IMMUNOLOCALIZATION AND CHANGES IN EXPRESSION LEVELS OF GLYCEROPORIN HC-3 IN SEVERAL TISSUES OF GRAY TREE FROGS, *HYLA CHRYSOSCELIS* UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Yaganti, Sushmita. M.S. Program in Biological Sciences, Wright State University, 2009. Immunolocalization and changes in expression levels of glyceroporin HC-3 in several tissues of gray tree frogs, *Hyla chrysoscelis* under different physiological conditions.

Previous studies show that aquaporin HC-3 in gray tree frogs, *Hyla chrysoscelis*, a homolog of mammalian AQP3 is an aqua glyceroporin also responsible for freeze tolerance. Our study tried to localize and compare the protein expression levels of aquaporin HC-3 in seven different organs of gray tree frogs under five different physiological conditions using immunohistofluorescence and western blot. HC-3 was immunolocalized in the epidermis and dermis of the skin, basolateral portion of the collecting ducts in the kidney, red blood cells in the liver and other tissues, muscle Schwann cells and the basolateral portion of the large intestine epithelial membrane. HC-3 cannot be immunolocalized in the stomach and the small intestine tissues. The basolateral expression of aquaporin HC-3 in the epidermis of the skin is similar to the expression in the mammalian skin. The intensity of the labeling was different in the various physiological conditions. There was a noticeable increase in the intensity of the HC-3 expression in warm (hydrated, 23°C, control) skin, kidney and liver compared to dehydrated
(loss of 20% of standard body mass over one week at 23° C) and the cold
(hydrated 4° C). The intensity of labeling in large intestine had varied
pattern with more expression in the warm, slightly less in the cold and
absolutely negligible in the other conditions. The up-regulation of the
aquaporin HC-3 protein expression in the dehydrated skin and the kidney
may be due to its regulated role in the water conservation whereas its up-
regulation in the cold liver can be due to synthesis and release of glycerol in
this organ.

Keywords: Aquaporin, aquaglyceroporin, skin, kidney, liver, muscle,
stomach, intestine, *Hyla chrysoscelis* (Gray tree frog).
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1.0 Literature Review

1.1 Introduction

Discovery of aquaporin channels was a matter of chance when during isolation of the 32 kD transmembrane component of the red cell Rh blood group antigen, a 28 kD polypeptide was found (Denker et al., 1988). This protein had a hydrophobic composition and was found in N-glycosylated form. This channel was highly water permeable and found in abundance in erythrocytes and renal membranes. It was designated AQP1 (Agre, 1997). Functional characteristic of the first aquaporin was reported in 1992 by Dr. Peter Agre for which he was awarded the Nobel prize in 2003.

Aquaporins are a large family of integral membrane proteins that have a similar structure and transport water across cell membranes. There are a large number of members in aquaporin family which are found in a wide variety of organisms, including humans, bacteria and plants. In humans, 13 different aquaporins (AQP0-12) have been discovered. This ubiquitous presence suggests that aquaporins are involved in fundamental biological processes. Many aquaporins like human AQP-1, -2, -4, -5, -6 are exclusive water channels and are not permeable to ions or other small molecules. Other aquaporins, like AQP-3, -7, -9, -10 are called
aquaglyceroporins, and can transport water along with glycerol and some other small molecules like urea (Ishibashi et al. 2000; Agre, 2006).

These water channels play a very important role in osmoregulation. Thus it is not surprising that several aquaporins are expressed in kidneys. In mammals, AQP1, originally isolated in red blood cells (Preston & Agre, 1991), has been found on the renal tubules, apical and basolateral membranes and Bowman’s capsule of the kidney (Brown et al., 1995). AQP2 expression is localized in the apical membranes of the principal cells in the renal collecting ducts (Fushimi et al., 1993). On the other hand, AQP3 (an aquaglyceroporin) and AQP4 are expressed in the basolateral membrane of renal collecting ducts (Frigeri et al., 1995). AQP3 coexists with AQP2 in the same cells of the renal collecting ducts and helps in the basolateral exit of water which enters the principal cell apically through AQP2 (Rojek et al., 2008).

Studies proved the presence of various aquaglyceroporins like AQP3, -7, -9 and -10 in different cells of the mammalian skin (Boury-Jamot et al., 2006). Studies of AQP3-knockout mice showed significant reduction in both osmotic water permeability in the epidermal cells and glycerol permeability of keratinocytes with normal glycerol concentration in the dermis suggesting reduced glycerol transport from the blood into the epidermis in AQP3-
knockout mice (Hara & Verkman, 2003). AQP7 which was originally cloned from testis (spermatocytes) and human white adipose tissue was also localized in many other tissues like the brush border of the proximal tissue in kidney (Ishibashi et al., 2000; Skowronski et al., 2007) and pancreatic β-cells (Matsumura et al., 2007). In muscle tissue, AQP7 was localized in the capillaries of skeletal and the cardiac muscle but not as extensive as in the adipose tissue (Skowronski et al., 2007). In vivo studies suggested an increase in AQP7 mRNA in adipose tissues after a long period of fasting along with decrease in insulin levels which accelerates lipolysis and in turn increases glycerol and free-fatty-acid release from the adipose tissue (Kishida et al., 2000). All these metabolic changes in AQP7 dysregulation lead to an increase glycerol levels for hepatic gluconeogenesis and hence increase glucose levels in type II diabetes (Rojek et al., 2008). Studies of AQP7-knockout mice suggested increased triglyceride concentrations and glycerol levels in the islet cells of pancreas, increased β-cell glycerol kinase activity and decreased insulin content (Matsumura et al., 2007).

Another aquaglyceroporin, AQP9, is expressed in the liver, epididymis and skin in many species. Additionally, in humans AQP9 mRNA was detected in peripheral leucocytes and organs which accumulated leucocytes like lung, spleen and bone marrow (Rojek et al., 2008; Ishibashi
et al., 1998). Immunolocalization studies showed the presence of AQP9 in perivenous hepatocytes and in periportal hepatocytes (Rojek et al. 2007; Carbrey et al. 2003). Unlike many other aquaporins expressed in the liver hepatocytes, AQP9 is the only one localized basolaterally (Huebert et al., 2002). The various studies on the expression of AQP9 mRNA in rat and mouse liver under different metabolic conditions suggested an increase in the AQP9 protein and mRNA levels after prolonged fasting and a decrease after refeeding (Kuriyama et al., 2002; Carbrey et al., 2003). AQP9 is also considered an important aquaporin like AQP3 in maintaining proper skin hydration and elasticity (Rojek et al., 2007; Hara et al., 2002). The basolateral localization of AQP9 in the mouse small intestine is in contrast to its apical localization in hepatocytes, keratinocytes and epididymis (Okada et al., 2003).

Amphibians like Cope’s gray tree frogs (Hyla chrysoscelis) are likely to be at risk of dehydration and so we might expect a role for aquaporins in conservation of water. For many years researchers have known that the water transport in the pelvic skin and the urinary bladder of amphibians are arginine vasotocin (AVT) dependent. More recently, studies of another tree frog, Hyla japonica, have revealed that AVT induces expression of two
aquaporins, designated AQP h2 and AQP h3 in the pelvic skin and AQP h2 in the urinary bladder (Hasegawa et al., 2003).

Cope’s gray tree frogs express aquaporins homologous to mammalian AQP1, AQP2 and AQP3. Like AQP3, HC-3 is an aquaglyceroporin channel that can transport both water and glycerol along with some other solutes like urea. Researchers detected of AQP3 homologs in eight other mammalian species and bacteria (Rojek et al., 2008). Aquaporin HC-3 found in *Hyla chrysoscelis* has 94% amino acid and 82% nucleotide similarity with the mammalian AQP3 (Zimmerman et al. 2007). AQP3 expression in the kidney is localized in the principal cells of collecting ducts, basolateral plasma membranes of connecting tubule and inner medullary collecting duct cells (Coleman et al., 2000; Ecelbarger et al. 1995). We hypothesized that the aquaglyceroporin channels in addition to the above osmoregulatory role may help in the distribution of glycerol. Glycerol acts as cryoprotectant (Layne & Jones, 2001) in this species that tolerates freezing as a key adaptation for surviving subfreezing body temperatures during winter. Studies showed a threefold decrease in the water permeability of the basolateral membrane of the collecting ducts in AP3-knockout mice compared to the wild-type control mice (Ma et al., 2000). There was a tenfold increase in the daily
urine output of the knockout mice compared to the controls (Rojek et al., 2008).

