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Mechanisms of Freeze Avoidance in Eggs of the Antarctic Naked Dragonfish (*Gymnodraco acuticeps*) and Atlantic Tomcod (*Microgadus tomcod*)

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Accepted: _______________

INTRODUCTION

A remarkable number of marine teleosts belonging to the perciform suborder Notothenioidei and the paracanthopterygian suborder Gadoidae (cods and their allies) have evolved biophysical and physiological adaptations that promote freeze avoidance to temperatures as low as 1°C below the equilibrium freezing point of their body fluids (Cheng et al. 2003; Costanzo et al. 1995; Howes 1991; Raymond and DeVries 1977). Among the Notothenioids endemic to the Antarctic Ocean, the naked dragonfish (*Gymnodraco acuticeps*, Figure 1) inhabits the ice-laden waters near McMurdo Sound where the annual water temperature remains stable at -1.87 ± 0.1°C (Komatsu et al. 1970; Bargelloni et al. 1994). In the north Atlantic region, fish fauna along the coastal waters from southern Labrador and northern Newfoundland to Virginia include the Atlantic tomcod (*Microgadus tomcod*, Figure 2) which is primarily found in shallow, low-salinity estuaries and brackish or freshwater river mouths where water temperatures reach -1.2°C during winter seasons (Pearcy and Richards 1962).

Biogeography and Life History of *Gymnodraco acuticeps*

Antarctic teleosts are phylogenetically distinct from fishes in the northern hemisphere (Figure 3; Cheng et al. 2003). Traditional phylogenetic hypotheses suggest that the Antarctic Notothenioids arose within the Antarctic over 15 million years ago as a result of the Antarctic Circumpolar Current (ACC) posing a significant oceanographic barrier to northward migration (Cheng et al. 2003). Near the Ross Ice Shelf at McMurdo Sound, Antarctica, female dragonfish deposit their eggs on rocky substrate in the vicinity of anchor ice where spawning generally occurs in September (Faranda et al. 2000). Freshly laid eggs are guarded by the male dragonfish until hatching occurs in the spring (Gon and Heemstra 1990). Since the eggs are hypoosmotic to
seawater and therefore have the potential to freeze (Bargelloni 1994), survival in this environment is highly dependent on the ability of eggs to avoid freezing in the vicinity of ice.

**Figure 1.** The Antarctic naked dragonfish (*Gymnодraco acuticeps*) guarding its eggs on rocky substrate near McMurdo Sound, Antarctica.

**Biogeography and Life History of *Microgadus tomcod* (Walbaum)**

Ecological parameters including differences in spawning behavior and wintering areas, temperature and salinity barriers to egg fecundity, and the influence of hydrogeological movements on prey dispersal served as the basis by which Svetovidov (1948) first recognized geographical groups of gadoid fishes including the Arctic-Circumpolar, Atlantic-boreal and Pacific-boreal populations. The latitudinal separation and antiequitorial distribution of gadoids supports the theory that these warm-intolerant poikilotherms underwent a southward dispersal from the Arctic basin, ultimately resulting in isolation of the currently recognized ancestral stocks.
Most Atlantic tomcods spawn between the months of November and February in low salinity waters, preferably freshwater streams where females produce an average of 20,000 eggs each season (Stewart and Auster 1987). Egg development is confined to low salinity waters (less than 15 ppt) and is highly stenothermal with a narrow temperature range of -1.2 to 6.0°C (Howe 1971). At Shinnecock Bay, Long Island, NY, the local tomcod population lacks access to freshwater and is exposed to mild salinity resulting from tidal mixing and freshwater runoff (Reisman et al. 1984). The study of spawning sites at Shinnecock Bay is unique considering that most other populations of tomcod have access to freshwater rivers or mixed-water estuaries along the east coastal region of North America (Figure 2). Despite the geographical separation and lack of phylogenetic similarity between the tomcod and dragonfish, their survival in subzero marine environments is similar and depends upon the presence of small ions, organic osmolytes and a unique family of eight structurally-related glycoproteins and glycopeptides collectively known as “antifreeze glycoproteins” (AFGPs; O’Grady et al. 1982).

