_2. Cells were passaged every 6-7 days using a 1:7 splitting ratio. *mRNA* expression analysis showed that these cells do not natively express any of the three peptide transporters (Supplementary 1).

2.2. Establishing a stable PepT2-expressing transgenic TmB line

The coding region of Mozambique tilapia PepT2 (accession number: KX034111), was amplified from cDNA sample of posterior intestinal (primers are listed in Supplementary Table 1). The ORF of PepT2 was cloned into the N1-EGFP (Clontech Takara Bio USA) plasmid using XhoI and NotI restriction enzymes. Subsequently, a P2A-puromycin sequence was cloned into the N1-PepT2 plasmid downstream of the PepT2 sequence (without stop codon) using the Restriction-Free (RF) Cloning method (Peleg and Unger, 2014) with Q5 High-Fidelity DNA Polymerase (NEB, MA, USA). This construct allows the expression of both transcripts under the same constitutive CMV promotor. The final plasmid sequence (pN1-PepT2-P2A-Puromycin) is presented in supplementary 2. TmB Cells were transfected with pN1-PepT2-P2A-Puromycin (10 μg per 2.6 $*10^6$ cells), using Viafect transfection reagent (Promega, CA, USA). 48 h post transfection, full L15 medium was replenished with selection medium (1 μg/ml puromycin addition to full L15). Transfected cells were cultured in selection medium for 6 weeks. Selection medium was routinely replenished to discard dead cells form the culture. PepT2 mRNA expression in the cells was validated using PCR (primers are listed in Supplementary Table 1) and transport function was verified using fluorescence microscopy after incubating TmB and PepT2-TmB for 2 h with 125 μM of fluorescent di-peptide solution, β-Ala-Lys-AMCA (β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid) (Bio Trend Chemicals, FL, USA), a known substrate for the peptide transporters (Alghamdi et al., 2018; Dieck et al., 1999).

2.3. Preparation of experimental media

All uptake assays were conducted in Hanks buffered salt solutions (HBSS) with 20 mM Hepes to eliminate pH changes and serum-originated peptide effects on substrate uptake. Hypo- and hypersaline Hanks buffered salt solutions (HBSS) were prepared by reduction or supplementation of NaCl. NaCl was added to HBSS until the solution osmolality reached 900 mOsmol/kg. 600 mOsmol/kg solution was prepared by diluting 900 mOsmol/kg solution with 300 mOsmol/kg (normal HBSS). In order to create hyposaline HBSS, NaCl-free HBSS was prepared, with an osmolality of 45 mOsmol/kg. The NaCl-free HBSS was then used to dilute the 300 mOsmol/kg HBSS until the osmolality reached 150 mOsmol/kg. Osmolality of all HBSS media was validated using an Osmometer basic M (Löser Messtechnik, Germany). All experimental solutions were filtered through a 0.22 μm filter.

2.4. Peptide-uptake assay

PepT2-TmB cells were grown in full L15 medium using 75T flasks until 50-60% confluence and transfected with 10 μg pN1-GFP plasmid (Clontech Takara Bio USA) using 1:4 DNA to Viafect transfection reagent ratio to allow fluorescence normalization that accounts for differences in cell numbers between treatment and control wells. 48 h post-transfection, cells were trypsinized and centrifuged at 700g for 5min at RT in 15-ml tubes. The cells were counted and washed with normal HBSS-20mM Hepes to eliminate serum residuals, centrifuged again and resuspended in experimental HBSS (according to experiment) at a final concentration of 0.8-1 * 10^6 cells/ml. All experiments were conducted with cells at passage numbers between 40 and 50.

Prior to the assay, concentrated β-ala-Lys-AMCA solutions were prepared in appropriate assay solutions to create the desired final concentration (final concentration range of 0-500 μM). Peptide stock solutions (20 μl) were aliquoted into a 96 V-shape well plate. 100 μl of cell suspension were added to each well and incubated at 300 rpm on a benchtop shaker, at the appropriate time and temperature according to experimental protocol. The assay was stopped by addition of 100 μl of ice cold HBSS. Plates were centrifuged at 500g for 5 min at 4 °C. After centrifugation, 200 μl of the supernatant were carefully removed from each well to prevent cell aspiration and cells were washed using 200 μl of fresh ice cold HBSS. Centrifugation and washing steps were repeated for 3 additional times. Following the fourth centrifugation, 100 μl of the supernatant was carefully transferred to 96well fluorimeter plate (168055; Nunc, Thermo Fisher Scientific) in order to measure the background fluorescence of the medium of each well. The cells were then resuspended in the original V shape well using a pipette and 100 μl of the cell suspension was transferred into a fluorimeter plate for the measurement of accumulated fluorescence. Background and cell fluorescence was measured using a Synergy Neo2 plate reader (Agilent, CA, USA) for AMCA (350/450) and for GFP (485/528) (Excitation/Emission). The peptide uptake assay is summarized in Fig. 1.

2.5. Time optimization assay

In order to determine the appropriate incubation time of the cells with the labeled di-peptide we performed an uptake assay that compared different incubation time. This calibration was conducted using a fixed concentration of 125 μM of β-ala-Lys-AMCA. Cells were incubated with the di-peptide for 15, 30, 60, and 120 min. All incubations were stopped by addition of 100 μl of ice cold HBSS. Further washing and measurements were conducted as described above.

2.6. Temperature effect

Temperature is a key element affecting protein activity, especially regarding active and secondary active transport. In order to understand the effect of environmental temperature, which fish are experiencing, on the transporter activity, we adjusted the temperature during the uptake assay in our *in-vitro* system. We tested the activity of PepT2 by performing uptake assays at three different temperatures: 15 °C, 25 °C, and 35 °C. These three temperature conditions were chosen to represent the wide range of temperatures that tilapia commonly experience in their natural habitats, as well as in aquaculture environments.

2.7. Salinity effect

Although PepT2 is a proton-dependent transporter, ions levels affect peptide transport (Ganapathy and Leibach, 1983). In order to study the salinity effect on the PepT2 activity, we conducted uptake assays using a range of salinities. We tested the effect of three salinities: hyposaline (150 mOsmol/kg HBSS) and hypersaline (600 and 900 mOsmol/kg HBSS), in addition to control (300 mOsmol/kg HBSS). These levels were chosen to represent a wide range of water salinities which can enter into the intestine of Mozambique tilapia, which was shown to be able to acclimate to salinities as high as three times seawater (Stickney, 1986). Freshwater osmolality is less than 1 mOsmol/kg, while seawater is around 900 mOsmol/kg. In addition, the TmB cell line has been characterized as highly osmotolerant (Gardell et al., 2014). To this end, cell suspensions and β-ala-Lys-AMCA concentrations were prepared in four different NaCl-adjusted HBSS representing hyper- and hyposaline conditions. Aiming to avoid cell volume effects due to the osmotic pressure of the HBSS, final washes and fluorescence reads of all treatments were done with ice-cold, 300 mOsmol/kg HBSS.