The current study hypothesized that glyceroporin HC-3 plays an important role in the water conservation and maintains cell integrity in different physiological conditions such as dehydration, cold and freezing and its expression levels vary accordingly in Cope’s gray tree frogs, *Hyla chrysoscelis*. In situ hybridization and immuno histochemistry were used to test this hypothesis.

**1.2 Structure and classification of aquaporins**

Aquaporins possess six membrane spanning regions with five connecting loops; the N- and C-termini are located intracellularly. There are two highly conserved sequence motifs, asparagine-proline-alanine (NPA) in the loops “B” and “E” (Jung et al., 1994b). They are part of short 10-15 amino acid long hydrophobic regions. These fold back partially and build together as two half-loops to form the actual water pore. At position 189 in AQP-1 (also in AQP-2, -5 and -6) a cysteine residue is present near to the second NPA-motif. Binding of mercury ions at this cysteine leads to a blockade of the pore (Preston et al., 1993).
Based on the gene structure aquaporins are divided into three subfamilies:

Class 1: In this group all the aquaporins are water selective- AQP0, 1, 2, 4, 5, 6, and 8. Some studies showed that AQP6 and 8 also transport anions and free radicals like H$_2$O$_2$ (Ishibashi et al., 2008).

Class 2: Also named aquaglyceroporins, this class of
aquaporins which includes AQP3, 7, 9, 10 is permeable to small neutral solutes such as glycerol and urea in addition to water (Bienert at al., 2008).

**Class 3:** This class of AQPs which includes AQP11 and AQP12 belongs to a new Subfamily called as “Superaquaporins” and has two deviated NPA motifs in its gene structure. Studies about their functions show that AQP11 has limited water transport activity (Yakata et al., 2007).

### 1.3 Different Types of Aquaporins and Their Functions

<table>
<thead>
<tr>
<th>AQUAPORIN</th>
<th>EXPRESSED IN</th>
<th>ROLE</th>
<th>CLINICAL IMPORTANCE?</th>
<th>NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP0</td>
<td>Lens</td>
<td>Confers low permeability</td>
<td>Mouse mutants suffer congenital cataracts</td>
<td>Not inhibited by Hg</td>
</tr>
<tr>
<td>AQP1</td>
<td>Kidney nephrons; eye; capillary endothelia and almost in all the tissues with few exceptions</td>
<td>Primarily water reabsorption</td>
<td>No apparent change in phenotype in deficient humans</td>
<td>The first AQP found</td>
</tr>
<tr>
<td><strong>AQP2</strong></td>
<td>Principal cells of the renal collecting duct</td>
<td>not constitutive; regulated by vasopressin through short-term exocytosis</td>
<td>Nephrogenic DI has been associated with AQP2 mutants; may often be an indicator of other disease states</td>
<td>Vasopressin action involves adenylyl cyclase and phosphorylation of the C-terminus of AQP2</td>
</tr>
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<td>---</td>
</tr>
<tr>
<td><strong>AQP(G)P3</strong></td>
<td>Kidney, skin liver, muscle, GI tract, eye, brain, heart, ovary, salivary gland, spleen, erythrocytes</td>
<td>Renal water reabsorption?</td>
<td>Water conservation, maintain skin hydration and elasticity</td>
<td>Is somewhat permeable to urea; may be vasopressin-regulated</td>
</tr>
<tr>
<td><strong>AQP4</strong></td>
<td>Brain; optic nerve; fast-twitch muscle in rats, eye, salivary gland, GI tract, kidney, muscle</td>
<td>As an exit port for excess brain water; to restore osmotic balance in other tissues</td>
<td>Excess brain water can be lethal in cerebral edema</td>
<td>Is not affected by Hg; may be actively regulated by PKC</td>
</tr>
<tr>
<td><strong>AQP5</strong></td>
<td>Apical membranes of respiratory pathways, eye, GI tract, ovary</td>
<td>Involved in airway humidification, and the release of saliva and tears</td>
<td>Possible gene therapy agent; its gene has been engineered into an adenoviral vector</td>
<td></td>
</tr>
<tr>
<td><strong>AQP6</strong></td>
<td>Kidney, brain</td>
<td>?</td>
<td>?</td>
<td>Shows low permeability to water</td>
</tr>
<tr>
<td><strong>AQP(G)7</strong></td>
<td>Spermatids and seminiferous</td>
<td>A port for water and glycerol</td>
<td>?</td>
<td>The human homologue may be</td>
</tr>
<tr>
<td>Protein</td>
<td>Localization</td>
<td>Function</td>
<td>Expression</td>
<td></td>
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<td>---------</td>
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</tr>
<tr>
<td>AQP8</td>
<td>Testis; pancreas; liver; colon; trachea, salivary gland, ovary</td>
<td>Unclear. May be permeable to water and urea</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>AQ(G)P9</td>
<td>Leucocytes, liver, spleen, ovary, muscle</td>
<td>Appears to transport urea but not glycerol</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>AQ(G)P10</td>
<td>Duodenum and jejunum</td>
<td>Transports urea, glycerol and water</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>AQP11</td>
<td>Kidney, liver, testis, brain, pancreas, spleen, GI tract, ovary, muscle, leukocyte</td>
<td>Unknown</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>AQP12</td>
<td>Pancreas (acinar cells)</td>
<td>Unknown</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

(Ishibashi et al., 2008)
1.4 Classification of Tree Frogs

KINGDOM-ANIMALIA  
PHYLUM-CHORDATA  
SUBPHYLUM-VERTEBRATA  
CLASS-AMPHIBIA  
ORDER-ANURA (FROGS AND TOADS)  
FAMILY-HYLIDAE  
GENUS-HYLIA  
SPECIES- *Hyla versicolor* (common gray treefrogs) and *H.chrysoscelis*  
(Cope’s gray treefrogs)

1.5 Freeze Tolerance in Tree Frogs

Freeze tolerance is a key adaptation for species that routinely experience subfreezing body temperatures during winter. This physiological adaptation normally helps animals to survive extremely low temperatures of the body during normal winter seasons during which ice formation occurs within the body fluids (Croes & Thomas, 2000). Among the small number of freeze-tolerant vertebrates that have been identified are several species of frogs. The ability of these frogs to survive freezing is aided by the accumulation of cryoprotectants. All of the freeze-tolerant frogs use glucose liberated at the onset of freezing as the primary cryoprotectant. In addition, the sister species, *H.versicolor* and *H.chrysoscelis* use glycerol. Glycerol is used in many biomedical procedures as a cryoprotectant for freezing of cells.
at ultralow temperatures without damaging the cell integrity (Krane & Goldstein, 2007).

Freeze-tolerant frogs derive their cryoprotectants primarily from liver glycogen (Schmid, 1982). In response to freezing frogs mobilize glucose directly from hepatic glycogen stores, although limited glycogenolysis in frogs is also induced by the dehydration stress. Their freeze tolerance with respect to the low temperature and freeze duration is promoted by massive hyperglycemia (Storey & Storey, 1984; Costanzo et al., 1993a). Additional freeze tolerance is seen by selective gene expression (Storey, 1998). Freeze-tolerant frogs universally mobilize glucose upon freezing of their body tissues, whereas *H. versicolor* and *H. chrysoscelis* can mobilize glycerol with some changes in their internal conditions.