![Figure 2. The Atlantic tomcod (Microgadus tomcod) and its distribution on the east coastal region of North America.](image)
Figure 3. Taxonomic relationships among antifreeze-producing fishes in the division Teleostei (Modified illustration from Cheng 1998).
**Osmolytes in Nature**

Cellular osmolytes in vertebrates typically include sugars, amino acids and their derivatives, polyhydric alcohols, intracellular methylamines and occasionally urea (Timasheff 1992). Excluding urea, these naturally occurring substances are all known to be protein stabilizers (Lin and Timasheff 1994). Methylamines are vital to the survival of many marine vertebrates since they are capable of suppressing the detrimental effects of ammonia toxicity, high concentrations of NaCl, hydrostatic pressure and temperate stress on the organism (Seibel and Walsh 2002). The distribution of these beneficial compounds varies among species in different habitats and by season and ontogeny within a species (Seibel and Walsh 2002).

In many vertebrates, trimethylamine-N-oxide (TMAO) is found at high concentrations (Bedford et al. 1998) in the muscle, heart and spleen, particularly when urea is present, and is usually found at low concentrations in the brain, liver and kidneys (Bedford et al. 1998). Urea is known to have a destabilizing effect on protein structure as well as cellular structure and function and is considered an “incompatible solute” on the basis that it disrupts enzymatic activity within the cell (Timasheff 1992). Yet, in adult fishes urea is retained in the blood as part of a strategy to elevate blood osmolality relative to seawater without raising the concentration of ions in the body fluids (Bedford et al. 1998).

In most cells, the perturbing effects of urea are largely compensated by the presence of TMAO, betaine, sarcosine and glycerophosphorylcholine (Lien et al. 1993). These compounds are considered “compatible solutes” and are capable of preserving a stable rate of enzymatic activity within the cell (Yancey and Somero 1979). According to Withers and colleagues (1996), urea and methylamines also contribute toward the positive buoyancy of fishes since the partial molar volume of these compounds is relatively large compared to their molecular mass. Whether tomcod and dragonfish eggs preserve enzymatic activity using compounds similar to those found in adults is unknown.

**Antifreeze Glycoproteins**

DeVries (1970) was the first to perform a complete characterization of AFGPs in the Antarctic fish. The presence of AFGPs in the Atlantic tomcod was later discovered by Duman and DeVries (1974) in specimens obtained from Nova Scotia. In contrast to compounds such as electrolytes and small organic osmolytes which colligatively depress the equilibrium freezing point of body fluids, AFGPs kinetically lower the temperature at which ice grows in a non-
colligative manner based more on the chemical structure of AFGPs rather than their concentration in solution. Electrolytes and organic osmolytes depress the freezing point of pure water by a normal colligative effect (a 1M solution colligatively depresses the freezing point of pure water by 1.86°C), based on their concentration in solution (Raymond 1993).

Synthesizing AFGPs as an alternative to increasing the intracellular osmolyte concentration allows cells to lower their non-equilibrium freezing temperature without perturbing osmotic balance. The configurational dynamics of AFGPs determined through high field NMR spectroscopy and molecular studies have shown that although AFGPs are randomly oriented in solution, each AFGP consists of a similar backbone with an (Ala-Ala-Thr)\textsubscript{n} repeat (with minor sequence variations in which the first alanine is occasionally replaced by a proline) glycosidically attached from the hydroxyl oxygen of the threonine residues, to the carbohydrate moiety β-D-galactosyl-(1→3)-α-N-acetyl-β-D-galactosamine (Figure 4; Harding et. al. 2003).

**Figure 4.** Representative structure of an antifreeze glycoprotein. There are eight distinctive glycopeptide classes based on relative rates of electrophoretic migration and molecular masses ranging from 2.6 kDa (n = 4) to 33.7 kDa (n=50), where n is the number of Ala-Ala-Thr repeats in the AFGP backbone (Modified from Harding et. al. 2003).

AFGPs are completely soluble in most protein-precipitating agents, stable at 100°C for 5 min and are resistant to irreversible denaturation when precipitated with ethanol or acetone (DeVries et al. 1970). The resilience of AFGPs suggests that they may also avoid destabilization by urea. On a weight basis, AFGPs are capable of depressing the freezing point of water to the
same extent as NaCl (DeVries et al. 1970). Although the precise mechanism by which AFGPs inhibit the growth of ice crystals remains controversial, prevailing theories suggest that AFGPs bind to prism faces on an ice crystal (Figure 5) causing the formation of highly curved fronts and an increase in free energy that prevents any further growth of the crystal (Eastman 1993).