2.8. Uptake rate calculation and statistical analysis

Background well measurements at emission wavelengths were subtracted from the corresponding cell-containing well measurements for each replicate. AMCA fluorescence was normalized by calculating the AMCA to GFP fluorescence ratio for each well to account for variation in cell number, and the average ratio of the control group (no substrate) was subtracted from each cell-containing well ratio. Non-linear regressions were used to fit the Michaelis-Menten curves (Equation (1)) for the transport-rate data.

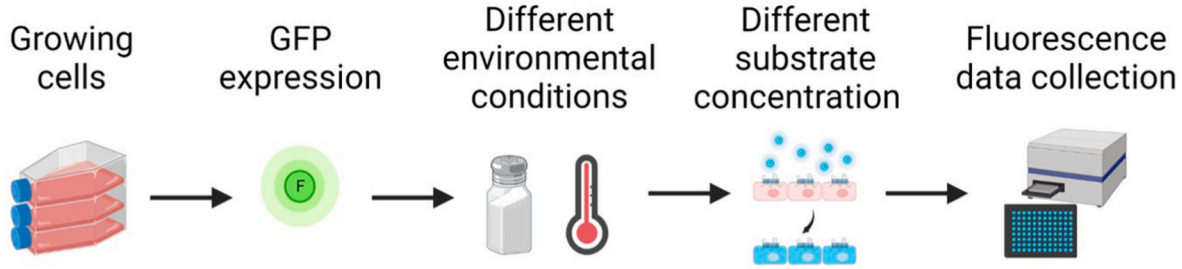

Fig. 1. Graphical illustration of the uptake assay protocol. Created with BioRender.com.

TheMichaelis-Menten modelformula : $V_{(S)} = \frac{Vmax * S}{Km + S}$ Equation 1

Where $V_{(S)}$ is the transport rate at specific substrate concentration; S is the concentration of the substrate; *Vmax* is the maximum transport rate (transport under saturation substrate conditions; indicator for transporter capacity); and *Km* is the Michaelis constant (indicator for transporter affinity to its substrate).

Analysis of means (ANOM) was performed for curves parameters comparison. Prism (Graphpad) and JMP programs were used for data visualization.

3. Results

3.1. PepT2 functionality verification

In order to verify PepT2 functionality in the transgenic cells we conducted an uptake test under optimal conditions. Naïve TmB (non-transfected) and PepT2-TmB (transgenic) cells were exposed to HBSS and β-ala-Lys-AMCA (125 μM)-containing HBSS for 2 h and visualized using a fluorescence microscope. While TmB cells did not show any fluorescence accumulation, PepT2-tmb cells exhibited high cytoplasmic fluorescence supportive of peptide accumulation (Fig. 2).

3.2. Calibration of incubation time for the uptake assay

In order to determine the appropriate incubation time of the cells with the fluorescent di-peptide we performed an uptake assay under normal conditions (300 mOsmol/kg; 25 °C) for different incubation periods. Fluorescence accumulation in the cells significantly increased with incubation time (Fig. 3A). However, uptake rate calculations showed a significantly decreased uptake rate at 30, 60, and 120 min treatments compared to the 15 min treatment (Fig. 3B) suggesting a saturation effect. Aiming to avoid experimental bias by saturation, and to perform the assay at an optimal substrate to the transporter ratio (according to Michaelis-Menten model assumptions), we selected the 15 min incubation time for all subsequent experiments.

3.3. Temperature effect

The transport rate curves for all three temperatures tested strongly fitted the Michaelis-Menten model ($R^2 = 0.94$). Temperature treatments affected the maximal transport rate of β-Ala-Lys-AMCA by the PepT2 expressing cells (Fig. 4). Vmax significantly increased with higher incubation temperature (0.25 ± 0.05, 0.47 ± 0.06 and 0.69 ± 0.07 RFU*minute^{-1} for 15 °C, 25 °C and 35 °C treatments respectively. Fig. 4B), while Km was not significantly affected by incubation temperature (184.8 ± 74.4, 178.2 ± 40.3 and 223.8 ± 39.5 μM for 15 °C, 25 °C and 35 °C treatments respectively. Fig. 4B).

3.4. Salinity effect

The transport rate curves for 150, 300, and 600 mOsmol/kg treatments significantly fitted the Michaelis-Menten model ($R^2 = 0.97$; Vmax: 0.5 ± 0.12, 1.36 ± 0.06 and 1.45 ± 0.11 RFU*minute^{-1}, Km: 347.2 ± 155.5, 178.78 ± 19.78 and 341.4 ± 51.89 μM, respectively), while the data from the 900 mOsmol/kg treatment did not significantly fit this model (Fig. 5). While hypersalinity (600 mOsmol/kg) significantly elevated the Km but not Vmax, hyposaline HBSS (150 mOsmol/kg) media significantly affected the Vmax but not the Km of the transporter (Fig. 5B).

4. Discussion

Mechanisms that underlie different physiological acclimation and adaptation pathways to environmental changes remain largely unknown, due to the complexity of their study at the whole-organism level. This complexity is especially apparent in fish, due to the dynamic nature of the aquatic environment, and their distinct physiological characteristics, such as osmoregulatory ability and being poikilothermic organisms. Many teleost fishes are subjected to various environmental changes throughout their life, such as water salinity and temperature, which induce many different physiological responses to maintain homeostasis, setting them as interesting research subjects for evolutionary and ecophysiology research. However, most fish are considered as "non-model" organisms and, therefore, there are limited scientific resources that can support advanced ecophysiological research. Hence, it is

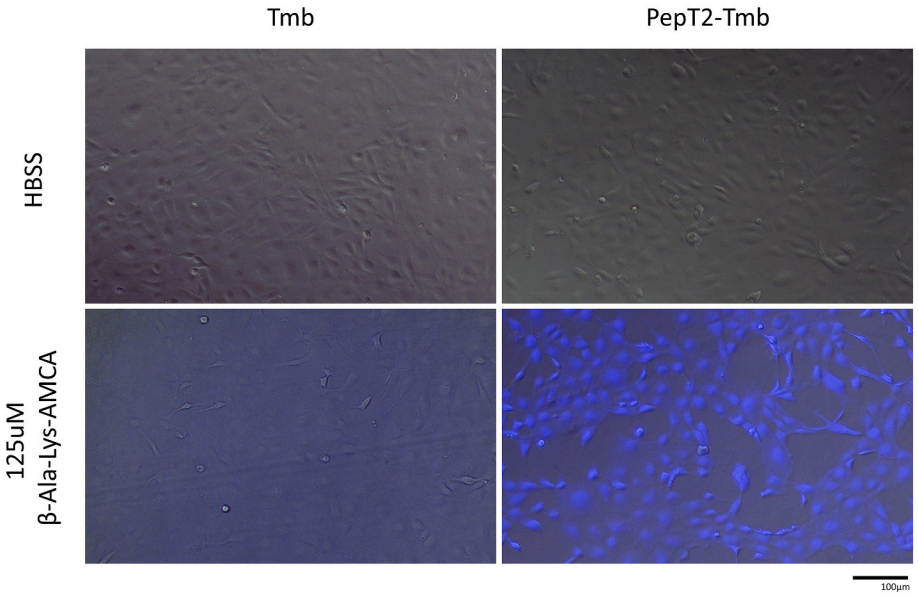

Fig. 2. PepT2 functional validation test. TmB-cells and PepT2-TmB cells post 2 h incubated with or without 125 μM β-Ala-Lys-AMCA in HBSS (20 μM hepes). Fluorescent peptide accumulation is seen in PepT2-TmB cells in blue (bottom right panel). Scale bar 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