Gray tree frogs have the ability to retain high plasma glycerol levels for weeks before and after freezing in contrast to wood frogs in which glycogenolysis is stimulated by the onset of freezing and glycogenesis starts immediately upon thawing (Krane & Goldstein, 2007). It was seen that the increase in glucose at the time of freezing was accompanied by decline in liver glycogen. The increase in plasma glucose, along with liver glucose and glycerol at the time of freezing, suggests the cryoprotectant role of these compounds, with glucose as the primary component (Costanzo et al., 1995;
Storey and Storey, 1996). Freeze-tolerant frogs accumulate cryoprotectants during the initial period of freezing and ice formation and the cryoprotectants formed in the liver are rapidly distributed to all the body organs via blood (Storey, 1987).

Cryoprotectants, like glucose and glycerol, are increased in the freeze-tolerant frogs during the process of freezing (Costanzo et al., 1995; Storey and Storey, 1996). Exposure to subfreezing temperature results in a significant increase in almost all the enzymes in the liver. This can be due to two reasons: 1) an overall increase in the protein synthesis in the liver of freeze-tolerant frogs which is a long term process; or 2) an overall dehydration of the tissue (Churchill & Storey, 1993). However, the large increase in the rate of glycogen breakdown to polyols and mono-saccharides seen at low temperatures is mediated by temperature effects on enzyme kinetic properties, rather than by changing expression levels of enzymes or expression of new isoforms (Lee, 1991).

1.6 Hypothesis

During cold acclimatization, H.chrysoscelis anticipates freezing and utilizes glycerol as a cryoprotectant. Glycerol helps maintain the protein structure and cellular integrity at the time of freezing. Liver acts as a source
of cryoprotectant by providing glycerol during the time of freezing. Skin helps in maintaining the water balance in the body by moving water in and out with the help of aquaporin channels present in the ventral pelvic skin. The kidney also helps in osmoregulation by movement of water molecules through the aquaporin channels. Some aquaporin channels allow the movement of both water and glycerol through them.

We hypothesize that there is an up-regulation of aquaporin channels by new translation and transcription during cold acclimatization and freezing. AQP3 is an aqua glycerol channel which is present in both the kidney liver and many other organs. It helps in the transport of glycerol and water and is up regulated during cold acclimatization and freezing. We used in situ hybridization and immunohistochemistry to test the localization and difference in the expression level of HC-3 different tissues of gray tree frogs. The intention was to use in situ hybridization to examine expression and localization of mRNA in response to cold acclimatization, and to use immunohistochemistry to test whether protein expression is increased in cold acclimatization and dehydrated state.
1.7 Methods used to test the hypothesis

1.7.1 In Situ Hybridization

In situ Hybridization is a method of detecting and localizing in tissues or cells specific mRNA sequences by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest. The sensitivity of the technique is such that the threshold levels of detection are in the region of 10-20 copies per mRNA per cell.

1.7.2 Immunohistochemistry

Immunohistochemistry helps in the localization of antigens in tissue sections with the help of antibodies as specific reagents through antigen-antibody interactions which can be visualized by a marker like enzyme, fluorescent dye, radioactive element or colloidal gold. The antibodies used for specific detection can be monoclonal or polyclonal. Monoclonal antibodies have more specificity than polyclonal. There are two methods used in immunohistochemistry, the direct method and the indirect method. By using immunohistochemistry, the aquaporin protein expression in tubules of kidneys, skin, liver, muscle and other tissues can be localized. Specific antibodies for AQP HC-3 were generated in tree frogs. These antibodies
were purified by using sulpholink/immunopure; aliquots were made and stored in -80 °C until further immunofluorescence studies.
2.0 Materials and methods

2.1 Animals

Cope’s gray tree frogs were supplied by vendor Tennessee (The Sullivan company, Nashville, TN), a colleague in Alabama (Stephen Secor, Ph.D University of Alabama, Tuscaloosa, AL) and caught locally in Ohio (Caesar Creek State Park, Waynesville, OH). Three frogs were used in each of the physiological condition.

2.2 Frog conditioning procedures

a. Warm acclimated frogs

Cope’s Gray tree frogs, *Hyla chrysoscelis*, were warm acclimated using standard housing procedures which required a 16:8 hour light cycle in a 22-24°C room. Two to three frogs were housed per cage measuring 2ft×1ft×1 ft with a vented top and free access to water. Each frog was fed with a minimum of three crickets twice a week. All studies with these animals were carried out according to the protocols approved by the Wright State University Laboratory Animal Care and Use committee.
b. Slowly dehydrated frogs

Cope’s Gray tree frogs, *Hyla chrysoscelis*, were dehydrated by first inserting a glass capillary tube into their cloacae and removing the bladder water. This is a standard procedure used for collecting amphibian urine. The animals were weighed to determine “standard mass,” and the frog was transferred to a pre-weighed plastic container measuring approximately 5in×5in×5in with a vented top, taking care not to induce urination. The plastic container was then placed within a humidified chamber (approximately 1ft×1ft×0.5ft rat cage lined with saturated gauze) so that dehydration would occur slowly. To reduce stress leading to urination, the cage and the frog together were weighed daily and the mass of the frog was calculated by subtracting the cage mass from the total. Deprivation of food was also done during this period to increase the effect of dehydration of the frogs as the insect food fed to the frogs provides a rich source of water and so restricts the effects of dehydration. A Loss of 20% body mass required approximately two days and the animal was sacrificed when the body weight of the animal was 80% of the initial body weight.
c. Fast dehydration in gray tree frogs

To achieve rapid dehydration the steps mentioned above (slow dehydration) were carried out; the only difference was that for fast dehydration, the animal was placed in a dry container free of moisture. A plastic container with a vented top and a bed of calcium sulphate (CaSO4) desiccant was used as a cage for these animals. A wire mesh was used approximately one inch above the desiccant so as not to allow any direct contact between the frog and the desiccant. The animal was weighed three times daily until the mass was reduced to 80% of the initial body weight.

d. Cold acclimation in gray tree frogs

To cold acclimatize the animals they were moved into a temperature-controlled room at Wright State University and the temperature was gradually decreased from room temperature (20 °C) to 4 °C. The decrease in temperature was carried out gradually over a period of about two months during which the temperature and the light cycle were progressively dropped to simulate the natural transition to colder temperatures. This gradual acclimation to cold is important to develop cold acclimation including glycerol accumulation, a key basis for freeze tolerance in this species. During this cold acclimation process the frogs were fed as mentioned in the
standard housing protocol with access to water until the frogs ceased feeding (8-10 °C). When the frogs stopped feeding they were transferred to plastic containers (4in×4in×4in) with vented tops and water placed in small containers for humidity. The temperature was reduced to 4 °C over an additional 2-4 weeks and remained at 4 °C until the frogs were sacrificed for further studies or conditioned for freezing.

**e. Freezing in gray tree frogs**

Freezing was induced in cold acclimated gray tree frogs by decreasing their temperature further as described by Croes and Thomas (2000). Frogs were transferred from the temperature controlled cold room to a low temperature incubator set at 2 °C. The temperature was lowered by 1 °C every 24 hours until it reached -2 °C. After 24 hours at -2 °C, temperature was further lowered to -2.5 °C and freezing was triggered by placing an ice chip on the frog’s back. Frogs were sacrificed after being frozen for 24 h.
2.3 In situ hybridization

2.3.1 Preparation of organs

Organs were removed from the warm, cold and frozen tree frogs. These tissues were then immersed in 4% Paraformaldehyde and refrigerated at 4 °C for one day. These tissues were then placed in 4% Paraformaldehyde and 20% sucrose for 3-4 days. After fixation, the tissues were wrapped in foil and frozen at -80 °C until sectioned.

2.3.2 Sectioning of tissue

20µm sections were cut with a cryostat and mounted on gelatin coated slides.

2.3.3 Labeling of oligonucleotide

1) The temperature block was set to 37 °C before starting the procedure.

