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BARD Project Number: IS-4296-10

Date of Submission of the report: July 14, 2014

Project Title:

Gastrointestinal osmoregulatory activity in tilapia and its effect on growth, an opportunity for fish diet development

Investigators

Institutions

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Prolactin, Transporters, Intestine, Salinity, *Oreochromis*

Abbreviations commonly used in the report, in alphabetical order:

CFTR	cystic fibrosis transmembrane conductance regulator
FW	freshwater
GO	gene ontology
GH	growth hormone
HL	hepatic loop
IGF	insulin growth factor
IGFBP	insulin growth factor binding proteins
NKA $\alpha 3$	Na ⁺ -K ⁺ -ATPase alpha3
NCC	Na ⁺ -Cl ⁻ co-transporter
NKCC	Na ⁺ -K ⁺ -Cl ⁻ co-transporter

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PRL	prolactin
PRLR	prolactin receptor
PepT	peptide transporter
PMC	proximal major coil
SMR	standard metabolic rate
SW	seawater
TS	terminal segment
V-ATPase	vacuolar type H ⁺ -ATPase

Budget: IS: \$160,000

US: \$155,000

Total: \$315,000

C. 2nd

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (Numbers of publications, and in square brackets are the numbers of items in the list of publication)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	1 [7]	6 [1-6]		7
Submitted, in review, in preparation	1 [11]	4 [9,13-15]	3 [8,10,12]	8
Invited review papers				
Book chapters				
Books				
Master theses			2 [16,17]	2
Ph.D. theses		1 [18]		1
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Enbal Ben-Tal Cohen

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	2	1		3
Longer Visits (Sabbaticals)				None

Description Cooperation:

Experimental fish feeds designed and produced by the Israeli team that had been shipped to the American team in Hawaii were employed in the experiments designed to evaluate the effects of NaCl on growth rate. While visiting in the Hawaii Institute of Marine Biology Avner Cnaani learned the technique of hypophysectomy, and presented the genomic analysis in a departmental seminar. During October 2013, a jointly organized workshop entitled “Endocrinology of Cichlids” was carried out in Ein Harod, Israel, and at the 10th International Symposium on Tilapia in Aquaculture was carried out in Jerusalem. During this period, results from both Israeli and American teams were presented to a wide audience from the academia and the industry, and further directions on collaborative research were discussed. This project

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promoted an open communication route between the two groups, with mutual visits that facilitated interactions with students and post-docs from both sides.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				None
Issued (allowed)				None
Licensed				None

Abstract

Fish living in freshwater and seawater environments experience constant osmotic pressure between their internal body and the surrounding water. Regulation of ion and water balance under these conditions is highly energetic demanding, and eventually, affects the fish growth. While the role of the gills in osmoregulation was extensively studied, the osmoregulatory activity of the gastrointestinal tract is less known. In this study we characterized the tilapia intestine as a multifunctional organ, having a role in both nutrition and in ion regulation. We studied the pituitary endocrine regulation of intestinal salinity adaptation, the salinity-dependent physiological activity along different intestinal sections, and specific genes that are linking nutrient absorption with ion and acid-base regulation. The results of this study indicate that different intestinal sections developed various specific activities. Their endocrine regulation is now better understood, a large data-set of salinity dependent gene transcript was developed, as well as new tools and methods to study new aspects of intestinal physiology.

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Evaluation of major achievements

In order to study the relationship between osmoregulatory activity and fish growth two different directions were taken in this research. The American partners conducted in depth studies on pathways and genes previously known to have a role in tilapia's osmoregulation, while the Israeli partners screened for novel genes and conducted first studies to characterize their salinity dependent expression.