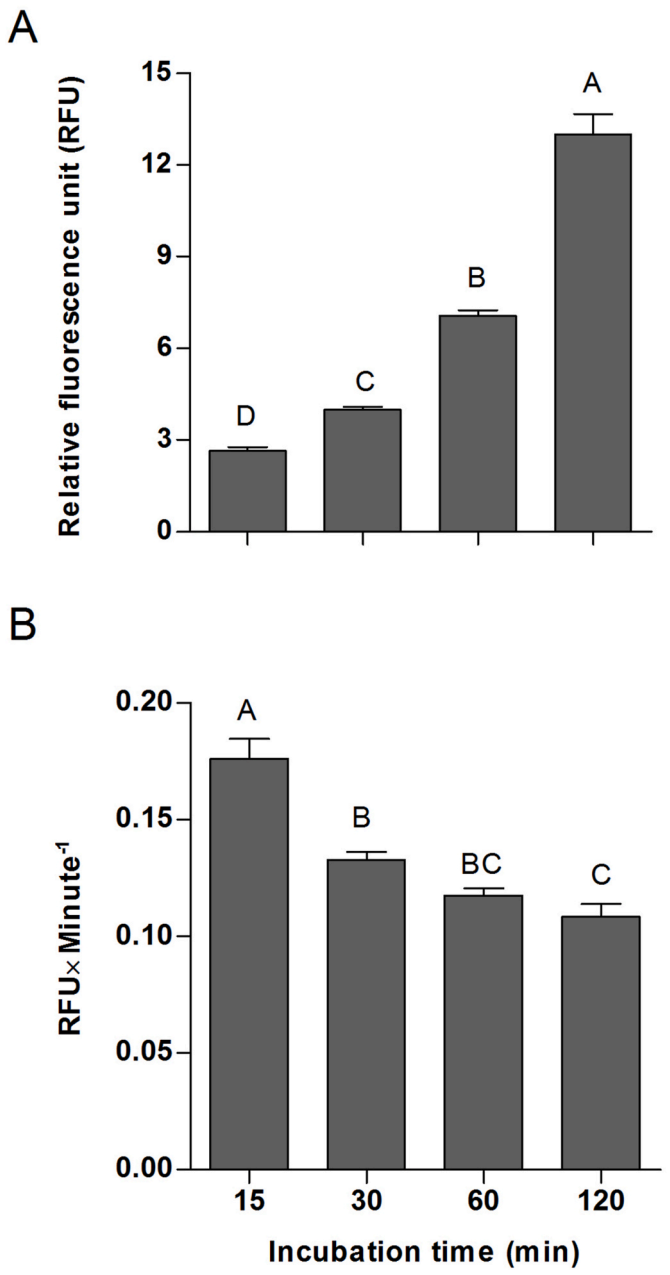

Fig. 3. Incubation time optimization. The effect of incubation-time on fluorescence accumulation (A) and transport rate (B) of PepT2 incubated with 125 μM of β-Ala-Lys-AMCA under normal conditions (300 mOsmol/kg; 25 °C).

necessary to establish appropriate *in-vitro* experimental systems that will allow to uncover molecular and cellular mechanisms that underlie different acclimation and adaptation phenomena relevant to "non-model" organisms (Kültz et al., 2007). These *in-vitro* systems will provide accurate and reliable approaches to address physiological questions in the relevant context of environment-physiology interactions, based on the natural genomic background of the studied species. Our proposed *in-vitro* system provides an experimental set-up within a cellular context, which mimics the natural environment in which most proteins function, thus, serving as an optimal system for evaluating physiological and molecular adaptations. Furthermore, our demonstrated pipeline could be in use for other fish-originated cytosolic or transmembrane recombinant proteins. Most of the recombinant proteins used in fish research are translated and processed in heterologous expression systems, such as yeast, insects, and mammalian cell lines (Mohammadzadeh et al., 2022). Since protein structure, and consequently its function, are greatly affected by the nature of the expression system, due to translational, post-translational, folding and membrane-integrations processes, our system could provide better conditions for recombinant protein expression.

4.1. *In-vitro vs. in-situ experimental systems*

The homologous cell-based expression system described here, based on a Mozambique tilapia cell line, is an optimal research tool to study transporter activity under various fish-relevant environmental conditions and for characterization of transporter paralogs. This expression system allows a more controlled environment and higher experimental reproducibility than *in-vivo* or *in-situ* methods. While the usage of *in-situ* systems, such as the previously used inverted gut sac (Bucking and Schulte, 2012; Con et al., 2017), provide a homologous system to test different conditions, some difficulties persist as obstacles to molecular studies. For example, expression and post-expression variations can create large differences in functional proteins, thus masking transport results. Overcoming these biases will require further normalization, according to the specific functional protein unit, an option which is not always available, especially in non-model organisms that have limited experimental tools and resources (Kültz et al., 2007). Additionally, different conditions may affect the expression of the transporter along the intestine and change the location of expression, as seen for the PepT2 transporter (Con et al., 2017). Controlling experimental conditions in *in-vivo* or *in-situ* systems is inherently, more complex than in *in-vitro* systems. Individual variations at multiple regulatory levels, coupled with environmental effects, necessitates an increased number of sacrificed animals to ensure both statistical accuracy and experimental consistency. Furthermore, if the transporter of choice is part of a multiple-transporters absorption mechanism, such as PepT2 that co-expresses with PepT1 variants in the intestine of fish, the ability to study the function of a specific transporter in *in-situ* systems without cross-effects of other transporters is difficult or impossible. Our system enables the exogenously expression and separate transporters variants in order to study their activity. The presented expression system effectively addresses and eliminates these challenges, making it an optimal controlled experimental system for characterizing fish transporters.

4.2. *Homologous vs. heterologous expression systems*

While some heterologous expression systems serve as adequate *in-vitro* systems that allow the functional characterization of a single specific transporter (Gomes et al., 2020; Vacca et al., 2022), they lack the ability to mimic the natural cellular environment of the transporter. These differences are more pronounced when addressing environmental stress responses and their effects on cellular processes, since they differ greatly between species and cell types (Kültz, 2005). For example, in MDCK cells, high levels of heat-shock protein 27 (HSP27) correlated with higher survival percentage under osmotic stress (Beck et al., 2000), and in murine inner medullary collecting duct 3 cells (mIMCD-3), osmotic tolerance was correlated with higher HSP70 expression (Santos et al., 2023). In addition, the natural membrane environment and its response to stressors affect transporters activity (Kültz, 2012). It has been shown that membrane lipid composition affects the ATP-binding cassette (ABC) transporter OpuA of bacteria and that this transporter's osmosensing is dependent on the protein-lipid interactions (van der Heide et al., 2001). Our system provides a controlled experimental system that allows constant and specific expression, proper post-translational processing (i.e. integration in the plasma membrane), adequate membrane environment and integrity, and relevant cellular physiological traits, all of which are highly important for transmembrane transport studies with the relation to environmental effects under the physiologically relevant conditions that apply to fish.