2) To label the aquaporin oligonucleotide the following was combined in a sterile eppendorf tube in the order listed below:
   a. 5µl of autoclaved /nuclease free water
   b. 1µl of terminal deoxynucleotide transferase DTT (activity=15-25 U/ml)
   c. 5µl of 5X terminal transferase buffer
d. 5µl of probe concentration 4pmol/ul. The probe can be either the sense (control) or the antisense at the defined position in the sequence. (The probes are DNA oligonucleotides which are synthesized chemically, which allows for the incorporation of amino-modified nucleotides (amino-allyl T). The resulting free amines were chemically coupled to fluorophores after synthesis. The modified Ts should be about 10 bases apart to prevent quenching and high background. The GC (Guanine-Cytosine) content of the oligo should be around 50%, its length can be variable, but 40-50 bases (5 fluorochromes per molecule of probe) works well in most cases.

e. 4µl of S$^{35}$ dATP, activity=1000-1500 CL/mMol

Centrifuge briefly and incubate in a 37°C temp block for at least 90 minutes.

3) After 90 minutes the reaction was stopped by removing the tube and adding 50µl of 0.1M TRIS-HCL/TEA/EDTA. Tapping the tube helped to mix the solution. (Total=75µl)

2.3.4 Purifying the oligonucleotide

(Using Mini-Quick spin DNA columns, BOEHRINGER MANNHEIM)

1) 2µl 0f the probe mixture was taken and added to 5ml of scintillation mixture. This was the raw mixture.
2) Mini-quick spin column is now resuspended and placed in an eppendorf tube and centrifuged for 1 min at 3200 rpm.

3) Place the column in another eppendorf tube, add 73µl of the probe mixture to the center of the column bed and centrifuge it for 4 minutes at 3200rpm.

4) The solution you get after centrifugation is the purified probe. Add 2ml of this solution to 5ml of the scintillation fluid for counts.

5) Calculations: raw cpm x dilution factor = actual cpm. Percentage binding should be calculated. The ideal binding is 20-70%.

6) The purified probe in second tube should be diluted with hybridization buffer to yield approximately 1.0-1.5 x 10⁶/100µl and placed at -20 ºc until used for hybridization.

2.3.5 Hybridization

1) Sections are placed in a slide boxes and washed with 0.01M (PH=7.4) for 15 minutes.

2) Wash slides for 30 minutes in 2X SSC (suspended sediment concentration) at room temperature.

3) Dry the slides on a slide warmer and add the hybridization buffer.

4) Incubate in a heat block for 18-20 hours at 37 ºC.
2.3.6 Post-hybridization

(SSC is suspended sediment concentration)

1) Wash slides for 1 hour in 1X SSC at room temperature.
2) Wash for 15 minutes in 1X SSC at room temperature.
3) Wash for 15 minutes in 1X SSC at room temperature.
4) Wash for 30 minutes in 1X SSC at 50°C.
5) Dry slides on slide warmer and place in the Fuji film cassette. Scan after 3-4 days.

2.4 Immunohistochemistry

2.4.1 Antibody production

Polyclonal rabbit antiserum was raised in New Zealand white rabbits against a synthetic peptide consisting of 16 amino acids from the carboxyl terminus of aquaporin HC-3 (CQENVKLSNVKHKRI) (Sigma-Genosys, The Woodlands, Texas). The antibody sequence of aquaporin HC-3 mentioned above was probed against the GenBank protein database using the BLAST program. No close homology was found to any other mammalian/amphibian protein (Accession No: ABC 98210; is the sequential number assigned to each protein as it is added in the database and also indicates the chronological order of its acquisition). KLH (Keyhole
Limpet Hemocyanin)-peptide conjugate was used for immunization, and three bleeds were collected from the animals on different post-immunization days. Antibody was affinity purified by an immunoadsorbent using SulfoLink purification kit (Product No: 44995, Pierce, Rockford, IL). The purified antibodies were aliquoted and stored at -80 °C until further use for immuno histochemistry. Cy2 conjugated goat anti-rabbit secondary antibody was purchased from Jackson ImmunoResearch Laboratories (Pennsylvania).

2.4.2 Tissue processing

Organs were collected from frogs in the various physiological conditions (warm, dehydrated, cold and frozen). Tissues were fixed in Periodate-Lysine Paraformaldehyde (PLP) fixative made up of 4% paraformaldehyde, 75 mM lysine, 37.5 mM sodium periodate, and 10 mM Na$_2$HPO$_4$ (pH = 7.2) (Mclean & Nakane, 1974) for 8-12 hours at 4 °C. Tissues were dehydrated in an increasingly concentrated series of ethanol solutions and embedded in paraffin (Humason, 1979). 8-10 µm sections were cut with a microtome, mounted on a gelatin coated slides and stored at 4 °C for later use. For basic histology, some sections were stained with hematoxylin and eosin and photographed at 40x using a digital camera attached to a Nikon light microscope.
2.4.3 Immunohistochemistry Procedure

Day 1

Deparaffinizing and Hydration

- Xylene 1: 5 min
- Xylene 2: 5 min
- ETOH 100%: 2 min
- ETOH 90%: 2 min
- ETOH 70%: 2 min
- ETOH 30%: 2 min

Immunofluorescence

- PBS 1 (0.1M, pH=7.2): 5 min
- PBS 2: 5 min
- PBS 3: 5 min
- TX-100 (0.1%): 15 min (Made in PBS pH= 7.2)
- Glycine (1%): 15 min (Made in PBS pH= 8)
- NaBH4 (0.1%): 15 min (Made in PBS pH= 8)
- PBS 1: 5 min
- PBS 2: 5 min
- Blocking Serum 10%: 1 hr at room temp
- Primary Ab: Overnight at 4°C (Made in 1% blocking serum)
  (1:10 dilutions of 10% blocking serum in PBS)
  10% blocking serum = 10% goat serum, 4%
  bovine serum albumin, 0.05% tween-20)

Day 2

- PBS 1: 5 min
- PBS 2: 5 min
- PBS 3: 5 min
In Dark

Sec Ab 5 min (Made in 1% blocking serum)
PBS 1 5 min
PBS 2 5 min
PBS 3 5 min

Add mounting solution with DAPI on the slides or stain with

Propidium Iodide in PBS 15 min
PBS 1 5 min
PBS 2 10 min
PBS 3 15 min

Cover with cover slip
Seal with nail polish
Store at 4°C overnight

Day 3

Photographs and Confocal imaging.

Slide mounted sections of skin, kidney, liver, muscle, stomach and intestine were deparaffinized in xylene and rehydrated using ethyl alcohol in decreasing concentrations. Serial washes of the sections were carried out in PBS (phosphate buffer solution, pH=7.2, 0.02 M sodium monophosphate, 0.08 M sodium dibasic phosphate, and 150 Mm NaCl) to remove the PLP fixative, tissue sections were then permealized with 0.2% Triton X-100 in PBS, treated with 1% glycine in PBS (pH=8) and 0.1% sodium borohydrate in PBS (pH=8). Tissues were then blocked with 10% normal goat serum
(10% goat serum, 4% bovine serum albumin, and 0.05% Tween-20) for one hour at room temperature and incubated overnight at 4 °C with HC-3 primary antibody diluted in 1% normal goat serum (1:100 dilutions). On day two sections were rinsed in several changes of PBS and incubated for 90 minutes at room temperature with Cy2 labeled goat anti-rabbit IgG in 1% normal goat serum (1:200 dilutions). After two washes in PBS, sections were counterstained for 15 minutes with 0.1µg of propidium iodide (Sigma) for cell nuclei. The sections were again rinsed in three changes of PBS, coverslipped and stored at 4 °C until photographed.

2.4.4 Confocal imaging

Sections were examined under Leica/Zeiss Microsystems Confocal Microscope at 40X (Product Serial No: 190246) using the Heidelberg GmbH Leica Confocal Software. All images were taken at a standard setting for Cy2 labeled secondary antibody and propidium iodide nuclear stain and the pictures were digitally enhanced by improving the background-to-fluorescence signal ratio. The expression level of HC-3 in the various tissues under different physiological conditions was compared visually by comparing their immunofluorescence intensity under the same standard settings of the Confocal microscope.
3.0 Results

3.1 In situ hybridization results with HC-1

The in situ results with HC-1 in different tissues of gray tree frogs, *Hyla chrysoscelis*, were difficult to read as signals with both antisense and sense (control) slides (Fig. 1, 2) were observed. Initially, a DTT concentration of 10mM was used which had previously been described as the standard concentration but this resulted in binding signal in both sense and antisense. Further experiments with different concentration of DTT (stabilizer for the probe) ranging from 0.5 mM to 50 mM in liver tissues suggested that the most successful results occurred in tissues treated with 1mM DTT. However, continuing efforts with this technique in liver and other tissues with 1mM concentration of DTT, failed to yield consistent results. Because this process did not produce successful results, an immunohistochemistry procedure was initiated.

