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

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**Abstract**

All organisms encounter environmental changes that lead to physiological adjustments and drive evolutionary adaptations. These, in turn, induce behavioral, physiological and molecular changes that 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 are prime targets for molecular studies of environmental effects, as they facilitate the ability of cells to interact with the external surrounding. Fish are subjected to fluctuations of environmental factors of their aquatic surrounding and exhibit different coping mechanisms. To study the molecular adjustments of fish proteins to their unique external surrounding, suitable experimental systems must be established. Mozambique tilapia (*Oreochromis mossambicus*) is an excellent model for environmental stress studies due to its extreme osmotolerance. We established a homologues cellular-based expression system, and an uptake assay, that allowed us to study effects of environmental conditions on transmembrane transport. We applied it to study the effects of environmental conditions on the activity of PepT2, a widely studied transporter due to its importance in absorption of dietary peptides and drugs. We created a stable, modified fish cell-line, exogenously expressing the tilapia PepT2 and tested the effects of temperature and water salinity on the uptake of fluorescent di-peptide, β-Ala-Lys-AMCA. While temperature affected the Vmax of the transport, salinity affected both the Vmax and the Km. These assays demonstrate the importance of suitable experimental systems for fish ecophysiology studies. The presented tools and methods can be adapted to study other transporters *in-vitro.*

**1. Introduction**

Organisms routinely experience changes in environmental conditions. These can result from diurnal and seasonal cycles, moving between habitats, or climatic events. Environmental changes can perturb the organisms' 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), function and metabolism of tissues and cells (Kültz et al., 2013; Nitzan et al., 2019; Root et al., 2021a; Vargas-Chacoff et al., 2009), as well as 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 allow organisms to react to environmental stress and maintain homeostasis (Hoffmann and Hercus, 2000). Deciphering the underlying molecular adjustments that occur during environmental changes endurance will contribute to a broader understanding of the environmental adaptations mechanism.

Various aquatic environments exhibit unique fluctuations of environmental factors such as water salinity and pH levels, 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 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 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., 2021b). These tissues are responsible for continuously, yet dynamic interchanging fluxes of different molecules with the surrounding environment. For these special responses to environmental conditions, it is important to establish tools and experimental systems that address fish ecophysiology.

The intestine is one of the fish organs exposed to the surrounding environment. 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 at their apical membrane facing the intestinal lumen, a dynamic environment that changes according to feeding regime, feed content and water salinity (Genz et al., 2008; Taylor and Grosell, 2009, 2006). 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 nutrients, endogenous metabolites and drugs across cell plasma and organellar membranes (Almén et al., 2009). Their importance in metabolism and homeostasis is mirrored in their involvement in many pathological states (Milne, 1964). In addition, they are responsible for the selective permeability of cell membranes, 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 great influence on enzymatic reactions and protein folding, it also affects the membrane environment which is significant for the integrity of transmembrane proteins activity (Lee, 2004; Saita and De Mendoza, 2015). As poikilothermic animals, fish have specific adaptations to temperature in compare to mammalians cells (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 (PepT) 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, they 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). PepT 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 that 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 correlation between their abundances, functional properties and the substrate availability in the lumen (Con et al., 2019, 2017; Gomes et al., 2020; Vacca et al., 2022). Their expression pattern allows for a high efficiency of small peptide absorption along the intestinal tract. Their expression was also found to be affected by water salinity (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 have aimed to establish 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.

**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 L-15 medium containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1.25 U/ml Nystatin ("full L15") and were incubated at 25°C with no supplemental CO2. Cells were passaged every 6-7 days using a 1:7 splitting ratio. *mRNA* expression analysis (primers are listed in supplementary Table 1) showed that these cells do not natively express any of the three peptide transporters (unpublished data).

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, Massachusetts, USA). This construct allow the expression of both transcripts under the same constitutive CMV promotor. The final plasmid sequence (pN1-PepT2-P2A-Puromycin) was deposit in Addgene. TmB Cells were transfected with pN1-PepT2-P2A-Puromycin (10µg per 2.6 \*106 cells), using Viafect transfection reagent (Promega, USA). 48 hours post transfection, full L15 medium was replenished with selection medium (1µg/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 two hours with labeled 125µM di-peptide, β-Ala-Lys-AMCA (β-Ala-Lys-N-7-amino-4-methylcoumarin-3-acetic acid), a known substrate for the peptide transporters (Alghamdi et al., 2018; Dieck et al., 1999) (Bio Trend Chemicals, Destin, USA).