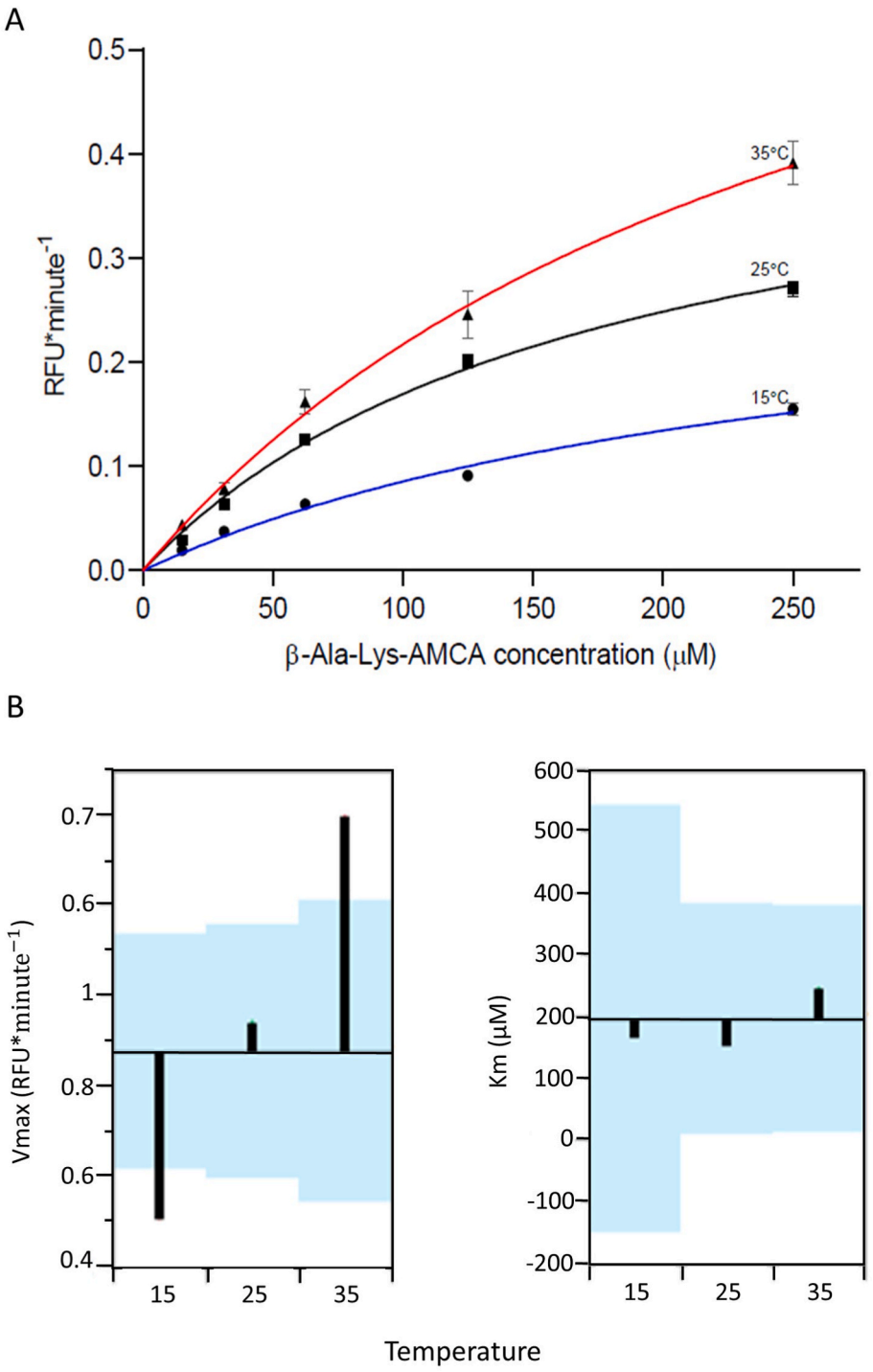

Fig. 4. Transport rate curves of β-Ala-Lys-AMCA by PepT2-TmB cells at different incubation temperatures (A). Statistical comparison of Vmax and Km (ANOM charts) (B). Blue area limits represent decision limits at $\alpha = 0.05$ of the ANOM analysis. Bars exciding the decision limits (i.e., out of the blue area in the graphs) indicate a significant difference of the parameters between treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.3. Nutrient transport under the poikilothermic context

Temperature is known to affect metabolism, physiological, and biochemical processes, such as enzymatic reactions, and considered as one of the major factors that drive evolutionary adaptations. The temperature range of fish habitats is very wide, from extreme cold of −1.5 °C in the Southern ocean to over 40 °C in some African lakes (DeVries and Wohlschlag, 1969; Reite et al., 1974). Hence, when addressing temperature effects on fish physiology, and especially enzymatic reactions, it is important to address biological questions in an appropriate experimental design using tools that minimize confounding variables. Tilapias are widely spread in tropical and sub-tropical areas, but do encounter cold temperatures below 10 °C both in their natural habitat and under aquaculture conditions. It has been shown that differences in cold resistance lead to differential expression of temperature-related pathways associated with glucose and amino-acid metabolism in tilapia gills and liver (Nitzan et al., 2019). Here we tested the temperature effect on the activity of the Mozambique tilapia