**Figure 5.** Antifreeze glycoproteins are thought to inhibit the growth of ice by adsorbing to peripheral facets on the crystal surface, forming highly curved fronts.

Many studies have examined physicochemical nature of freeze avoidance in adult cold-water fishes (reviewed by DeVries 1983), but a complete characterization of the eggs has never been completed. To answer the question of whether tomcod and dragonfish eggs utilize mechanisms of freeze avoidance similar to adult fishes, the present study was undertaken to characterize and quantify compounds including electrolytes, organic osmolytes and AFGPs in the eggs of both species.
MATERIALS AND METHODS

Specimen Collection

Fresh samples of eggs from *G. acuticeps* were harvested (October – December, 2003) at the McMurdo Sound station in Antarctica. Atlantic tomcod eggs were collected at Shinnecock Bay, Long Island, NY (January-March, 2004). Ooplasm was extracted from the eggs via syringe and was stored between –60°C and -80°C prior to use.

Osmolality, Electrolytes and Thermal Hysteresis

The total osmolality of ooplasm from *G. acuticeps and M. tomcod* was measured using a Wescor vapor pressure osmometer (Model: VAPRO; Logan, UT). Pooled samples of ooplasm were measured in triplicate using 10µl volumes. To determine the extent of thermal hysteresis, defined as the difference between the melting point and the non-equilibrium freezing point of a solution, sub-microliter volumes of ooplasm were exposed to freeze/thaw cycles using a nanoliter osmometer (Clifton Technical Physics, Hartford, USA). Thermal hysteresis is considered an indicator of AFGP activity. Electrolyte concentrations including Na⁺, Cl⁻ and K⁺ were measured using an electrolyte analyzer (Bayer/Ciba-Corning, type 644; East Walpole, MA) with a 40-second throughput.

High-Pressure Liquid Chromatography (HPLC)

Antifreeze glycoproteins (AFGP) I-VIII were characterized and quantified with HPLC, using a 300 × 7.8 mm Bio-Sil SEC type 125 gel filtration column (flow rate = 1mL/min) and an ISCO V4 absorbance detector. The deuterium lamp was set at 220nm to a sensitivity of 0.1% T. Ooplasm from *G. acuticeps and M. tomcod* was measured in duplicate and in triplicate, respectively, with 10µl injection volumes. Spectra of standardized fractions of AFGP I-V, VI and VII-VIII were also acquired. Chemical shifts were used to identify different AFGP groups, while the relative areas under the peaks in the spectra were used to determine their concentration, relative to the standards.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Proton and phosphorus nuclear magnetic resonance (¹H and ³¹P-NMR) spectra were acquired using a 300MHz G.E. QE300 FT-NMR to characterize and quantify different osmolytes
in ooplasm from both species. $^1$H-NMR spectra were acquired continuously over 64 scans with 16,384 data points using a recycle delay of 1.0 sec and pulse width of 3.10 µsec with an average probe temperature of 22.0°C. Standard 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS, 200.2 mM) was used as an external chemical shift and concentration reference.

$^{31}$P-NMR spectra were acquired continuously over 1,000 scans with 16,384 data points using a recycle delay of 0.5 sec and pulse width of 10 µsec with an average probe temperature of 22.8°C. Methylene diphosphonic acid (MDPA, 103.2 mM) was used as an external reference. All spectra were acquired using 5 mm NMR tubes. Phosphate compounds were identified based on their chemical shift in the spectrum using a technique known as “spiking.” Glycerophosphorylcholine (GPC), glycerophosphorylethanolamine (GPE) and o-phospho-L-serine were added individually to the ooplasm and their peak position was observed relative to MDPA. Each free induction decay (FID) was Fourier transformed and baseline corrected before integration. Chemical concentrations were determined by comparing the integral of the external standard to the integral of each osmolyte peak.