2.3 Preparation experimental mediums:

All functional uptake assays were conducted in Hanks buffered salt solutions (HBSS) with 20µM Hepes to eliminate pH changes and serum originated peptides effects on substrate uptake. Hypo- and Hyper-osmotic Hanks buffered salt solutions (HBSS) were prepared by reduction or supplementation of NaCl. NaCl was added to HBSS until osmolality reached 900mOsmol/kg. 600 mOsmol/kg solution was prepared by diluting 900 mOsmol/kg solution with 300 mOsmol/kg (normal HBSS). In order to create hypo-osmotic HBSS, NaCl-free HBSS was prepared and its osmolality was measured for 45 mOsmol/kg. The NaCl free HBSS was then used to dilute the 300 mOsmol/kg HBSS solution 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 0.22µm 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 10µg 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 hours 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 solutions (according to experiment) at a final concentration of 0.8-1 \* 106 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 0-500µM). Peptide stock solutions (20µl) were aliquoted into a 96 V-shape well plate. 100µl 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 100µl of ice cold HBSS. Plates were centrifuged at 500g for 5 min at 4°C. After centrifugation, 200µl of the supernatant were carefully removed from each well to prevent cell aspiration and cells were washed using 200µl of fresh ice cold HBSS. Centrifugation and washing steps were repeated for 3 additional times. Following the fourth centrifugation, 100µl 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 100µl 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 Figure 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 125µM of β-ala-Lys-AMCA. Cells were incubated with the di-peptide for 15, 30, 60 and 120 minutes. All incubations were stopped by addition of 100µl 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, in 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 Osmolality effect:

Although PepT2 is a proton dependent transporter, ions levels had different effects on peptide transport (Ganapathy and Leibach, 1983), In order to study the effect of osmolality on the PepT2 activity, we conducted uptake assays at various osmolality conditions. To this end, cell suspensions and β-ala-Lys-AMCA concentrations were prepared in four different osmolality-adjusted HBSS solutions representing hyper- and hypo-osmotic conditions. Aiming to avoid cell volume effects due to the osmotic pressure of the HBSS solutions, 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. Michaelis Menten curves were fitted for transport rate data, using non-linear regression in JMP and analysis of means (ANOM) was performed for curves parameters comparison. Graph 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 (untransfected) and PepT2-Tmb (transgenic) cells were exposed to HBSS and β-ala-Lys-AMCA (125µM)-containing HBSS for 2 hours 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 (Figure 2).

3.2 Calibration of incubation time for the uptake assay:

In order to determine the appropriate incubation time of the cells with the labeled di-peptide we performed an uptake assay under normal conditions (300 mOsmol/kg; 25°C) for different incubation periods. Florescence accumulation in the cells significantly increased with incubation time (Figure 3A). However, uptake rate calculations showed a significantly decreased uptake rate at 30, 60 and 120 minute treatments compared to the 15 minutes treatment (Figure 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 minutes 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 (R2=0.94). Temperature treatments affected the maximal transport rate of β-Ala-Lys-AMCA by the PepT2 expressing cells (Figure 4). Vmax of the transport 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. Figure 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. Figure 4B).

3.4 Salinity effect:

The transport rate curves for 150, 300 and 600 mOsmol/kg treatments significantly fitted the Michaelis-Menten model (R2=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 of the 900 mOsmol/kg treatment did not significantly fit this model (figure 5). While hyper-osmotic conditions (600 mOsmol/kg) significantly elevated the Km but not Vmax, hypo-osmotic HBSS (150 mOsmol/kg) media significantly affected the Vmax but not the Km of the transporter (Figure 5B).

**4. Discussion:**

Mechanisms that underlay 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 further exceeds in fish due to the aquatic environmental conditions and their distinct physiological characteristics. Many teleost fishes are subjected to various environmental changes throughout their life, such as water salinity and temperature that 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 ecophysiology research. Hence, it is necessary to establish appropriate *in-vitro* experimental systems, that will allow to uncover molecular and cellular mechanisms that underlay 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.

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 characterization of transporter paralogs. This expression system allows a more controlled environment and higher experimental reproducibility than *in-vivo* or *in-situ* methods. When using *in-situ* systems such as the previously used inverted gut sac (Bucking and Schulte, 2012; Con et al., 2017), genetic and expression variations arise from individual differences at multiple regulatory levels. These, in turn, increase the number of sacrificed animals necessary to ensure correct statistical analyses and to maintain consistency between experiments. Moreover, 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. The presented expression system address and eliminate these problems and serves as optimal controlled experimental system for fish transporters characterization.

4.2 Homologous vs. heterologous expression systems

While some heterologous expression systems, serve as an adequate *in-vitro* system that allows 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 degree was correlated with higher HSP70 expression (Santos et al., 2023). In addition, the natural membrane environment and its responses to stressors affect transporters activity (Kültz, 2012). It has been shown that membrane lipids composition affected the ATP-binding cassette (ABC) transporter OpuA of bacteria and that this transporter 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.