3.2 Immunolocalization of glyceroporin HC-3 using Texas red

Immunolocalization of glyceroporin HC-3 using Texas red gave good results in some tissues like skin and muscle, but with other tissues like kidney and liver, there was background and localization of HC-3 in these tissues was not clear (Fig. 5, 8). The other concern was that not much could
be commented on the difference in the immunofluorescence intensity in different tissues under different physiological conditions was observed. In the skin tissue HC-3 was co-localized in the stratum granulosum and stratum spinosum of the epidermis and also in the glands present in the dermis. In the kidney HC-3 was found in the collecting ducts and also in the other tubules present in the kidney (Fig. 6). It was unclear about the exact localization of HC-3 in the kidney structures using Texas red because of non-specific binding. In the liver tissue, HC-3 was apparently expressed in the erythrocytes (Fig. 7) present in the hepatic sinusoids and also in the liver tissue itself; however, it was not clear if the immunofluorescence signal in the liver tissue was a specific binding, as it was also present in “control” tissue treated with anti-HC-3 antibody preabsorbed with the corresponding antigen peptide. In skeletal muscle, HC-3 was co-localized in the Schwann cells adjacent to the muscle tissue (Fig. 8). In all of these tissues, the diffuse nature of the fluorescence signal, in addition to undesirably high levels of background or non-specific fluorescence, made it difficult to successfully localize HC-3 protein, and also to comment on differences in immunofluorescence intensity in tissues collected from tree frogs under different physiological conditions. To address these problems, Cy2-labeled
antibody was initiated and Confocal imaging to achieve more accurate results that would allow the original to be tested hypothesis.

3.3 Immunolocalization of glyceroparin HC-3 in using Cy2

Skin

The histological structure of the gray tree frog skin consists of an epidermis made up of seven to eight layers of granular cells and dermis with different types of glands (serous and mucous). Stratum corneum is the outer most layer of the epidermis which consists of dead cells and does not show any activity. The lower granular layers of the epidermis are the stratum granulosum, stratum spinosum and stratum basale which contributes to the major thickness of the epidermis. Immunofluorescence labeling of HC-3 was seen in the multilayered granular cells in the epidermis of the ventral pelvic skin of these animals (Fig. 10a). In the dermis the labeling was concentrated in the exocrine glands which included both serous and mucous glands (Fig. 10a, b). The intensity of immunofluorescence labeling was increased in fast dehydrated (FD) (Fig. 9d); slow dehydrated (SD) (Fig. 9f) and cold (Fig. 9h) compared to the warm (control) (Fig. 9b) tissue. There is less difference in the intensity of the frozen (Fig. 9j) from the warm tissue. No labeling was
seen in the tissue blocked with the antiserum preabsorbed with the corresponding HC-3 antigen peptide (Fig. 9k).

**Kidney**

The kidney of gray tree frogs is made up of different structures like Bowman’s capsule with glomerulus, proximal tubule, distal tubule and collecting ducts. HC-3 was immunolocalized in the basolateral portion of the columnar epithelium of the collecting tubules (Fig. 12a, b). The intensity of immunofluorescence labeling was increased in fast dehydrated (FD) (Fig. 11d), slow dehydrated (SD) (Fig. 11f) and cold (Fig. 11h) compared to the warm (control) (Fig. 11b) tissue. There was not much difference in the intensity of immunofluorescence labeling between the frozen (Fig. 11j) and warm tissue. Collecting ducts consist of a single layer of columnar epithelial cells which functions in the removal of water from the filtrate. This whole process of removal of water is vasotocin or ADH (anti diuretic hormone) dependent. Studies show that HC-2 is present on the apical portion of the epithelium of the collecting tubules (Fig 12a). HC-1 is immunolocalized in the parietal layer of Bowman’s capsule in the kidney. The immunofluorescence labeling was blocked with the antiserum preabsorbed with the corresponding HC-3 antigen peptide (Fig. 11k).
Liver

The histological structure of the gray tree frog liver is complex. Liver capsule covers the whole of the liver. Sheets of connective tissues divide the liver into many lobules which are hexagonal in shape and have portal triads at the vertices and a central vein in the center. Hepatic acinus is the functional unit of the liver made up of parenchymal cells called hepatocytes arranged around the hepatic arterioles and portal venules which anastomose to form sinusoids. Sinusoids are vascular channels which contain blood in them and all sinusoids together drain into the central vein. HC-3 is immunolocalized in the erythrocytes present in the sinusoids surrounded by the liver hepatocytes (Fig. 14a, b). The intensity of immunofluorescence labeling was increased in fast dehydrated (FD) (Fig. 13d), slow dehydrated (SD) (Fig. 13f) and cold (Fig. 13h) compared to the warm (control) (Fig. 13b) tissue. The intensity was highest in the cold tissue erythrocytes compared to all other conditions. The intensity of the immune labeling in SD tissue erythrocytes (Fig. 13f) was less than the cold tissue but greater than the rest. There were no erythrocytes seen in the frozen tissue (Fig. 13i,j) so no immunofluorescence activity was seen except some nonspecific binding with the liver tissue which was also seen in the peptide slide (Fig. 13k). No immune labeling was detected in erythrocytes of the liver when anti-HC3
was preabsorbed with the corresponding antigen peptide (Fig. 13k). In all the liver sections, there was a minimum amount of immunolabeling of the liver tissue itself apart from the high intensity of labeling of the erythrocytes. Compared to the sections of other tissue like skin (Fig. 9a-j) and kidney (Fig. 11a-j), the liver tissue itself had some immunofluorescence.

**Muscle**

Skeletal muscle in gray tree frogs contains large numbers of muscle fibers running parallel to each other and bundled into fascicles. The interstitial space between the fascicles contains capillaries and arterioles containing blood. Skeletal muscle is innervated by nerves of the somatic nervous system. HC-3 was immunolocalized in the nerve bundles present in the muscle adjacent to the muscle fibers (Fig. 15) presumably, the somatic motor neurons (Schwann cells). No immune labeling was detected in any part of the muscle when anti-HC3 was preabsorbed with its corresponding antigen peptide (Fig.15k). No difference in the intensity of immunofluorescence labeling in fast dehydrated (FD) (Fig. 15d), slow dehydrated (SD) (Fig. 15f) and cold (Fig. 15h) compared to the warm (control) (Fig. 15b) tissue based on immuno histochemistry results could be determined.
**Stomach and Small intestine**

The histological structure of stomach and small intestine tissue in gray tree frogs is divided into three parts: mucosa, submucosa and muscularis externa. The mucosa consists of the epithelium, lamina propria and the muscularis mucosa. No HC-3 immuno labeling was detected in the epithelium or the glands present in the stomach (Fig. 16 a-j) or small intestinal (Fig. 17 a-j) tissues except for a small amount of non-specific bindings also seen when anti-HC3 was preabsorbed with the corresponding antigen peptide (Fig. 16k, 17k). Immuno localization of HC-3 was seen in the erythrocytes (Fig. 16i, j) present in the vessels of the stomach tissue.