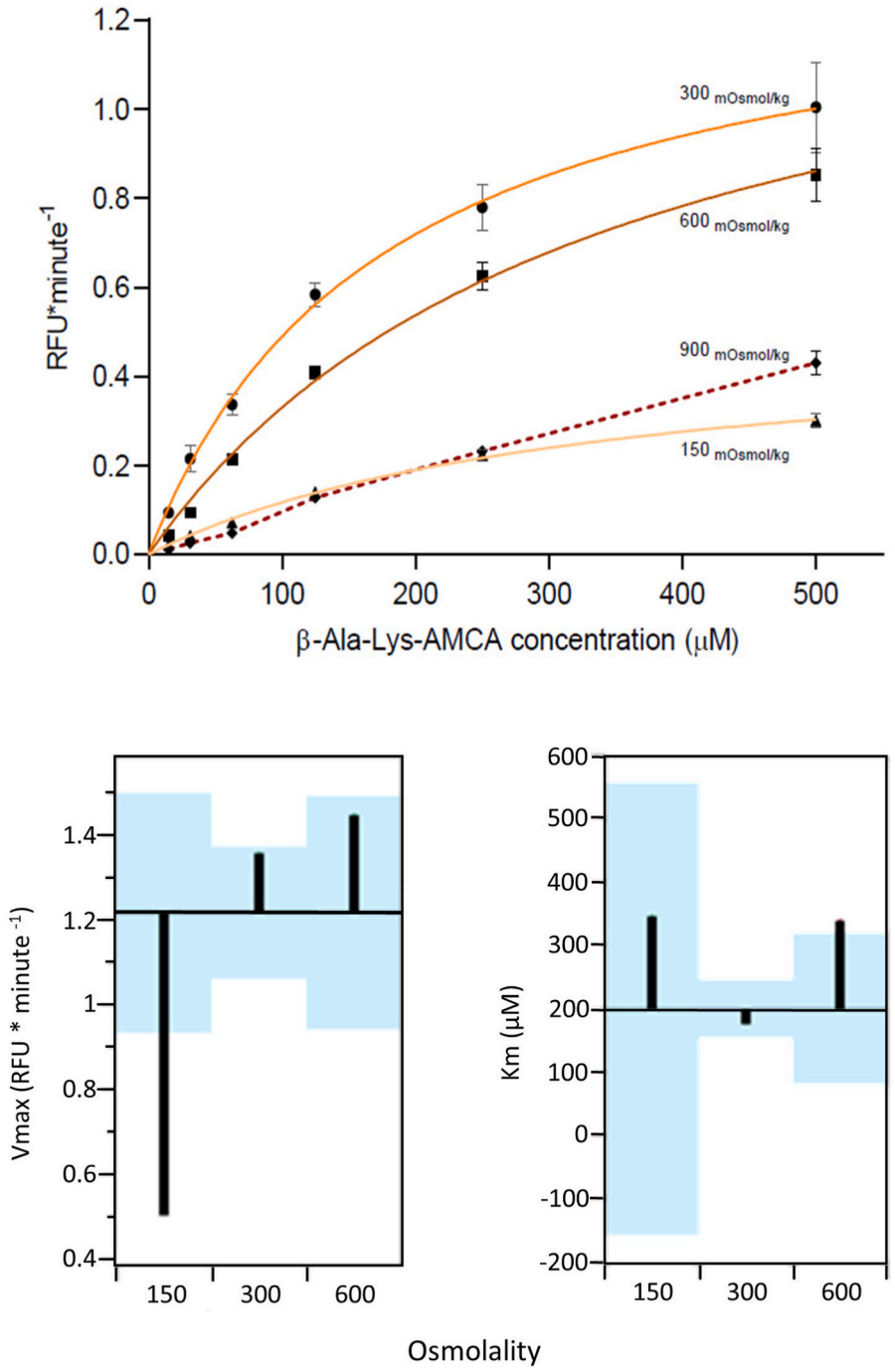

Fig. 5. Transport rates curves for β-Ala-Lys-AMCA into PepT2-TmB cells under different osmolality conditions (A) Statistical comparison of Vmax and Km (ANOM charts) (B). Blue area limits represent decision limits at $\alpha = 0.05$ of the ANOM analysis. Bars exciding the decision limits (i.e., out of the blue area in the graphs) indicate a significant difference of the parameters between treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

PepT2 transporter and found that it affects only Vmax, which indicates on the transporter's capacity. Although capacity is reduced at low temperatures, the integrity of the Mozambique tilapia PepT2 activity is maintained. It had been shown that tilapia actively lowering their metabolism levels in response to cold challenges (Segev-Hadar et al., 2021). Our results indicate that peptide transport at low temperature is feasible and can continuously supply nutrients to support metabolic function. Our results also emphasize that environmental tolerance manifests at multiple biological organization levels.

The importance of homologous expression system was clearly demonstrated by Maffia et al. (2003), which showed a significant effect of the experimental system on the transport of small peptides. In their study on the peptide transporter 1 (variant not indicated) of the Antarctic teleost *Chionodraco hamatus*, the authors showed that, while absorption measured by the BBMV (brush border membrane vesicles) method was significantly higher at 0 °C, transport assay conducted with the same transporter in *Xenopus laevis* oocytes did not show the same trend. In addition, the pre-incubation temperature of the oocytes was shown to affect the results as the transport was abolished completely when the oocytes were pre-incubated at 4 °C. These effects of

temperature on the transport of small peptides in the oocytes could result from a cellular stress response and from changes in membrane integrity under non-physiological temperatures (Maffia et al., 2003). Our homologous expression systems offer a suitable membrane and intracellular environments, together with the natural wide range of temperature in which the cells grow and function, allowing precise evaluation of the nutrient transport under different environmental conditions.

4.4. Nutrient transport under the context of osmotolerance

Nutrient absorption into cells is driven by electrochemical and in particular ions gradients. In higher eukaryotes, many nutrients are absorbed by utilizing Na^+-dependent transport systems (Daniel et al., 2006). However, the "Archaic" proton-dependent transporters that utilize a proton gradient across the cell membrane are still integral in nutrient-absorption mechanisms, although they are much more common in bacteria. While the transport of small peptides by PepTs is not dependent on Na^+ directly (Fei et al., 1994), Na^+ movement across the cell membrane does affect the proton gradient and membrane potential, thus, indirectly affecting these proton-dependent transporters. The indirect Na^+ dependency of small-peptide absorption across the intestinal and renal epithelia was observed in *in-vivo* and *in-situ* systems, and attributed to the activity of Na^+/H^+ exchanger (NHE) that excretes protons from cells in exchange for Na^+ (Ganapathy and Leibach, 1983). Studies using *in-vitro* systems of BBMV showed that Na^+ concentration did not affect the transport of small peptides (Boll et al., 1996; Daniel et al., 1991). However, while BBMV allows transport studies in a natural membrane-setting, this experimental tool depends on the isolation of the brush border membranes, and thus, partially disconnects the environmental effect on the transport from the cellular context. Ganapathy and Leibach (1983) discussed this inconsistent dependence of small-peptide absorption on Na^+ and suggested that in studies conducted with tissues, the presence of Na^+/K^+-ATPase (NKA) at the basolateral membrane of epithelial cell continues to regulate the intracellular concentration of Na^+, thus, allowing the continued secondary function of NHE. In the BBMV method, NKA is not detected in the vesicles, and thus, the secondary activity of NHE is inhibited by the intra-vesicular Na^+ concentration (Ganapathy and Leibach, 1983). Therefore, to assess the correct effect of Na^+ on the fish-peptide transport, a cellular expression system that reflects proper transport conditions (including NKA function) is preferred. The TmB cell line was characterized in the past as highly osmotolerant and adequate to sustain very high osmolality levels, corresponding with the tilapia's high natural osmotolerance (Gardell et al., 2014). This ability and its homologous origin make this cell line a preferred expression system for testing salinity effects on fish transporters, particularly in tilapias. Indeed, in our study, changes in NaCl in the extracellular medium significantly altered PepT2 activity. Moreover, we have seen different effects of hyper- and hyposaline conditions. While the hyposaline conditions decreased Vmax, the hypersaline condition increased the Km value of the transporter, which indicates the transporter's lower affinity. This different effect likely implies that salinity influences PepT2 activity through the cellular osmosensing and osmoregulation responses. The observed salinity effects on PepT2 activity are correlated well with the observed cellular responses to directional osmotic stress (hyper- or hypo-osmotic stress) (Evans and Somero, 2008; Kültz, 2012). Our new findings, together with the previously discussed inconsistency of Na^+-dependent peptide transport, emphasizes the importance of cellular context when addressing the effect of salinity and osmotic conditions on nutrient transport.