**Lipid Extraction**

Lipid and water-soluble PDEs were separated using a modified version of the Bligh and Dyer lipid extraction procedure (1959). To 0.50 ml of dragonfish ooplasm, 0.50 ml of chloroform and 1.0 ml of methanol were added over ice and mixed gently every minute with a pipette over a period of 15 minutes. The mixture was vortexed for 20 seconds and kept on ice for 15 minutes. Following the extraction, 0.50 ml of chloroform and 0.40 ml of saline were added. Water and lipid-soluble phases were separated and stored on ice prior to use.

**Thin-Layer Chromatography**

Ooplasm from *G. acuticeps* and *M. tomcod* was analyzed with one-dimensional thin-layer chromatography (TLC). Using 3.0 µl microcaps, 2.0 µl of ooplasm from *G. acuticeps* or *M. tomcod* eggs was applied to a silica-coated aluminum TLC plate. Phosphatidylcholine (derived from soybean, 99% purity, 2 mg/ml, Sigma) and phosphorylcholine (type “XIII-E” from frozen egg yolk, 99% purity, 2 mg/ml; Sigma) were spotted as a chemical reference and control, respectively. For lipid migration based on the general separation of phospholipids by headgroup polarity, a solvent system consisting of chloroform/methanol/water (65:25:4 v/v) was used in a closed chamber lined with filter paper to aid in saturation. The chromatogram was briefly “dipped” in a mixture of phosphomolybdic acid (PMA) and ethanol (6g/120ml), dried using a hot
air gun and exposed to iodine vapor for approximately 11 minutes. The retention factor (R<sub>f</sub>) of each compound in the ooplasm was compared to the standards and referenced to values stated in the literature. The R<sub>f</sub> value for a given solvent and sample is described as the ratio of the sample migration distance to the solvent front.

**Quantitative Colorimetric Urea Assay**

The concentration of urea in pooled ooplasm from *G. acuticeps* was measured by quantitative colorimetric assay using the QuantiChromTM Urea Assay Kit (DIUR-01K, BioAssay Systems). Optical density was measured at a wavelength of 520 nm using a spectrophotometer and compared to a standard curve to determine the concentration of urea.

**RESULTS**

**Osmolality, Electrolytes and Thermal Hysteresis**

The total osmolar concentration of the ooplasm was significantly higher in dragonfish (832 ± 25 mmol · kg<sup>-1</sup>, n=6) than in tomcod (443 ± 55 mmol · kg<sup>-1</sup>, n=4). Electrolyte analysis revealed similar concentrations of Na<sup>+</sup> (176 mM, n=1) and Cl<sup>-</sup> (142 ± 25mM, n=4) in tomcod ooplasm (Table 1). However, the concentration of Cl<sup>-</sup> (316 ± 16mM, n=6) was significantly higher than Na<sup>+</sup> (167 ± 3mM, n=5) in dragonfish ooplasm. As shown in Table 1, the concentration of K<sup>+</sup> was higher in dragonfish than in tomcod ooplasm although K<sup>+</sup> makes a greater contribution to the total osmolality in tomcod. The electrolyte contribution to the total osmolality was approximately 68.4% in dragonfish and 85.8% in tomcod ooplasm.

<table>
<thead>
<tr>
<th>Species</th>
<th>Na&lt;sup&gt;+&lt;/sup&gt; (mM)</th>
<th>Cl&lt;sup&gt;-&lt;/sup&gt; (mM)</th>
<th>K&lt;sup&gt;+&lt;/sup&gt; (mM)</th>
<th>Contribution to total osmolality</th>
<th>Total osmolality (mmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. acuticeps</em></td>
<td>167 ± 3 (n=5)</td>
<td>316 ± 16 (n=6)</td>
<td>86 ± 5 (n=6)</td>
<td>68.4 %</td>
<td>832 ± 25 (n=6)</td>
</tr>
<tr>
<td><em>M. tomcod</em></td>
<td>176 (n=1)</td>
<td>142 ± 25 (n=4)</td>
<td>62 ± 23 (n=7)</td>
<td>85.8 %</td>
<td>443 ± 55 (n=4)</td>
</tr>
</tbody>
</table>