At the first part of the study we searched for the intestinal targets of pituitary hormones [7]. In light of the observed parallel expression of ion transporters and PRLRs along the intestine of FW- and SW-acclimated fish (Figs. 2, 3 and 4 in [7]), we tested whether PRL may regulate ionoregulatory genes. By employing our established experimental model of hypophysectomy and hormonal replacement, we examined the role of PRL on the mRNA expression of the ion transporters NKA $\alpha 3$, NKCC, V-ATPase, CFTR, PRLR1 and PRLR2 (Fig. 1). The expression of NKCC2 in the PMC and TS was diminished following hypophysectomy and restored by PRL injection (Fig. 1C). The regulation of NKCC2 by PRL, therefore, provides a new molecular target for PRL that participates in salt and water balance. On the other hand, although CFTR mRNA levels were suppressed by hypophysectomy in the HL and TS, they were not restored by PRL (Fig. 1D), suggesting that another pituitary-derived or pituitary-dependent factor regulates this Cl^- channel. It is worth noting that while intestinal NKCC2 gene expression was clearly dependent on pituitary PRL, this was not the case for other transcripts examined in this study, including PRLR1. This differs from the regulatory pattern that is observed in the gill, where PRL up-regulates PRLR1 gene expression. Overall, these differences in sensitivity to PRL underscore the complexity of organ-specific endocrine control of osmoregulation. This experiment has been recently submitted for publication to *General and Comparative Endocrinology*.

We also tested whether a high salt diet (3% NaCl) affects growth in FW- and SW-acclimated tilapia. Testing previous findings that SW fish grow faster than FW-acclimated tilapia, we hypothesized that tilapia fish fed a high salt diet would grow faster over time than fish that were fed a normal diet. Surprisingly, we found that FW fish fed a high salt diet did grow slower than those fed the control diet or those fish kept in SW (Fig. 2A). This suggests that the higher dietary salt intake may suppress

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metabolism. Based on these results, we tested whether the SMR of fish acclimated in SW or transferred from FW to SW was higher than that of fish kept in FW. The metabolic rate data shows higher SMR in SW or FW-SW groups compared with the FW and SW-FW groups, supporting the notion that the osmoregulatory cost in SW is higher than that of FW (Fig. 2B; [9]). It is possible, therefore, that the high-salinity induced increase in metabolic rate is associated with the lower growth rate observed in FW-fish fed a high salt diet. Nonetheless, growth in SW fish was not suppressed, whether they were fed a high salt diet or not. This apparent paradox with the high metabolic rate observed in SW-acclimated fish or those transferred from FW to 75% SW, warrants further investigation. Beyond the proposed objectives we also reported on: 1) the endocrine factors that regulate PRL gene expression and release in Mozambique tilapia [1]; 2) the pituitary control of branchial NCC, NKCC and NKA α -subunit in Nile tilapia [2]; 3) the effects of nutritional status and GH on the gene expression of IGFs and their IGFbps [3]; 4) the effects of rearing tilapia under a tidally-changing salinity on the functional morphology of branchial ionocytes [4]; 5) the inhibitory effect of PRL on leptin synthesis and secretion [5] and 6) the effects of acclimation salinity on the gene expression of selenoproteins in Mozambique tilapia [6].

Aiming to detect novel genes, not known before for their role in tilapia's osmoregulation, we sequenced the transcriptome of two intestinal sections (anterior and posterior intestine) in fish adapted to fresh and salt water. Furthermore, trying to understand the differential salinity tolerance among different tilapia species, this transcriptome sequencing was done comparatively in the high salinity tolerant *O. mossambicus* (Mozambique tilapia), and the low salinity tolerant *O. niloticus* (Nile tilapia). This comparative analysis revealed high similarity in gene expression response to salinity changes between species in the posterior intestine and large differences in the anterior intestine (Figures 1 and 4 in [8]). Furthermore, in the anterior intestine 68 genes were saltwater up-regulated in one species and freshwater up-regulated in the other species (Figure 2 in [8]). GO analysis showed a high proportion of transporters and ion channels activities among these genes. These results point to the anterior intestine, and the group of genes that differed in their salinity-dependent regulation pattern, as potentially having a role in the differential

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salinity tolerance of these two closely related species [8,16]. This experiment has been recently submitted for publication to *Open Biology*.