**Large intestine**

The large intestine constitutes the terminal part of the digestive system in the gray tree frogs. As in typical vertebrate gastrointestinal tissue, it contains three concentric layers: mucosa, submucosa, and the muscularis externa. The mucosa is made up of the epithelium, lamina propria and the muscularis mucosa. Goblet cells (GC) are seen in the surface epithelium of the colon (Fig. 19a). HC-3 localization was found in the basolateral portion of the columnar absorptive epithelium (EP) (Fig. 19a). The apical portion towards the lumen (L) of the colon did not show any labeling (Fig. 19a). As
evident in Figure 19b and 19c, immunolocalization of HC-3 was again (as in liver) seen in the erythrocytes (E) present in intestinal blood vessels. The intensity of immunofluorescence labeling in the basolateral surface of the colonic epithelium was highest in the warm (control) (Fig. 18b) tissue. The fast dehydrated (FD) (Fig. 18d) tissue had similar localization of labeling as in tissues from warm animals, but with much lower intensity on the basolateral surface of the epithelium. Tissues from slow dehydrated (SD) animals (Fig.18f) had no labeling except some non-specific binding also seen in the control (peptide pre-incubation) slide. Surprisingly, the cold (Fig.18h) tissues showed a noticeable increase in immuno activity on the basolateral epithelium compared to the FD and SD. Frozen (Fig. 18j) tissues showed much less activity in the epithelium, but a good amount of labeling in the erythrocytes (E) present in the intestinal vessels. Only a small amount of non-specific labeling was detected in any part of the large intestine when anti-HC3 was preabsorbed with the corresponding antigen peptide.
4.0 Discussion

This study observed the localization and difference in expression level of HC-3, a mammalian AQP-3 homolog, in skin, kidney, liver, muscle, stomach, small intestine and large intestine of gray tree frog, *Hyla chrysoscelis*, in five different physiological conditions. Previous studies show that HC-3, an aquaglyceroporin, is permeable to both glycerol and water in Xenopus oocytes (Zimmerman et al., 2007). Other studies on mammalian AQP-3 also confirm that it is permeable to both water and glycerol (Boury-Jamot et al., 2009; Chikuma & Verkman, 2006).

The immunofluorescence results of gray tree frog skin showed that HC-3 is localized in the granular cells of the epidermis and the glands in the dermis. Previous studies on mammalian AQP3 have shown similar results (Matsuzaki et al., 1999; Mochida et al., 2008) explaining the role of AQP3 in the epidermal cells as a pump which supplies water from the dermis at the time of water deprivation to maintain skin hydration and elasticity. Studies suggest that AQP-3 expression is increased in response to skin stress like dehydration etc (Chikuma & Verkman, 2008). The osmoregulatory property of AQP3 is very important for hydration of the epidermis and studies suggested that in AQP3-null mice both water and glycerol permeability is reduced in the epidermis (Ma et al., 2000). AQP3 plays the major role in
transporting glycerol across the plasma membrane in the basal layer of keratinocytes in the skin to maintain proper hydration and elasticity (Ishibashi et al., 2008; Hara & Verkman, 2003).

Our results show that the immunofluorescence activity in the skin of gray tree frogs is increased from the warm hydrated condition to the fast dehydrated, slow dehydrated and cold conditions. Based on the function of AQP3 in mammalian skin, these results suggest that under the stress of dehydration, aquaporin HC-3 helps gray tree frogs to maintain the normal skin properties. The expression of HC-3 protein increases to compensate for the water loss and to allow the skin to retain its normal hydrated state. HC-3 protein present in the epidermal keratinocytes absorbs more water from the cells and vessels underlying them and transports it to the superficial layers for hydration (Matsuzaki et al., 1999a; Sourgat et al., 2002).

In cold conditions, due to the cryoprotective responses of the liver more glycerol is produced in the body of *Hyla chrysoscelis* (Irwin & Lee, 2003). This large quantity of glycerol is absorbed into the blood and distributed throughout the body. As mentioned earlier, glycerol is also a key substance for maintenance of healthy skin and so in cold conditions the expression of HC-3 proteins present in the epidermal layers is increased in order to increase the uptake of glycerol from the dermal vessels and
transport it to the superficial epidermal layers of skin to maintain proper hydration and elasticity (Takata et al., 2004).

Our present study clearly shows the localization of HC-3 in the basolateral membrane of the principal cells of the collecting ducts in gray tree frogs. The collecting duct consists of a series of tubules which connect the nephron to the ureter. Its main function is to maintain the body’s fluid and electrolyte balance by reabsorption and excretion. In mammals, in collaboration with AQP2 which is present on the apical membrane of the collecting duct principal cell, AQP3 on the basolateral membrane helps in the transepithelial transfer of water from the cell to the interstitium of the collecting duct and plays a major role in concentration of urine (Ogushi et al., 2007). AQP3 localization is not affected by anti diuretic hormone unlike AQP2 which translocates to apical membrane from the cytoplasmic compartment on ADH stimulation (Yamamoto et al., 1995). It has been shown that the osmotic permeability of the collecting duct cell is decreased more than three times in the AQP3/AQP4 knockout mice (Verkman 1999, 2000).

Some studies on water deprived rats showed an increase in AQP3 expression in the basolateral principal cell of renal collecting duct (Ecelbarger et al., 1995; Ishibashi et al., 1997). Similarly, our results of
HC-3 protein expression at the basolateral membrane of collecting duct showed increased immunofluorescence intensity in dehydrated and cold tissue compared to the warm hydrated tissue. In the dehydrated condition, the kidney tries to compensate for less amount of water in the body and so conserves more water by producing more concentrated urine. In order to absorb more water from the collecting duct HC-3 protein expression is increased, which in collaboration with HC-2 reabsorbs more water from the urine to maintain body homeostasis. Other studies on AQP3 mRNA in rat kidney by northern blot showed that the levels of AQP3 mRNA increased with water deprivation (Ishibashi et al., 1997). Freezing is also a form of dehydration in which the body loses liquid water from intracellular and extracellular fluid. Thus, similarly as in dehydrated state, HC-3 protein expression is increased during freezing to reabsorb water into the cell from the collecting duct interstitium to maintain body fluid balance.

The immunofluorescence results for gray tree frog liver showed the localization of aquaporin HC-3 in the erythrocytes present in the hepatic sinusoids separating the hepatocytes in the liver acini. Studies have already shown the presence of AQP3 in mammalian erythrocytes (Zelenina et al., 2004). Immunohistochemistry studies on AQP3 in liver have shown its expression in the hepatocytes and lymphocytes in the liver tissue (Mobasheri
et al., 2003). In our results, the major part of the HC-3 immunofluorescence was localized in the erythrocytes and very mild expression was seen in the hepatocytes. Liver is the major organ for glycerol production during cold acclimation which acts as a cryoprotectant (Layne & Jones, 2001). This cryoprotectant stabilizes the protein and membrane structure and thus protects the organ damage due to ice crystal formation (Zimmerman et al., 2007). The production of the cryoprotectant is maximum during cold acclimation just before freezing and not further elevated during freezing (Zimmerman et al., 2007; Irwin & Lee, 2003).

Our studies on the HC-3 protein expression in the erythrocytes and minimally in the hepatocytes present in the gray tree frog liver showed increased immunofluorescence intensity in the dehydrated and cold tissues compared to warm (control hydrated) tissues. The intensity was maximum in the cold acclimated tissues compared to all other conditions. As stated above, glycerol production is maximum during cold condition and so the immunofluorescence intensity of HC-3 expression is maximum in cold tissue. The increase in the immunofluorescence intensity in our cold tissues corresponds with an increase in the HC-3 mRNA in cold acclimated frog liver (Zimmerman et al., 2007).
Previous studies of AQP3 in skeletal muscle of human tissue showed low expression levels (Mobasheri et al., 2005). Our studies showed localization of HC-3 in the nerve bundles adjacent to skeletal muscle of Gray tree frog, Hyla chrysoscelis. AQP3 was reported to be co-expressed with AQP4 on the plasma membranes of skeletal muscle fibers in human tissues which collectively functions in transporting both water and nonionic small solutes (Wakayama et al., 2002). HC-3 mRNA was present in skeletal muscle and an increase in glycerol concentration during cold acclimation also shows a marked increase in HC-3 mRNA in frog skeletal muscle (Zimmerman et al., 2007). Again, the present study suggests the presence of HC-3 in nerve bundles present in muscle tissue, but little difference observed in the immunofluorescence intensity under different physiological conditions. The mRNA results showed an abundant increase in the mRNA from warm to cold acclimated frogs (Zimmerman et al., 2007).