Thermal hysteresis was more pronounced in dragonfish (0.80 ± 0.05°C, n=2) than in tomcod (0.3 ± 0.1°C, n=2), suggesting that non-colligative freezing point-depression occurs in eggs from both species, although to a larger extent in dragonfish. Freezing point-depression
resulting from the action of AFGPs in dragonfish and tomcod ooplasm accounted for approximately 36.9% and 26.1% of the non-equilibrium freezing temperatures, respectively (Table 2). The difference between measured and calculated equilibrium freezing temperatures was approximately 2.5% in tomcod and 8.9% in dragonfish (Table 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Measured Melting T (°C)</th>
<th>Measured Freezing T (°C)</th>
<th>Measured Hysteresis* (°C)</th>
<th>Calculated Melting T (°C)</th>
<th>Calculated Freezing T (°C)</th>
<th>Calculated Hysteresis* (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. acuticeps</td>
<td>-1.37 ± 0.04 (n=2)</td>
<td>-2.17 ± 0.04 (n=2)</td>
<td>0.80 ± 0.05 (n=2)</td>
<td>-1.54</td>
<td>-1.15 ± 0.08 (n=3)</td>
<td>0.3 ± 0.1 (n=2)</td>
</tr>
<tr>
<td>M. tomcod</td>
<td>-0.8 ± 0.1 (n=3)</td>
<td>-1.15 ± 0.08 (n=3)</td>
<td>0.3 ± 0.1 (n=2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Temperatures of phase change in ooplasm from Gymnodraco acuticeps and Microgadus tomcod eggs

Nuclear Magnetic Resonance (NMR) Spectroscopy and Quantitative Colorimetric Urea Assay

1H-NMR spectra show 80 ± 20 mM TMAO in the ooplasm of dragonfish (Figure 8) but no TMAO was found in the ooplasm from tomcod (Figure 6). The concentration of urea in dragonfish ooplasm (20 ± 2 mM, n=4) was approximately 25% of the concentration of TMAO. Three prominent peaks arising from PDEs containing serine ethanolamine, choline and threonine ethanolamine were identified in the PDE region of 31P-NMR spectra of dragonfish and tomcod ooplasm by “spiking” with known PDEs. The ooplasm of dragonfish contained a total PDE concentration of 137±24 mM (n=6), while the ooplasm of tomcod contained only 75±11 mM (n=4). 31P-NMR spectra acquired following aqueous and organic extractions (Figure 10) suggest that lipid-soluble PDEs are present at significantly greater concentrations than cytosoluble PDEs in dragonfish ooplasm, but the fatty acid composition of the PDEs was not determined. Results from thin-layer chromatography confirmed the presence of phosphatidylcholine in the ooplasm of both species (Figure 11).

Comparing 31P-NMR spectra of tomcod (Figure 7) and dragonfish (Figure 9) ooplasm reveals that the concentration of GPC is significantly higher in the tomcod ooplasm than in dragonfish. Although urea was not assayed in tomcod ooplasm, the comparison is interesting considering that TMAO is absent from tomcod ooplasm, and GPC (which is both a PDE and a methylamine) is used in place of TMAO to counteract the effects of urea on protein destabilization in the kidneys of mammals (Lien et al. 1993). Comparing tomcod and dragonfish 31P-NMR spectra (Figure 7 and 9, respectively) also shows that SEP is found at half of the TEP concentration in tomcod where as in dragonfish ooplasm, SEP is twice the concentration of TEP.
Studies by Van den Thillart and colleagues (1996) suggest that SEP plays a significant role in neural function. SEP and other PDEs may also function in phosphorus mobilization during embryonic growth and development to increase soluble phosphates and contribute to neurological development (Van den Thillart et al. 1996).

**Figure 6.** $^1$H-NMR spectrum of pooled ooplasm from Atlantic tomcod (*Microgadus tomcod*) eggs. The ooplasm was frozen to -60°C after collection and thawed for study. Standard 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS, 200 mM) was used as an external chemical shift and concentration reference. Spectra were acquired continuously over 64 scans with 16,384 data points using a recycle delay of 1.0 sec and pulse width of 3.10 µsec with an average probe temperature of 11.1°C.

**Figure 7.** $^{31}$P-NMR spectrum of pooled ooplasm from Atlantic tomcod (*Microgadus tomcod*) eggs. Methylene-diphosphonic acid (MDPA, 100 mM) was used as an external chemical shift and
concentration reference. The ooplasm was frozen to -60°C after collection and thawed for study. Spectra were acquired continuously over 1,000 scans with 16,384 data points using a recycle delay of 0.5 sec and pulse width of 10 µsec with an average probe temperature of 11.0°C.