4.5. Biochemistry and ecophysiology integration

Our tilapia PepT2 case study emphasizes the tight interaction between the biochemical regulatory level and protein expression in the whole fish, and how studies on protein function provide new insights into known physiological phenomena. Our *in-vitro* system demonstrates the impact salinity has on PepT2 function and illustrates how changes in lumen conditions, due to fluctuations in environmental salinity or temperature, affect PepT2's function and, consequently, the absorption and utilization of peptides in fish. Our current results correlate, and further explain, the salinity-dependent expression and localization shifts of PepT2 along the tilapia intestine that is seen in the whole animal (Con et al., 2017). Saltwater-acclimated fish exhibit wider expression pattern of PepT2 along the intestinal tract. In light of our current results of lower affinity under hypersaline conditions, the wider expression pattern of PepT2 is likely a mechanism for preserving small-peptide absorption capacity. The high salinity tolerance of the Mozambique tilapia is seen across various regulatory and organizational levels, including the biochemical level, highlighting its complexity (Kültz, 2012). This intricate regulation affects other physiological mechanisms, such as nutrient absorption, which is vital to support the fish metabolism, as seen through our PepT2 case study.

The results obtained using this homologous expression system demonstrate the complex regulation on PepT2 transporter in fish and emphasize the impact environmental changes have on fish peptide transport. Our system provides a precise, controlled, and homologous experimental set-up for transport studies in fish, particularly concerning environmental effects and array of multi-transporter mechanisms. Considering the complexity of fish physiology and eco-physiological research, this system brings a new tool to study molecular and cellular mechanisms explaining environmental physiology, fitness, and adaptation to different habitats in fish.

CRediT authorship contribution statement

Pazit Con: conceived and designed the experiments, contributed to plasmids design, cultivate, modified and established the modified cell line, performed all uptake experiments, analyzed the data and preformed statistics analysis, wrote the paper. **Jens Hamar:** contributed to plasmids design. **Jakob Biran:** contributed to plasmids design, wrote the paper. All authors approved the final version of the manuscript. **Dietmar Kültz:** conceived and designed the experiments, wrote the paper. **Avner Cnaani:** conceived and designed the experiments, wrote the paper. All authors approved the final version of the manuscript.

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.

Data availability

All fluorescence measurements of all uptake assays and data analyses used in this presented research have been made publicly available in the Dryad data repository, doi: 10.5061/dryad.7sqv9s4xm.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2024.100118.

References

Alghamdi, O.A., King, N., Jones, G.L., Moens, P.D.J., 2018. A new use of β-Ala-Lys (AMCA) as a transport reporter for PEPT1 and PEPT2 in renal brush border membrane vesicles from the outer cortex and outer medulla. Biochim. Biophys. Acta Biomembr. 1860, 960–964. https://doi.org/10.1016/j.bbamem.2017.12.021.

Almén, M.S., Nordström, K.J.V., Fredriksson, R., Schiöth, H.B., 2009. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. BMC Biol. 7, 50. https://doi.org/10.1186/1741-7007-7-50.

Beck, F.X., Grünbein, R., Lugmayr, K., Neuhofer, W., 2000. Heat shock proteins and the cellular response to osmotic stress. Cell. Physiol. Biochem. 10, 303–306. https://doi.org/10.1159/000016362.

Bodinier, C., Lorin-Nebel, C., Charmantier, G., Boulo, V., 2009. Influence of salinity on the localization and expression of the CFTR chloride channel in the ionocytes of juvenile *Dicentrarchus labrax* exposed to seawater and freshwater. Comp. Biochem. Physiol., A 153, 345–351. https://doi.org/10.1016/j.cbpa.2009.03.011.

Boll, M., Herget, M., Wagener, M., Weber, W.M., Markovich, D., Biber, J., Clauss, W., Murer, H., Daniel, H., 1996. Expression cloning and functional characterization of the kidney cortex high-affinity proton-coupled peptide transporter. Proc. Natl. Acad. Sci. U. S. A. 93, 284–289. https://doi.org/10.1073/pnas.93.1.284.

Bucking, C., Schulte, P.M., 2012. Environmental and nutritional regulation of expression and function of two peptide transporter (PepT1) isoforms in a euryhaline teleost. Comp. Biochem. Physiol. Mol. Integr. Physiol. 161, 379–387. https://doi.org/10.1016/j.cbpa.2011.12.008.

Chew, S.F., Hiong, K.C., Lam, S.P., Ong, S.W., Wee, W.L., Wong, W.P., Ip, Y.K., 2014. Functional roles of Na^+/K^+-ATPase in active ammonia excretion and seawater acclimation in the giant mudskipper, *Periophthalmodon schlosseri*. Front Physiol 5 APR 1–17. https://doi.org/10.3389/fphys.2014.00158.

Chourasia, T.K., D'Cotta, H., Baroiller, J.F., Slosman, T., Cnaani, A., 2018. Effects of the acclimation to high salinity on intestinal ion and peptide transporters in two tilapia species that differ in their salinity tolerance. Comp Biochem Physiol -Part A Mol Integr Physiol 218, 16–23. https://doi.org/10.1016/j.cbpa.2018.01.004.

Con, P., Nitzan, T., Cnaani, A., 2017. Salinity-dependent shift in the localization of three peptide transporters along the intestine of the Mozambique tilapia (*Oreochromis mossambicus*). Front. Physiol. 8, 1–12. https://doi.org/10.3389/fphys.2017.00008.

Con, P., Nitzan, T., Slosman, T., Harpaz, S., Cnaani, A., 2019. Peptide transporters in the primary gastrointestinal tract of pre-feeding Mozambique Tilapia larva. Front. Physiol. 10 https://doi.org/10.3389/fphys.2019.00808.

Cossins, A.R., Macdonald, A.G., 1989. The adaptation of biological membranes to temperature and pressure: fish from the deep and cold. J. Bioenerg. Biomembr. 21, 115–135. https://doi.org/10.1007/BF00762215.

Daniel, H., Morse, E.L., Adibi, S.A., 1991. The high and low affinity transport systems for dipeptides in kidney brush border membrane respond differently to alterations in pH gradient and membrane potential. J. Biol. Chem. 266, 19917–19924.

Daniel, H., Spanier, B., Kottra, G., Weitz, D., 2006. From bacteria to man: archaic proton-dependent peptide transporters at work. Physiology 21, 93–102. https://doi.org/10.1152/physiol.00054.2005.

DeVries, A.L., Wohlschlag, D.E., 1969. Freezing resistance in some antarctic fishes. Science 163, 1073–1075. https://doi.org/10.1126/science.163.3871.1073.

Dieck, S.T., Heuer, H., Ehrchen, J., Otto, C., Bauer, K., 1999. The peptide transporter PepT2 is expressed in rat brain and mediates the accumulation of the fluorescent dipeptide derivative β-Ala-Lys-Nε-AMCA in astrocytes. Glia 25, 10–20. https://doi.org/10.1002/(SICI)1098-1136(19990101)25:1<10::AID-GLIA2>3.0.CO;2-Y.