The main function of the digestive system in mammals is fluid secretion and absorption. AQP3 was present in the epithelium of the upper digestive system from the oral cavity to the fore stomach and of the lower digestive tract from the distal colon to anus (Matsuzaki et al., 1999). AQP3 was present in the luminal surface of the fore stomach covered with stratified epithelium and also in the basolateral surface of the mucous cells.
present in the luminal fundic stomach covered with simple columnar epithelium (Matsuzaki et al., 1999). AQP3 expression was relatively low in the glandular portion compared to the non-glandular part of the stomach (Takata et al., 2004). Other than in the erythrocytes present in the gastric vasculature, immunolabeling in stomach tissue from gray tree frogs did not indicate expression of HC-3, either in the epithelium or the glands of stomach tissue.

AQP3 was more prominent in large intestine, where final water absorption is accomplished, compared to the small intestine, where the largest proportion, 80-90% (Fordtran et al., 1965) of total intestinal water absorption occurs (Selub, 1995). In most of the mammalian small intestine, no AQP3 was detected in the epithelial lining or the glands of the duodenum and jejunum, but in some cases weak AQP3 immunolabeling was found in the basolateral surface of the epithelial cells at the villous tip (Matsuzaki et al., 1999; Ramirez-Lorca et al., 1999). No immunolocalization of HC-3 was found in any part of the small intestine in the present study. In distal colon, AQP3 was localized in the basolateral plasma membrane facing the lumen of the absorptive columnar epithelial cells facing the lumen of the large intestine (Mochida et al., 2008; Matsuzaki et al., 1999; Takata et al., 2004).
Studies suggested that AQP3 plays an important role in the hydration of the colonic epithelial cells that are directly facing the lumen of the colon and come into contact with the fecal content (Takata et al., 2004; Matsuzaki et al., 1999). Another suggested role of AQP3 is its contribution in water absorption in the intestine by transepithelial fluid movement (Matsuzaki et al., 1999). The expression level of AQP3 is up regulated by low osmolality of the feces in the colonic lumen (Matsuzaki et al., 1999). The fluid movement in the intestine occurs secondary to the osmotic driving forces created by active salt transport and to hydrostatic pressure differences of the feces and causes fecal dehydration (Ma & Verkman, 1999). Studies showed that another principal role of AQP3 at the colonic plasma membrane is to function as a route of water entry from the sub epithelial side into the epithelial cells and thus maintain intracellular osmolality and cell volume whenever cells undergo water loss (Matsuzaki et al., 1999).

In our study, HC-3 was immunolocalized in the basolateral surface of the large intestinal absorptive epithelial cells facing the lumen of the colon and at the neck of the crypts. The intensity of the immunolabeling was maximum in the warm hydrated tissues compared to all other condition. In the warm hydrated tissue, the fecal osmolality is low, so the HC-3 expression is the highest and as the fecal osmolality goes on increasing from
fast dehydrated to slow dehydrated condition the HC-3 expression level also goes down accordingly. Freezing is also a state of dehydration in which the fecal osmolality goes up (if any fecal matter present), as it is likely that the colon is empty in the frozen state, so the HC-3 expression is less in this condition. HC-3 tries to compensate for the extreme water loss during prolonged cold acclimation by providing a route of water entry into the epithelial cells from the subepithelial side in order to maintain cell volume and intracellular osmolality, this explains why there is an increase in HC-3 expression in cold tissue compared to the dehydrated tissue. Also, in the cold condition a large amount of glycerol is synthesized by the animal for cryoprotection. The glycerol present in the subepithelial blood vessels is absorbed into the epithelial cells to maintain the cell integrity and prevent damage due to cold. Due to this increased uptake of both water and glycerol in the large intestine of the cold acclimated tissue, the expression level of HC-3 is increased. This physiological change does not occur in the dehydrated condition as it is a relatively fast process compared to cold condition which occurs in a period of three to six weeks. Erythrocytes present in the vessels in the intestinal tissue were immunolabeled with HC-3. In summary, our study showed localization of HC-3 in different tissues of gray tree frogs, *Hyla chrysoscelis*, under different physiological conditions.
The varied expression level of HC-3 in different conditions has a physiological basis.
5.0 Conclusion

The results of the present study concluded that aquaglyceroporin HC-3 has an important physiological role in different tissues of gray tree frogs under different physiological conditions. The hypothesis stated that the expression level of HC-3 is up-regulated during cold acclimation and freezing and our results showed a similar pattern in some of the tissues tested. Gray tree frog skin, kidney and liver showed results supporting our hypothesis with an increased intensity of immunolabeling in cold tissues compared to the warm tissues. The results of muscle, stomach, and intestine HC-3 immunolabeling were inconclusive.

The pattern of expression of HC-3 in the epidermis and dermal glands of ventral skin of gray tree frogs was similar to the expression of its mammalian counterpart AQP-3. Similarly, in the kidney and the large intestine, HC-3 was expressed in the basolateral surface of the collecting ducts and the basolateral surface of the large intestinal epithelium respectively which was similar to the pattern of expression of AQP-3 in human tissue. In the rest of the tissues tested, the results of the HC-3 localization were not similar to mammalian AQP-3. In muscle, the expression of HC-3 in gray tree frogs was localized to the nerve bundles adjacent to the muscle tissue, whereas the AQP-3 was co-expressed with
AQP-4 on the plasma membrane of human muscle tissue. In the stomach and small intestine, HC-3 was not localized anywhere in the tissue except in the erythrocytes present in the blood vessels innervating the tissue. In the human stomach tissue, AQP-3 was localized in the luminal epithelial surface of fore stomach and the mucous glands in the fundic stomach. The mammalian small intestine results were very similar to ours with few exceptions where weak immunolabeling was seen in the basolateral surface of the epithelial villous tip. The gray tree frog liver results showed strong immunolocalization of HC-3 in the erythrocytes present in the hepatic sinusoids and a weak immunolabeling of the hepatic tissue itself, whereas the human liver tissue showed the localization of AQP-3 in the hepatocytes and the lymphocytes in the liver tissue.

The expression levels of HC-3 in the tissues of gray tree frog under different physiological conditions had varying intensity. In tissues like skin, kidney and liver of gray tree frogs, the intensity of immunolabeling was highest in the cold condition compared to all other conditions. Also, the intensity was increased in the dehydrated tissues compared to the warm (control) tissues, but not as high as the cold tissues. The highest intensity in the cold tissues might result from HC-3 up-regulation in response to synthesis and circulation of glycerol as a cryoprotectant. As HC-3 is an
aquaglyceroporin channel, it also transports glycerol along with water in the cold conditions, in contrast to the transport of only water during the dehydrated conditions. All the results show that the expression of HC-3 in gray tree frogs is up-regulated during the cold and the dehydrated conditions in skin, kidney and liver compared to the warm frogs.