From the results of the transcriptome study we have selected several genes for further investigation of their dual role in salinity adaptation and food digestion. These genes included three peptide transporters: PepT1a (SLC15A1a), PepT1b (SLC15A1b), PepT2 (SLC15A2), and two amino-acid transporters: B^{0,+}AT1 (SLC7A9), B⁰AT1 (SLC6A19). We have characterized their salinity-dependent expression along different intestinal sections, in different time points of the food digestion cycle [12,17]. The PepT1 genes were mainly located in the anterior and middle intestine and with the highest expression a few hours after feeding, and PepT2 were mainly located in the posterior intestine and the kidney, with the highest expression 24 hours after feeding (Figure 3). Antibodies for these genes were developed and used for immunohistochemical analysis that physically localized them on the brush borders of the enterocytes (Figure 4). Additionally, we have found a correlation between expression patterns of these genes and those of sodium and proton transporters, leading to future plans on studying the effect of pH on nutrient absorption.

The above-described transporters, which are involved in nutrient absorption, are dependent on the transfer of proton and sodium ions across the enterocyte membrane. These ions transfer depends, in part on the electric potential between the gut lumen, the epithelial cell, and the inter-cellular matrix. Potassium ions have a key role in regulation of membrane potential. Several genes involved in maintaining membrane potential were detected in the transcriptome sequencing. We have further characterized the gene expression and protein localization of two inward rectifier potassium channels (KCNJ15, KCNJ16) that were not yet studied in teleost [10], and of an array of cation-chloride transporters (NKCC, NCC, KCC) which are more known for their role in osmoregulation [11]. Expression patterns of KCNJ15 and two variants of KCNJ16 were analyzed along the digestive tract (esophagus, stomach, and different intestinal sections), as well as in other osmoregulatory organs, such as the gills, kidney and opercular membrane. The results demonstrated the salinity-dependent expression, and the organ specific expression of these two potassium channels, and of the two variants of KCNJ16 (Figure 5). Antibodies for the two potassium channels genes were developed and used for immunohistochemical

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analysis that physically localized the KCNJ15 gene on apical membrane and the KCNJ16 on the basolateral membrane of epithelial cells (Figure 6).

Summary

This research expanded the knowledge on the interaction between the pituitary control on osmoregulatory pathways and intestinal physiology. The effects of PRLs secreted from the pituitary on receptors, transporters, and ion channels, were characterized under different environmental conditions related to osmotic stress. Additionally, by adopting new technology (deep-sequencing) that was not available for us when this project was initiated, novel genes that were not previously studied for their role in osmoregulation were characterized and analyzed for their salinity-dependent activity. This study is one of the first to characterize in details the salinity-dependent physiology of the tilapia intestine, and its endocrine regulation.

Following our objective to study the relationship between intestinal osmoregulatory activity and fish growth, we have focused on genes related to nutrition (peptide and amino acid transporters), and to regulation of metabolism (GH, IGF, and their related peptides). We anticipate that the tools developed to study these genes, and the knowledge gained on their regulation under different environmental conditions in specific intestinal sections, will further promote research and development aiming for increased food utilization, as was the original aim that governed this study. The results of this study were already published in 7 manuscripts in peer-reviewed scientific journals, with 8 more submitted or under preparation. This research supported 3 graduate students and one post-doc, and was a basis for a jointly organized international workshop that attracted researchers, students, and representatives from the aquaculture industry.

List of publications

Papers in refereed journals

1. Seale A.P., Yamaguchi Y., Johnstone W.M., Borski R.J., Lerner D.T., Grau E.G., 2013. Endocrine regulation of prolactin cell function and modulation of osmoreception in the Mozambique tilapia. *Gen Comp Endocrinol*, 192:191-203.