4.3 Nutrients 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, ranging 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 test the 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 had been shown that differences in cold resistance led to differential expression of temperature-related pathways associated to glucose and amino acids metabolism in tilapia’s gills and liver (Nitzan et al., 2019). Here we tested the temperature effect on the activity of the Mozambique tilapia PepT2 transporter and found that it affects only the Vmax of the transporter. The importance of homologous expression system was clearly demonstrated by Maffia et al. (2003), that showed a significant effect of the experimental system on the transport of small peptides. In their study on the peptide transporter 1 (variant is 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 cellular stress response and from changes in membrane integrity under non-physiological temperatures (Maffia et al., 2003). Our homologues expression systems offer a suitable membrane and intracellular environments, together with the natural wide range of temperature in which the cells grow and function (data not shown), allowing precise evaluation of the nutrients transport under different environmental conditions.

4.4 Nutrients transport under the context of osmotolerance.

Nutrient absorption into cells is driven by 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 part of the nutrients absorption mechanism, although much more common in bacteria. While the transport of small peptides by PepTs isn’t dependent on Na+ directly (Fei et al., 1994), Na+ ion 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 intestine and the kidney epithelia was observed in *in-vivo* and *in-situ* systems, and attributed to the activity of Na+/ H+ antiporter (NHE) that excrete protons from the cells in exchange to Na+ ions (Ganapathy and Leibach, 1983).Studies using *in-vitro* systems of BBMV showed that Na+ ion 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 sodium ions 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-vesicle Na+ concentration (Ganapathy and Leibach, 1983). Therefore, to assess the correct effect of sodium ions 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 organismal 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, the changes of NaCl in the extracellular medium significantly altered PepT2 activity. Moreover, we have seen different effects of hyper- and hypo-osmotic conditions. While the hypo-osmotic conditions decreased the maximal transport rate, the hyper-osmotic condition increased the Km value of the transporter, which indicates a lower affinity of the transporter to the substrate. This different effect likely implies that NaCl influences the PepT2 activity through the cellular osmoregulation responses. These osmotic effects on PepT2 activity are correlated well with the differences of cellular responses to directional osmotic stress (hyper- or hypo-osmotic stress) (Evans and Somero, 2008; Kültz, 2012). Moreover, osmotic stress affects membrane integrity and also had been shown to affect ligand binding of enzymes (Somero, 1986). Our new findings, together with previously discussed inconsistency of peptides transport dependency on Na+ ions, emphasizes the importance of cellular context when addressing the effect of osmotic conditions on nutrients transport.

4.5 Biochemistry and ecophysiology integration

The tilapia PepT2 case study emphasize the tight interaction between the biochemical regulatory level and protein expression in the whole fish and demonstrate how protein function studies raises new insights regarding known physiological phenomena. The effect of the osmotic conditions on the functionality of PepT2 shown in our *in-vitro* system demonstrates how changes in the intestinal lumen conditions further affect the functional properties of PepT2 transporter, and by that, the fish’s peptide absorption and utilization. Our current results correlate and further explain salinity induced distribution changes that we have previously seen in the whole animal (Con et al., 2017). We have demonstrated how salt-water acclimated fish had wider expression patterns of PepT2 along the intestinal tract. In light of the lower affinity of PepT2 under higher salinity levels, this wider expression pattern could be an efficiency preserving mechanism of small peptide absorption in the intestine under these conditions. The osmotoleransce ability of the Mozambique tilapia is reflected in numerous regulation and organizational levels, as well as in the biochemical levels and in their integration (Kültz, 2012). This complex regulation further perturbed into other physiological mechanism such as nutrients absorption, as seen through the PepT2 case study, which will energetically support the fish metabolism and physiology.

The results obtained using this homologous expression system demonstrates the complex regulation on PepT2 transporter in fish, and emphasize the impact that environmental changes have on fish peptide transport. Considering the complexity of fish physiology and ecophysiology, this system brings a new tool to study molecular and cellular mechanisms explaining environmental physiology, fitness and adaptation to different habitats.

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**Author contribution:**

P.C, A.C, and D.K conceived and designed the experiments. P.C, J.H and J.B contributed to plasmids design. P.C cultivate, modified and established the modified cell line. P.C performed all uptake experiments, analyzed the data and preformed statistics analysis. P.C, A.C, D.K and J.B wrote the paper.

**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 Part 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 - A 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, 19917–24.

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 (80- ) 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 A Mol Integr Physiol 162, 245–51. 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

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

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

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., 2021a. 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

Root, L., Campo, A., MacNiven, L., Con, P., Cnaani, A., Kültz, D., 2021b. 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, 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

Somero, G.N., 1986. Protons, osmolytes, and fitness of internal milieu for protein function. Am J Physiol 251. https://doi.org/10.1152/ajpregu.1986.251.2.r197

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

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 - A 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

**Data accessibility**

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.

**Figure 1:** Graphical illustration of the uptake assay protocol. Created with BioRender.com.

**Figure 2:** PepT2 functional validation test. Tmb-cells and PepT2-Tmb cells post 2 hours 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.

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

**Figure 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 α=0.05 of the ANOM analysis.

**Figure 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 α=0.05 of the ANOM analysis.