Further studies can be done to quantify the intensity of immunolabeling to confirm these results. Also, studies should be done under post freezing recovery conditions to determine if the frog regains its normal physiological condition after the freezing. Western blotting should be done to see if we get similar results are obtained and the same conclusions can be drawn from them.
IN SITU HYBRIDIZATION

Fig 1: AQPhc1c sense and antisense probes were used. Hybridization buffer with 10mM concentration DTT was added to the sections. Liver and lung tissues were hybridized. Binding was seen in both sense (control) and antisense sections.
Fig 2: AQPhc1c sense and antisense probes were used. Hybridization buffer with 1mM DTT concentration was added to sections. Liver, lung, kidney and skin tissues were hybridized. Binding was seen in both sense (control) and antisense sections.
Fig 3: AQPhc1d sense and antisense probes were used. Hybridization buffer with different DTT concentrations ranging from 0.5mM to 50mM (0.5, 1.0, 10.0, 20.0, 50.0) were added to sections. Binding was seen in all the antisense sections and also all the sense (Control) sections except the section with 1mM concentration.
Fig 4: AQPhc1d sense and antisense probes were used. Hybridization buffer with 1mM DTT concentration was added to sections. Liver and kidney tissues were hybridized. Binding was seen in both antisense sections and sense sections randomly.
IMMUNOHISTOCHEMISTRY RESULTS WITH TEXAS RED

Fig 5: AQP HC-3 is localized in the stratum granulosum and stratum spinosum of the warm frog skin.
- Fig. 5a shows layers of skin in normal light.
- Fig. 5b shows nuclei stained with DAPI stain.
- Fig. 5c shows immunofluorescence with Texas red at 270 ms.
- Fig. 5d shows immunofluorescence with Texas red at 496 ms.
Fig 6: AQP HC-3 localization in the collecting ducts of the warm frog kidney
- Fig. 6a shows kidney tissue in normal light.
- Fig. 6b shows nuclei stained with DAPI stain.
- Fig. 6c shows immunofluorescence with Texas red at 270 ms.
- Fig. 6d shows immunofluorescence with Texas red at 496 ms.
Fig 7: AQP HC-3 localization in the erythrocytes of the warm frog liver
- Fig. 7a shows liver tissue in normal light.
- Fig. 7b shows nuclei stained with DAPI stain.
- Fig. 7c shows immunofluorescence with Texas red at 270 ms.
- Fig. 7d shows immunofluorescence with Texas red at 496 ms.
Fig 8: AQP HC-3 localization in the nerve bundles (Schwann cells) of the warm frog muscle
- Fig. 8a shows muscle tissue in normal light.
- Fig. 8b shows nuclei stained with DAPI stain.
- Fig. 8c shows immunofluorescence with Texas red at 270 ms.
- Fig. 8d shows immunofluorescence with Texas red at 496 ms.
IMMUNOHISTOCHEMISTRY WITH Cy2 & CONFOCAL MICROSCOPE

SKIN TISSUES:

Fig 9: Confocal microscopic images showing co-localization of HC-3 receptors in the keratinocytes of stratum granulosum, stratum spinosum and dermal glands in the ventral skin of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No immune labeling was detected in any cell of the skin when anti-HC3 was preabsorbed with the corresponding antigen peptide. The intensity of immunofluorescence labeling was increased in fast dehydrated (FD), slow dehydrated (SD) and cold compared to the warm (control) tissue. There is less difference in the intensity of the frozen from the warm tissue. Scale bar = 75µm
Fig 10: H & E staining of the skin showing the epidermis (EP) and the exocrine glands in the dermis (Fig. a). Enlarged view of HC-3 localization in the epidermis (EP) and mucous glands (MG) and serous glands (SG) of the gray tree frog skin dermis (Fig b, c). Scale = 75µm
KIDNEY TISSUES:

Fig 11: Confocal microscopic images of kidney showing localization of HC-3 receptors in the basolateral surface of the kidney collecting ducts of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No immune labeling was detected in any cell of the kidney when anti-HC3 was preabsorbed with the corresponding antigen peptide. The intensity of immunofluorescence labeling was increased in fast dehydrated (FD), slow dehydrated (SD) and cold compared to the warm (control) tissue. There is not much difference in the intensity of the frozen from the warm tissue. Scale bar = 75µm
Fig 12: H & E staining of the kidney showing the glomerulus and the collecting ducts (CD) (Fig. a). Enlarged view of HC-3 localization on the basolateral (BL) surface of the renal collecting duct (CD). Studies show that HC-2 receptors are localized on the apical (A) surface of the renal collecting duct (Fig. b, c). Scale = 75µm
LIVER TISSUES:

Fig 13: Confocal microscopic images of liver showing localization of HC-3 receptors in the erythrocytes present in the hepatic sinusoids separating the hepatocytes in the liver acini of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No immune labeling was detected in erythrocytes of the liver when anti-HC3 was preabsorbed with the corresponding antigen peptide. The intensity of immunofluorescence labeling was increased in fast dehydrated (FD), slow dehydrated (SD) and cold compared to the warm (control) tissue. The intensity was highest in the cold tissue erythrocytes compared to all other conditions. The intensity of the immune labeling in SD tissue erythrocytes was less than the cold tissue but greater than the rest. There were no erythrocytes seen in the frozen tissue so no immunofluorescence activity was seen expect some nonspecific binding with the liver tissue which was also seen in the peptide slide. Scale bar = 75µm
Fig 14: H & E staining of the liver showing the capsule, the hepatocytes (H) and the erythrocytes (E) in the sinusoids (Fig. a). Enlarged view of HC-3 localization in the erythrocytes (E) present in the hepatic sinusoids which separate the hepatocytes (H) in the liver acini (Fig. b, c). Scale = 75µm
MUSCLE TISSUES:

Fig 15: Confocal microscopic images showing co-localization of HC-3 receptors in the nerve bundles (NB) (Schwann cells) present in the skeletal muscle of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No immune labeling was detected in any part of the muscle when anti-HC3 was preabsorbed with the corresponding antigen peptide. We cannot comment on the difference in the intensity of immunofluorescence labeling in fast dehydrated (FD), slow dehydrated (SD) and cold compared to the warm (control) tissue based on immuno histochemistry results. H & E staining of the muscle showing muscle fibers (Fig. 1). Western blot results will be more suggestive. Scale bar = 75µm
STOMACH TISSUES:

(STOMACH TISSUES:)

Fig 16: Confocal microscopic images for co-localization of HC-3 receptors in the stomach tissue of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No HC-3 immuno labeling was detected in the epithelium or the glands present in the stomach tissues except few non specific bindings also seen when anti-HC3 was preabsorbed with the corresponding antigen peptide. Immuno labeling was seen in the erythrocytes (E) present in the vessels in the tissue. H & E staining of the stomach showing the surface epithelium, the lumen and the sub mucosa (Fig. 1). Scale bar = 75µm

(Wiechmann & Wirsig-Wiechmann, 2003)
SMALL INTESTINE TISSUES:

Fig 17: Confocal microscopic images for co-localization of HC-3 receptors in the small intestine tissue of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No HC-3 immuno labeling was detected in the epithelium or the glands present in the small intestine tissues except few non specific bindings also seen when anti-HC3 was preabsorbed with the corresponding antigen peptide. H & E staining of the small intestine showing the lumen, the villi and the blood vessels (Fig. l). Scale bar = 75μm
LARGE INTESTINE TISSUES:

Fig 18: Confocal microscopic images of large intestine showing localization of HC-3 receptors in the basolateral surface of the large intestinal epithelium of Gray tree frog, *Hyla chrysoscelis* under different physiological conditions. HC-3 was localized using rabbit anti-HC3 primary antibody and Cy2 labeled goat anti-rabbit secondary antibody (Green) while the cell nuclei was counterstained using propidium iodide (Red). No immune labeling was detected in any part of the large intestine when anti-HC3 was preabsorbed with the corresponding antigen peptide except some non specific binding. The intensity of immunofluorescence labeling was highest in the warm (control) tissue. The fast dehydrated (FD) tissue had very less labeling seen on the basal surface of the epithelium. The slow dehydrated (SD) tissue showed absolutely no labeling except some non specific binding also seen in the peptide slide. Surprisingly the cold tissues showed a noticeable increase in immuno activity on the basal epithelium compared to the FD and SD. Again the frozen tissues showed almost negligible activity in the epithelium but a good amount of labeling in the erythrocytes (E) present in the intestinal vessels. Scale bar = 75µm
Fig 19: Enlarged view of the large intestine showing HC-3 localization in the basolateral (BL) portion of the columnar absorptive epithelium (EP) (Fig. a). The apical portion towards the lumen (L) of the colon does not show any labeling. Goblet cells (GC) are also visible in Figure a. Figure b and c shows Immunolocalization of HC-3 in the erythrocytes (E) present in the intestinal blood vessels. H & E staining of the large intestine showing surface epithelium and the lumen (Fig. b). Scale = 75µm
REFERENCES


control of expression in liver. Proceedings of the National Academy of Sciences, USA 100:2945–50.


expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. Biochemical and Biophysical Research Communications 244:268–74.