Figure 8. $^1$H-NMR spectrum of pooled ooplasm from Antarctic naked dragonfish (*Gymnodraco acuticeps*) eggs. The ooplasm was frozen to -60°C after collection and thawed for study. Standard 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS, 200.2mM) was used as an external chemical shift and concentration reference. Spectra were acquired continuously over 64 scans with 16,384 data points using a recycle delay of 1.0 sec and pulse width of 3.10 µsec with an average probe temperature of 22.0°C.

Figure 9. $^{31}$P-NMR spectrum of pooled ooplasm from Antarctic naked dragonfish (*Gymnodraco acuticeps*) eggs. Methylene-diphosphonic acid (MDPA, 103.2mM) was used as an external chemical shift and concentration reference. The ooplasm was frozen to -60°C after collection and
thawed for study. Spectra were acquired continuously over 1,000 scans with 16,384 data points using a recycle delay of 0.5 sec and pulse width of 10 µsec with an average probe temperature of 22.8°C.

**Figure 10.** $^{31}$P-NMR stacked spectrum of pooled ooplasm from Antarctic naked dragonfish (*Gymnodraco acuticeps*) eggs. Methylene-diphosphonic acid (MDPA, 103.2mM) was used as an external chemical shift and concentration reference. Organic and aqueous extractions were performed according the method of Bligh and Dyer (1959). Spectra were acquired continuously over 1,000 scans with 16,384 data points using a recycle delay of 0.5 sec and pulse width of 10 µsec with an average probe temperature of 22.8°C.

**Thin-Layer Chromatography**

The phosphorylcholine control did not migrate in the phospholipid-selective solvent system (Figure 11). Phosphatidylcholine ($R_f = 28$) was identified in the ooplasm of both species by comparing band migrations to the standard.
**Figure 11.** One-dimensional, thin layer chromatograph of ooplasm from dragonfish (A) and tomcod (C) with phosphatidylcholine (2 mg/ml, B) and phosphorylcholine (2 mg/ml, D) standards. The phospholipid solvent system used consisted of chloroform/methanol/water (65:25:4 v/v). Phosphomolybdic acid (PMA) and ethanol (6g/120ml) was used as the “dipping reagent” and the chromatograph was exposed to iodine vapor for 11 minutes. Ooplasm from dragonfish and tomcod contained lipid-soluble PDEs including phosphatidylcholine ($R_f = 28$).

**High-Pressure Liquid Chromatography**

As indicated in Table 3, total concentration of AFGPs in dragonfish ooplasm ($23.1 \pm 0.7$ mg/ml, n=2) was nearly three-fold the concentration found in tomcod ($8 \pm 2$ mg/ml, n=3). In dragonfish, the sum of AFGP fractions I-VI represented approximately 40.7% of the total AFGP concentration whereas in tomcod this amounted to approximately 50.0%. Elution profiles of dragonfish and tomcod ooplasm are shown in Figures 12 and 13, respectively.

<table>
<thead>
<tr>
<th>Species</th>
<th>AFGP I-V (mg/mL)</th>
<th>AFGP VI (mg/mL)</th>
<th>AFGP VII, VIII (mg/mL)</th>
<th>Total AFGP (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$G. acuticeps$ (n=2)</td>
<td>$6.0 \pm 0.5$</td>
<td>$3.4 \pm 0.5$</td>
<td>$13.77 \pm 0.04$</td>
<td>$23.1 \pm 0.7$</td>
</tr>
<tr>
<td>$M. tomcod$ (n=3)</td>
<td>$3.1 \pm 0.6$</td>
<td>$0.9 \pm 0.5$</td>
<td>$4 \pm 2$</td>
<td>$8 \pm 2$</td>
</tr>
</tbody>
</table>

The combination of ions (Na+/Cl-/K+), TMAO, PDEs and urea in the ooplasm accounted for $96.9 \pm 3.0\%$ of the total osmolality in dragonfish and $103 \pm 14\%$ in tomcod ooplasm.
Figure 12. Elution profile of antifreeze glycoproteins (I-VI) and glycopeptides (VII, VIII) from ooplasm of Antarctic naked dragonfish (*Gymnodraco acuticeps*) eggs. The characterization was performed using a 300 × 7.8 mm Bio-Sil SEC type 125 gel filtration column (flow rate = 1ml/min) with an ISCO V4 absorbance detector and a deuterium lamp setting of 220 nm, calibrated to a sensitivity of 0.1%T. Trichloroacetic acid (TCA) was used as an internal reference.