Evans, T.G., Somero, G.N., 2008. A microarray-based transcriptomic time-course of hyper- and hypo-osmotic stress signaling events in the euryhaline fish *Gillichthys mirabilis*: osmosensors to effectors. J. Exp. Biol. 211, 3636–3649.

Fei, Y.-J., Kanai, Y., Nussberger, S., Ganapathy, V., Leibach, F.H., Romero, M.F., Singh, S. K., Boron, W.F., Hediger, M.A., 1994. Expression cloning of a mammalian proton-coupled oligopeptide transporter. Nature 368, 563–566. https://doi.org/10.1038/368563a0.

Ganapathy, V., Leibach, F.H., 1983. Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush-border membrane vesicles from the rabbit. Studies with L-carnosine and glycyl-L-proline. J. Biol. Chem. 258, 14189–14192.

Gardell, A.M., Qin, Q., Rice, R.H., Li, J., Kültz, D., 2014. Derivation and osmotolerance characterization of three immortalized tilapia (*Oreochromis mossambicus*) cell lines. PLoS One 9, e95919. https://doi.org/10.1371/journal.pone.0095919.

Genz, J., Taylor, J.R., Grosell, M., 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. J. Exp. Biol. 211, 2327–2335. https://doi.org/10.1242/jeb.016832.

Gomes, A.S., Vacca, F., Cinquetti, R., Murashita, K., Barca, A., Bossi, E., Rønnestad, I., Verri, T., 2020. Identification and characterization of the Atlantic salmon peptide transporter 1a. Am. J. Physiol. Cell Physiol. 318, C191–C204. https://doi.org/10.1152/ajpcell.00360.2019.

Gonçalves, A.F., Castro, L.F.C., Pereira-Wilson, C., Coimbra, J., Wilson, J.M., 2007. Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? Comp. Biochem. Physiol., Part D: Genomics Proteomics 2, 345–355. https://doi.org/10.1016/j.cbd.2007.08.002.

Guettou, F., Quistgaard, E.M., Trésaugues, L., Moberg, P., Jegerschöld, C., Zhu, L., Jong, A.J.O., Nordlund, P., Löw, C., 2013. Structural insights into substrate recognition in proton-dependent oligopeptide transporters. EMBO Rep. 14, 804–810. https://doi.org/10.1038/embor.2013.107.

Hallali, E., Kokou, F., Chourasia, T.K., Nitzan, T., Con, P., Harpaz, S., Mizrahi, I., Cnaani, A., 2018. Dietary salt levels affect digestibility, intestinal gene expression, and the microbiome, in Nile tilapia (*Oreochromis niloticus*). PLoS One 13, e0202351. https://doi.org/10.1371/journal.pone.0202351.

Hiroi, J., Yasumasu, S., McCormick, S.D., Hwang, P.-P., Kaneko, T., 2008. Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. J. Exp. Biol. 211, 2584–2599. https://doi.org/10.1242/jeb.018663.

Hoffmann, A.A., Hercus, M.J., 2000. Environmental stress as an evolutionary force. Bioscience 50, 217–226. https://doi.org/10.1641/0006-3568(2000)050[0217:ESAAEF]2.3.CO;2.

Ingersoll, S.a., Ayyadurai, S., Charania, M.a., Laroui, H., Yan, Y., Merlin, D., 2012. The role and pathophysiological relevance of membrane transporter PepT1 in intestinal inflammation and inflammatory bowel disease. AJP Gastrointest Liver Physiol 302, G484–G492. https://doi.org/10.1152/ajpgi.00477.2011.

Inokuchi, M., Kaneko, T., 2012. Recruitment and degeneration of mitochondrion-rich cells in the gills of Mozambique tilapia *Oreochromis mossambicus* during adaptation to a hyperosmotic environment. Comp. Biochem. Physiol. Mol. Integr. Physiol. 162, 245–251. https://doi.org/10.1016/j.cbpa.2012.03.018.

Killer, M., Wald, J., Pieprzyk, J., Marlovits, T.C., Löw, C., 2021. Structural snapshots of human PepT1 and PepT2 reveal mechanistic insights into substrate and drug transport across epithelial membranes. Sci. Adv. 7, 3259. https://doi.org/10.1126/sciadv.abk3259.

Kokou, F., Con, P., Barki, A., Nitzan, T., Slosman, T., Mizrahi, I., Cnaani, A., 2019. Short- and long-term low-salinity acclimation effects on the branchial and intestinal gene expression in the European seabass (*Dicentrarchus labrax*). Comp. Biochem. Physiol. Part A Mol Integr Physiol 231, 11–18. https://doi.org/10.1016/j.cbpa.2019.01.018.

Kültz, D., 2012. The combinatorial nature of osmosensing in fishes. Physiology 27, 259–275. https://doi.org/10.1152/physiol.00014.2012.

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67, 225–257. https://doi.org/10.1146/annurev.physiol.67.040403.103635.

Kültz, D., Fiol, D.F., Valkova, N., Gomez-Jimenez, S., Chan, S.Y., Lee, J., 2007. Functional genomics and proteomics of the cellular osmotic stress response in "non-model" organisms. J. Exp. Biol. 210, 1593–1601. https://doi.org/10.1242/jeb.000141.

Kültz, D., Li, J., Gardell, A., Sacchi, R., 2013. Quantitative molecular phenotyping of gill remodeling in a cichlid fish responding to salinity stress. Mol. Cell. Proteomics 12, 3962–3975. https://doi.org/10.1074/mcp.M113.029827.

Lee, A.G., 2004. How lipids affect the activities of integral membrane proteins. Biochim. Biophys. Acta Biomembr. 1666, 62–87. https://doi.org/10.1016/j.bbamem.2004.05.012.

Lewis, D.H., Marks, J.E., 1985. Microcultures of *Sarotherodon mossambicus* (Peters) cells: their use in detecting fish viruses. J. Fish. Dis. 8, 477–478. https://doi.org/10.1111/j.1365-2761.1985.tb01283.x.

Maffia, M., Rizzello, A., Acierno, R., Verri, T., Rollo, M., Danieli, A., Döring, F., Daniel, H., Storelli, C., 2003. Characterisation of intestinal peptide transporter of the Antarctic haemoglobinless teleost *Chionodraco hamatus*. J. Exp. Biol. 206, 705–714. https://doi.org/10.1242/jeb.00145.

McCormick, S.D., Sundell, K., Björnsson, B.T., Brown, C.L., Hiroi, J., Bjornsson, B.T., Brown, C.L., Hiroi, J., 2003. Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). J. Exp. Biol. 206, 4575–4583. https://doi.org/10.1242/jeb.00711.

Milne, M.D., 1964. Disorders of amino-acid transport. BMJ 1, 327–336. https://doi.org/10.1136/bmj.1.5379.327.

Minhas, G.S., Newstead, S., 2020. Recent advances in understanding prodrug transport through the SLC15 family of proton-coupled transporters. Biochem. Soc. Trans. 48, 337–346. https://doi.org/10.1042/BST20180302.

Mohammadzadeh, S., Ahmadifar, E., Masoudi, E., Milla, S., El-Shall, N.A., Alagawany, M., Emran, T. Bin, Michalak, I., Dhama, K., 2022. Applications of recombinant proteins in aquaculture. Aquaculture 561, 738701. https://doi.org/10.1016/j.aquaculture.2022.738701.