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2. Breves J.P., Seale A.P., Moorman B.P., Lerner D.T., Moriyama S., Hopkins K.D., Grau E.G., 2014. Pituitary control of branchial NCC, NKCC and Na⁺, K⁺-ATPase α -subunit gene expression in Nile tilapia, *Oreochromis niloticus*. *J Comp Physiol B*, 184:513-523.
3. Breves J.P., Tipsmark C.K., Stough B.A., Seale A.P., Flack B.R., Moorman B.P., Lerner D.T., Grau E.G., 2014. Nutritional status and growth hormone regulate insulin-like growth factor binding protein (igfbp) transcripts in Mozambique tilapia. *Gen Comp Endocrinol*, doi: 10.1016/j.ygcen.2014.04.034.
4. Moorman B.P., Inokuchi M., Yamaguchi Y., Lerner D.T., Grau E.G., Seale A.P., 2014. The osmoregulatory effects of rearing Mozambique tilapia in a tidally changing salinity. *Gen Comp Endocrinol*, doi: 10.1016/j.ygcen.2014.03.013.
5. Douros J.D., Baltzegar D.A., Breves J.P., Lerner D.T., Seale A.P., Grau G.E., Borski R.J., 2014. Prolactin is a major inhibitor of hepatic Leptin A synthesis and secretion: Studies utilizing a homologous Leptin A ELISA in the tilapia. *Gen Comp Endocrinol*, doi: 10.1016/j.ygcen.2014.03.007.
6. Seale L.A., Gilman C.L., Moorman B.P., Berry M.J., Grau E.G., Seale A.P., 2014. Effects of acclimation salinity on the expression of selenoproteins in the tilapia, *Oreochromis mossambicus*. *J Trace Elem Med Biol*, 28:284-292.
7. Seale A.P., Stagg J.J., Yamaguchi Y., Breves J.P., Soma S., Watanabe S., Kaneko T., Cnaani A., Harpaz S., Lerner D.T., Grau E.G., 2014. Effects of salinity and prolactin on gene transcript levels of ion transporters, ion pumps and prolactin receptors in Mozambique tilapia intestine. *Gen Comp Endocrinol*, under revision.

Submitted or in preparation

8. Ronkin D., Seroussi E., Doron-Faigenboim A., Cnaani A., 2014. Intestinal transcriptome analysis revealed differential salinity adaptation between two tilapiine species. Submitted.
9. Zikos A., Seale A.P., Lerner D.T., Grau E.G., Korsmeyer K.E., 2014. Effects of salinity on metabolic rate and branchial expression of genes involved in metabolism and mitochondrial biogenesis in Mozambique tilapia (*Oreochromis mossambicus*). Submitted.

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10. Ben-Tal Cohen E., Seroussi E., Genin O., Cnaani A., 2014. Expression and localization of the inward rectifier potassium channels KCNJ15 and KCNJ16 in tilapias' epithelial cells. In preparation.
11. Ben-Tal Cohen E., Ronkin D., Seroussi E., Seale A.P., Lerner D.T., Grau E.G., Harpaz H., Cnaani A., 2014. Salinity effect on tilapias chloride-cation transporters expression in the gastro-intestinal tract. In preparation.
12. Nitzan T., Rozenberg P., Cnaani A., 2014. Salinity-dependent expression of amino-acid transporters throughout the digestive cycle of Mozambique tilapia. In preparation.
13. Yamaguchi Y., Lerner D.T., Grau E.G., Seale A.P., 2014. Acute salinity tolerance and the control of Prolactin177 and Prolactin188 release and gene expression by extracellular osmolality in the Nile tilapia: a comparative approach. In preparation.
14. Inokuchi M., Breves J., Moriyama S., Watanabe S., Kaneko T., Lerner D.T., Grau E.G., Seale A.P., 2014. Prolactin 177, prolactin 188 and extracellular osmolality independently regulate the expression of ion transport effectors in the gills of Mozambique tilapia. In preparation.
15. Moorman B.P., Lerner D.T., Grau E.G., Seale A.P., 2014. The effects of acute salinity challenges on osmoregulation in Mozambique tilapia reared in a tidally changing salinity. In preparation.