Figure 13. Elution profile of antifreeze glycoproteins (I-VI) and glycopeptides (VII, VIII) from pooled ooplasm of Atlantic tomcod (*Microgadus tomcod*) eggs. The characterization was performed using a 300 × 7.8 mm Bio-Sil SEC type 125 gel filtration column (flow rate = 1mL/min) with an ISCO V4 absorbance detector and a deuterium lamp setting of 220 nm, calibrated to a sensitivity of 0.1%T. Trichloroacetic acid (TCA) was used as an internal reference.
Discussion

Despite the hypoosmotic nature of tomcod and dragonfish eggs, the presence of AFGPs, PDEs, ions and organic osmolytes depresses the freezing point of the eggs to a temperature well below the freezing point of their subzero marine environments. According to Timasheff (1992), nature has favored the selection of osmolytes which allow the cell to achieve thermodynamic stabilization and osmotic equilibrium on the basis of three criteria: (1) that they should not deleteriously affect enzymes or cellular metabolism; (2) that they should be electrically neutral so they do not to upset the electrostatic equilibrium of the cell; and (3) that they should be a native protein stabilizer and exhibit preferential ligand exclusion.

The 4:1 ratio of TMAO to urea in dragonfish ooplasm suggests that TMAO may have an osmoregulatory effect in the egg, in addition to its stabilizing effect on protein structure (Bedford et al. 1998). In the renal medulla of mammals, GPC acts as the counter-urea osmolyte in place of TMAO (Lien et al. 1993). Although urea was not quantified in tomcod ooplasm, comparing the GPC peak in the $^{31}$P-NMR spectrum of tomcod ooplasm (Figure 7) to dragonfish ooplasm (Figure 9) suggests that urea is likely present in tomcod eggs.

The Potential Role(s) of Noncyclic, Water-Soluble Phosphodiesters (WSPDEs) in Vertebrates

At least six noncyclic-WSPDEs are known to exist in living cells. These compounds include: 1.) glycerophosphorylcholine (GPC); 2.) glycerophosphorylethanolamine (GPE); 3.) glycerophosphorylglycerol (GPG); 4.) threonine ethanolamine phosphodiester (TEP); 5.) serine ethanolamine phosphodiester (SEP); and 6.) lombricine (Van den Thillart and Van Waarde 1996). The structures of known WSPDEs are illustrated in Figure 14. Lipid-soluble PDEs include phosphatidylcholine (PhCho), phosphatidylethanolamine (PhEth) and phosphatidylserine (PhSer).
Six potential roles of water-soluble phosphodiesters (WSPDEs) have previously been described: 1.) PDEs are potential products of phospholipid catabolism; 2.) PDEs are precursor metabolites in phospholipid biosynthesis; 3.) PDEs are endogenous (produced within the cell) inhibitors of lysophospholipase (e.g. lysolecithinase); 4.) Osmoregulation (the active regulation of osmotic pressure) is influenced by PDEs containing a methylamine moiety (a $1^\circ$ amine with a formula of $\text{CH}_3\text{NH}_2$); 5.) Certain phosphodiesters such as serine ethanolamine phosphate (SEP) participate in neural function(s); and 6.) Phosphodiesters may play a role in phosphorus mobilization during embryonic growth and development to increase soluble phosphates. Nonetheless, the metabolic origin and specific utility of phosphodiesters remains elusive (Van den Thillart and Van Waarde 1996).

**Cytosoluble Products of Phospholipid Catabolism**

As indicated in Figure 15, some phosphodiesters found in the NMR spectra are thought to arise by the deacylation of fatty acids from phospholipids by phospholipases A$_1$, A$_2$, and lysophospholipase (Zablocki et al. 1991, Burt et al. 1976). GPE and GPC are possible products of phospholipid degradation whereas SEP and TEP are formed by synthetase reactions (Van den
Although studies have indicated high levels of GPC in heart and skeletal muscle, Burt and colleagues (1976) found low phospholipase A\textsubscript{1} activity and negligible amounts of lysophospholipase - a required enzyme for complete phospholipid deacylation. Based on these findings, it was concluded that GPC likely arises from some mechanism other than phospholipid degradation in heart and skeletal muscle of certain species.