Nitzan, T., Kokou, F., Doron-Faigenboim, A., Slosman, T., Biran, J., Mizrahi, I., Zak, T., Benet, A., Cnaani, A., 2019. Transcriptome analysis reveals common and differential response to low temperature exposure between tolerant and sensitive Blue Tilapia (*Oreochromis aureus*). Front. Genet. 10, 1–11. https://doi.org/10.3389/fgene.2019.00100.

Nitzan, T., Rozenberg, P., Cnaani, A., 2017. Differential expression of amino-acid transporters along the intestine of Mozambique tilapia (*Oreochromis mossambicus*) and the effect of water salinity and time after feeding. Aquaculture 472, 71–75. https://doi.org/10.1016/j.aquaculture.2016.01.020.

Øverli, Ø., Sørensen, C., Nilsson, G.E., 2006. Behavioral indicators of stress-coping style in rainbow trout: do males and females react differently to novelty? Physiol. Behav. 87, 506–512. https://doi.org/10.1016/j.physbeh.2005.11.012.

Peleg, Y., Unger, T., 2014. Application of the Restriction-Free (RF) cloning for multicomponents assembly. DNA Cloning Assem Methods Methods Mol Biol, Methods in Molecular Biology 1116, 73–87. https://doi.org/10.1007/978-1-62703-764-8_6.

Reite, O.B., Maloiy, G.M.O., Aasehaug, B., 1974. pH, salinity and temperature tolerance of lake Magadi Tilapia. Nature 247. https://doi.org/10.1038/247315a0, 315–315.

Robertson, J.C., Hazel, J.R., 1995. Cholesterol content of trout plasma membranes varies with acclimation temperature. Am. J. Physiol. Regul. Integr. Comp. Physiol. 269 https://doi.org/10.1152/ajpregu.1995.269.5.r1113.

Rojas-García, C.R., Applebaum, S.L., Morais, S., Rønnestad, I., 2016. Trans-intestinal absorption rates differ between free amino acids during larval development in Atlantic herring (*Clupea harengus*). Aquaculture 464, 222–228. https://doi.org/10.1016/j.aquaculture.2016.06.029.

Root, L., Campo, A., MacNiven, L., Con, P., Cnaani, A., Kültz, D., 2021. Nonlinear effects of environmental salinity on the gill transcriptome versus proteome of *Oreochromis*

niloticus modulate epithelial cell turnover. Genomics 113, 3235–3249. https://doi.org/10.1016/j.ygeno.2021.07.016.

Rubio-Aliaga, I., Daniel, H., 2008. Peptide transporters and their roles in physiological processes and drug disposition. Xenobiotica 38, 1022–1042. https://doi.org/10.1080/00498250701875254.

Saita, E.A., De Mendoza, D., 2015. Thermosensing via transmembrane protein-lipid interactions. Biochim. Biophys. Acta Biomembr. 1848, 1757–1764. https://doi.org/10.1016/j.bbamem.2015.04.005.

Santos, B.C., Pullman, J.M., Chevaile, A., Welch, W.J., Gullans, S.R., Bento, C., Pullman, J.M., Welch, W.J., Gullans, S.R., 2023. Chronic Hyperosmolarity Mediates Constitutive Expression of Molecular Chaperones and Resistance to Injury 02139, pp. 564–574.

Sardella, B.A., Brauner, C.J., 2008. The effect of elevated salinity on "California" Mozambique tilapia (*Oreochromis mossambicus* x *O.urolepis hornorum*) metabolism. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 148, 430–436. https://doi.org/10.1016/j.cbpc.2008.05.006.

Schniers, B.K., Rajasekaran, D., Korac, K., Sniegowski, T., Ganapathy, V., Bhutia, Y.D., 2021. PEPT1 is essential for the growth of pancreatic cancer cells: a viable drug target. Biochem. J. 478, 3757–3774. https://doi.org/10.1042/BCJ20210377.

Segev-Hadar, A., Krispin, S., Olthof, A.M., Hyatt, K.C., Haller, L., Barki, A., Nitzan, T., Levkowitz, G., Kanadia, R.N., Cnaani, A., Biran, J., 2021. Oxytocin signaling regulates the homeostatic response to cold stress in poikilothermic vertebrates. bioRxiv, 2021.12.20.472748.

Spanier, B., Rohm, F., 2018. Proton coupled oligopeptide transporter 1 (PepT1) function, regulation, and influence on the intestinal homeostasis. Compr. Physiol. 8, 843–869. https://doi.org/10.1002/cphy.c170038.

Stickney, R.R., 1986. Tilapia tolerance of saline waters: a review. The Progressive Fish Culturist. Progress. Fish Cult. 48, 161–167.

Tai, W., Chen, Z., Cheng, K., 2013. Expression profile and functional activity of peptide transporters in prostate cancer cells. Mol. Pharm. 10, 477–487. https://doi.org/10.1021/mp300364k.

Taylor, J.R., Grosell, M., 2009. The intestinal response to feeding in seawater gulf toadfish, *Opsanus beta*, includes elevated base secretion and increased epithelial oxygen consumption. J. Exp. Biol. 212, 3873–3881.

Taylor, J.R., Grosell, M., 2006. Feeding and osmoregulation: dual function of the marine teleost intestine. J. Exp. Biol. 209, 2939–2951. https://doi.org/10.1242/jeb.02342.

Vacca, F., Gomes, A.S., Murashita, K., Cinquetti, R., Roseti, C., Barca, A., Rønnestad, I., Verri, T., Bossi, E., 2022. Functional characterization of Atlantic salmon (*Salmo salar* L.) PepT2 transporters. J. Physiol. 600, 2377–2400. https://doi.org/10.1113/JP282781.

van der Heide, T., Stuart, M.C.A., Poolman, B., 2001. On the osmotic signal and osmosensing mechanism of an ABC transport system for glycine betaine. EMBO J. 20, 7022–7032. https://doi.org/10.1093/emboj/20.24.7022.

Vargas-Chacoff, L., Arjona, F.J., Polakof, S., del Río, M.P.M., Soengas, J.L., Mancera, J. M., 2009. Interactive effects of environmental salinity and temperature on metabolic responses of gilthead sea bream *Sparus aurata*. Comp. Biochem. Physiol. Mol. Integr. Physiol. 154, 417–424. https://doi.org/10.1016/j.cbpa.2009.07.015.

Verri, T., Terova, G., Romano, A., Barca, A., Pisani, P., Storelli, C., Saroglia, M., 2012. The solute carrier (SLC) family series in teleost fish. In: Functional Genomics in Aquaculture. Wiley-Blackwell, Oxford, UK, pp. 219–320. https://doi.org/10.1002/9781118350041.ch10.

Weinrauch, A.M., Blewett, T.A., Glover, C.N., Goss, G.G., 2019. Acquisition of alanyl-alanine in an Agnathan: characteristics of dipeptide transport across the hindgut of the Pacific hagfish *Eptatretus stoutii*. J. Fish. Biol. 95, 1471–1479. https://doi.org/10.1111/jfb.14168.