Theses

16. Ronkin D., 2014. *Comparative genomic analysis of tilapias osmoregulation*. Thesis in preparation in partial fulfillment of the requirements for M.Sc. degree in Animal Sciences at the Hebrew University of Jerusalem.
17. Rozenberg P., 2014. *Salinity dependent peptide transporters activity in the tilapia gastro-intestinal tract*. Thesis in preparation in partial fulfillment of the requirements for M.Sc. degree in Animal Sciences at the Hebrew University of Jerusalem.
18. Moorman B., 2014. *Rearing Mozambique tilapia in a tidal environment: effects on osmoregulation and growth*. Thesis in preparation in partial fulfillment of the requirements for Ph.D. degree in the Department of Molecular Biosciences and Bioengineering at the University of Hawaii.

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Appendix

1. Published papers

Reprints of the six papers that were published, and on which BARD was acknowledged are included as *.pdf files.

2. Submitted papers

Copy of one submitted paper is also attached as *.pdf files.

3. Figures and tables of unpublished results

Figure 1: Effects of hypophysectomy (Hx) and PRL replacement (Hx: PRL) on (A) NKA α 3, (B) V-ATPase A-subunit, (C) NKCC2, (D) CFTR, (E) PRLR1, and (F) PRLR2 mRNA levels in the hepatic loop (HL), proximal major coil (PMC) and terminal segment (TS) of tilapia acclimated to BW (12‰). Bars represent means $\pm$ S.E.M. (n=6–7). Expression levels are presented as the ratio of target genes to the housekeeping gene 18S. Within each intestinal segment, differences among treatment groups were evaluated by one-way ANOVA followed by Fisher's LSD test (P<0.05). Means not sharing the same letter are significantly different from each other.

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Figure 2: (A) Effects of dietary salt content on growth of FW and SW-acclimated tilapia over a 33-day period (n= 14-24). B) Post-transfer Standard Metabolic Rates (SMR, n= 19-21). Metabolic rates were measured via respirometry as oxygen consumption rates (mg O₂/kg/h) recorded overnight, between 6 and 18 h following salinity transfer. Treatments for this experiment were (1) *FW-control*: FW-reared tilapia remained in FW (0.1 psu) (2) *FW-75% SW*: FW-reared tilapia following transfer to 75% SW (25 psu), (3) *SW-control*: SW-reared adult male tilapia remained in SW (35 psu), and (4) *SW-FW*: SW-reared tilapia following transfer to FW. Significant differences among treatments are indicated by different letters. Error bars shown represent 1 SE (One-Way ANOVA, Games-Howell test, $p < 0.05$).

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Figure 3: Gene expression analysis, using qPCR, of the PepT1a gene in the anterior intestine, and the PepT2 gene in the posterior intestine, at three time points after feeding.

Figure 4: Immunohistochemistry localization of the PepT2 gene on the apical side of the posterior intestine enterocytes.

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Figure 5: Comparative gene expression analysis, using qPCR, of the KCNJ15 gene and two variants of KCNJ16. Expression was measured if fresh (FW) and sea (SW) waters grown *O. mossambicus*, in the esophagus, stomach, anterior intestine, middle intestine, posterior intestine, kidney, gills, skin of the opercular membrane, and heart tissues.

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Figure 6: Immunohistochemistry localization in the anterior intestine of the KCNJ15 (A and B) and KCNJ16 (C and D) genes (red), double stained with antibodies to the NKCC (A and D) and $\text{Na}^+\text{-K}^+\text{-ATPase}$ (B and C) genes (green). KCNJ15 and NKCC are co-localized on the apical membrane, while KCNJ16 and NKA are co-localized on the basolateral membrane.