**Figure 15.** Phospholipid deacylation is thought to account for the formation of cytosoluble phosphodiesters. In this metabolic cycle, phosphates containing a choline (methylamine) moiety are shown as an example, based on a modified version of the phospholipid metabolism cycle proposed by Burt and Ribolow (1994).

**Phosphodiesters as Precursors to Phospholipid Biosynthesis**

According to Van den Thillart and Van Waarde (1996), only one study has focused on determining whether phospholipids can be directly synthesized from phosphodiesters. An initial condition necessary in all precursor-product relationships is a greater increase in precursor...
activity than that of the product (Chalovich and Bárány 1979). Using radioactive $[^3]H$ serine, Chalovich and Bárány (1979) demonstrated that SEP turnover in the dystrophic pectoralis muscles of chickens was significantly slower than the serine moiety of phospholipids, indicating that SEP is not a direct precursor of phosphatidylerine or phosphatidylethanolamine. Because SEP in avian muscle parallels the appearance of GPC in mammalian tissues (Burt and Chalovich 1978), it is conceivable along similar reasoning that GPC (which is a marker for human muscular dystrophy) is not a direct precursor to phosphatidylcholine.

**Lysophospholipase Activity is Inhibited by Phosphodiesters**

Glycerophosphorylcholine is formed during the catalyzed deacylation of lysophosphatidylcholine by the enzyme lysolecithinase (Ribolow et al. 1981). In semen, seminal plasma, and sperm fractions from salmon and humans, studies by Ribolow and colleagues (1981) indicate that lysolecithinase activity may be inhibited between 30% and 60% by GPC.

**TMAO from Phosphatidylcholine Hydrolysis**

According to Seibel and Walsh (2002), the hydrolysis of phosphatidylcholine (PhCho) is hypothesized to result in the formation of TMAO through the glycerophosphate pathway indicated in Figure 1. During the embryogenesis of tomcod and dragonfish eggs, a relationship may exist between the concentration of TMAO and PhCho. Since TMAO is considered a better counteracting osmolyte than betaine (Seibel and Walsh 2002) or PhCho, it would be advantageous for the egg to convert phosphatidylcholine to TMAO. In dragonfish eggs, the low concentration of PhCho relative to other PDEs may be an indicator of active TMAO synthesis. In tomcod ooplasm, the concentration of PhCho was significantly higher than in dragonfish ooplasm, but no TMAO was found. It is plausible that a counteracting osmolyte other than TMAO is used in tomcod eggs if urea is indeed present. Previous studies indicate that TMAO functions optimally as a counteracting osmolyte when in a ratio of 1:2 with urea (Barimo et al. 2004). Although TMAO is suspected of being synthesized from PhCho, it also is known to be obtained from dietary choline that is oxidized by bacteria in the gut of marine vertebrates (Seibel and Walsh 2002).
Figure 16. Hypothesized pathway for the synthesis of TMAO from the hydrolysis of phosphatidylcholine based on a modified version of the glycerophosphate pathway proposed by Seibel and Walsh (2002). During the formation of betaine from ethanolamine, choline may also be oxidized to TMAO and either stored or excreted. The concentration of phosphatidylcholine may be replenished by the trimethylation of ethanolamine.

Further study of tomcod and dragonfish ooplasm is necessary to determine if these PDEs are products of phospholipid catabolism or if they play a role as precursor metabolites in phospholipid assembly. Based on the findings of this study, it appears that tomcod and dragonfish eggs employ mechanisms of freeze avoidance similar to one another and also to adult fishes in each species. Future studies on PDEs and their metabolic significance in both species may provide further insight into a more intricate mechanism of freeze avoidance in marine teleosts.
References


21. Raymond, J.A. Seasonal variations of trimethylamine oxide and urea in the blood of a cold-adapted marine teleost, the rainbow smelt. *Fish Physiology and Biochemistry* 13:


