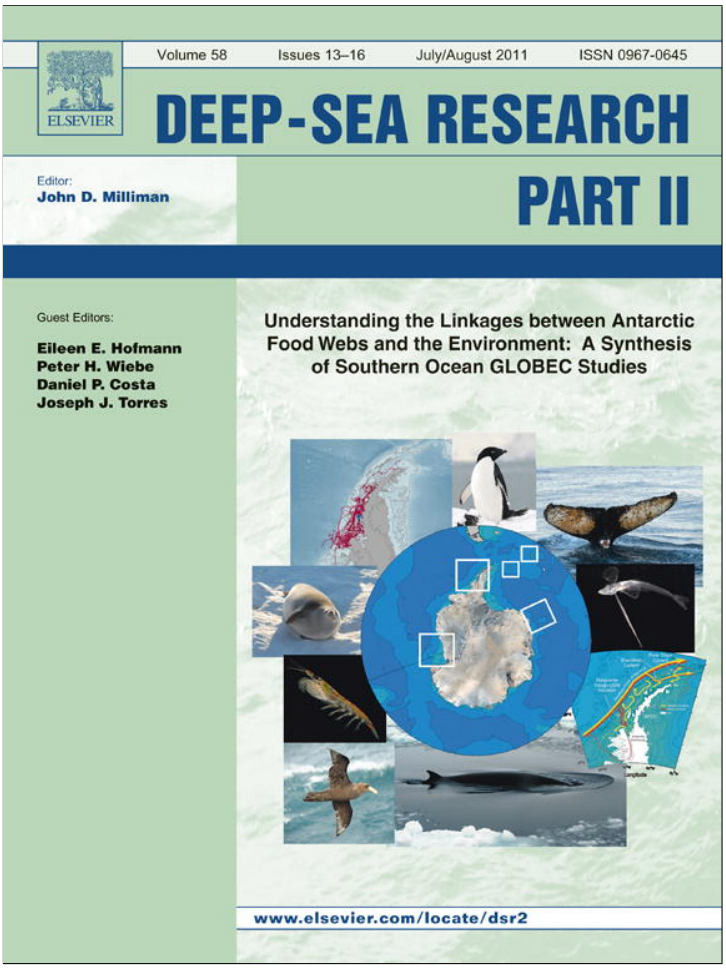

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Antifreeze proteins in pelagic fishes from Marguerite Bay (Western Antarctica)

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ABSTRACT

The Southern Ocean is home to two major types of fishes: the largely endemic suborder Notothenioidei and representatives of oceanic fish families that are widely distributed in the midwater and benthic environments elsewhere (e.g. bathylagids, myctophids, liparids, and zoarcids). In most regions of the coastal Antarctic, e.g. the Ross Sea, there is a distinct separation in the pelagic communities at the shelf break between the oceanics (off-shelf) and the endemics (on-shelf). Coincidentally, in much of the coastal Antarctic, the shelf break also marks the boundary between a water column entirely composed of the very cold (−2 °C) Ice Shelf Water and an oceanic profile that includes warmer circumpolar deep water (2 °C at 200 m) at intermediate depths. The distinct separation in pelagic communities observed in most coastal regions of the Antarctic is not seen on the western Antarctic Peninsula (WAP), where circumpolar deep water intrudes to form a warmer midwater and oceanic species are strongly represented. It was hypothesized that the cold ice-shelf water, lethal to fishes without antifreeze glycoproteins (AFGPs) in their blood, was excluding the oceanic species from most of the Antarctic continental shelf waters. To test the hypothesis, nine species of fish captured in WAP shelf waters were tested for the presence of AFGPs. The oceanic fish families analyzed: Myctophidae (*Electrona* and *Gymnoscopelus*), Zoarcidae (*Melanostigma*), Gempylidae (*Paradiplospinus*), Paralepididae (*Notolepis*), and Bathylagidae (*Bathylagus*) showed no antifreeze activity. Two endemic species captured in the same sampling program did show antifreeze activity: the important pelagic species *Pleuragramma antarcticum* (Nototheniidae) and the Bathydraconid species *Vomeridens infuscipinnus*. The absence of AFGPs in the blood of Antarctic oceanic species makes a strong case for temperature exclusion of oceanic fishes in the coastal Antarctic.

1. Introduction

The Southern Ocean is home to two major types of fishes: the largely endemic suborder Notothenioidei and representatives of oceanic fish families that are globally distributed in the midwater and benthos (e.g. bathylagids, myctophids, liparids, and zoarcids (Andriashev, 1965; DeWitt, 1965, 1970, 1971; Ekau, 1990; Hubold, 1991; Clarke and Johnston, 1996; Greely et al., 1999). The Notothenioidei are primarily benthic as adults. However, several species make substantial excursions into the pelagic realm either as part of their juvenile life history (e.g. *Cryodraco antarcticus*) or episodically throughout their life, such as *Aethotaxis mitopteryx* (Eastman, 1993). Others, such as *Pagothenia borchgrevinki*, use the underside of sea ice as a makeshift benthos and are classified as cryopelagic.

Two species of Antarctic notothenioids are notable for having adapted a completely pelagic lifestyle. The first, the Antarctic silverfish, *P. antarcticum*, is the most abundant pelagic fish in most Antarctic coastal waters. The second is the large piscine predator, the Antarctic toothfish *Dissostichus mawsoni*. Both play important roles in the trophic scheme of the Antarctic coastal pelagic zone, which in most cases is characterized by a temperature of about −2 °C from surface to bottom and a fish fauna consisting almost exclusively of *Pleuragramma* and *Dissostichus* (Andriashev, 1965, 1970; DeWitt, 1965, 1971; Hubold, 1991; Eastman, 1993, 1997; Clarke and Johnston, 1996). The coastal benthos is also dominated by the notothenioids with a few other more cosmopolitan families represented as well, e.g. Rajidae, Muraenolepidae, Zoarcidae, Liparidae, and Bothidae (Andriashev, 1965, 1970; DeWitt, 1965, 1971; Hubold, 1991; Eastman, 1993, 1997; Clarke and Johnston, 1996).

The shelf waters of the Western Antarctic Peninsula (WAP) have a very different faunal mix. Here there is a strong representation by the Myctophidae, a globally distributed oceanic family (Donnelly and Torres, 2008). The difference in faunal composition may be a result of the warmer profile found in the water column. Because of its position relative to the Antarctic Circumpolar Current (ACC), warm Circumpolar Deep Water (CDW) (2 °C) episodically intrudes onto the WAP shelf, making the intermediate depths (200–400 m) warmer here than in the remainder of the coastal Antarctic

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(Dinniman and Klinck, 2004; Klinck et al., 2004; Clarke et al., in press; Martinson et al., 2008). For example, the shelf regions of the Ross and Weddell Seas are both uniform in temperature (−2 °C) from top to bottom because of the very cold ice-shelf water that dominates the water column in both locations (Dinniman et al., 2003; Donnelly et al., 2003). The uniformly cold water likely has a profound influence on the faunal diversity in most of the coastal Antarctic.

Fishes in the perciform suborder Notothenioidei are able to survive in the cold shelf waters of the Ross and Weddell Seas because of a unique adaptation: the presence of biological antifreezes in their blood (DeVries and Wohlschag, 1969; DeVries, 1970, 1986; DeVries and Lin, 1977; DeVries and Cheng, 2005; Cziko et al., 2006). Like most vertebrates, fishes have an internal osmotic pressure (OP) of 300–500 m Osm, or about 30–50% that of seawater (DeVries and Lin, 1977; Hickman and Trump, 1969). Because of their low OP, without the aid of the antifreeze compounds their body fluids would rapidly freeze at the ambient temperature of −2 °C. The biological antifreezes found in the notothenioids prevent ice crystals from propagating in their blood and other body fluids (DeVries and Wohlschag, 1969; DeVries, 1970, 1986; DeVries and Lin, 1977), allowing them to survive in ice-laden waters that would be rapidly lethal for most fishes.

Antifreezes are fairly small molecules (2600–33,700 daltons), which can be either glycoproteins (AFGPs) or peptides (AFPs). There are at least eight different size classes of glycoproteins with "1" being the largest (33,700 daltons) and "8" being the smallest (2600 daltons). All of the AFGPs consist of an alanine/proline–alanine–threonine backbone with threonine O-glycosylated to a disaccharide (DeVries and Cheng, 2005). The two smallest size classes are normally the most abundant in the body fluids.

Antifreeze glycoproteins are found not only in the notothenioids, but also in several northern Arctic gadids. Though highly similar in structure (Chen et al., 1997a,b) and molecular masses, the AFGPs in the Arctic and Antarctic fishes evolved at different times and used different genetic "raw material" to create the biological antifreezes (Hochachka and Somero, 2002). The Antarctic has been cold enough to be a threat to ichthyofauna for 5–14 million years, with the formation of persistent ice sheets and coastal temperatures below −1 °C (Chen et al., 1997a). In contrast, the Arctic glaciation was a more recent event: 1–2 million years ago. The difference in time of appearance and genetic coding for the AFGPs in the two different groups of fishes argues strongly for two separate evolutionary origins of the AFGP structure, an elegant example of convergent evolution (Chen et al., 1997b).

Antifreeze peptides were first identified in the winter flounder, *Pseudopleuronectes americanus* (Duman and DeVries, 1974). Subsequently, three more types of antifreeze peptides have been found in fishes, making a total of four recognized types (Hochachka and Somero, 2002). They can be found in Arctic fishes, northern temperate fishes, and two Antarctic zoarcid fishes (DeVries and Cheng, 2005). Antifreeze peptides differ in amino acid sequence as well as secondary and tertiary structures, whereas AFGPs only differ in the number of Ala/Pro–Ala–Thr repeats (DeVries and Cheng, 2005).

Both types of antifreezes (AFGP and AFP) work by binding to the face of a growing ice crystal and inhibiting its growth (DeVries, 1986). When the antifreeze binds to a small seed crystal of ice, no growth is observed until the non-equilibrium freezing point or the thermal hysteresis freezing point is reached (DeVries,1971). At that point, growth occurs as a rapid burst of spicules from the ice crystal. The spicules grow in the non-preferred growth axis or *c*-axis (DeVries, 1986) with the concentration of AFGP determining its character. Samples containing more AFGP have smaller spicules and those with lower AFGP concentrations develop hexagonal bipyramids.

The antifreezes of Antarctic notothenioids allow them to dwell in shelf waters throughout the Antarctic, where they are the overwhelmingly dominant group (Andriashev, 1965; DeWitt, 1970). The fact that oceanic fishes like the myctophids, paralepidids, and bathylagids are not found in most of the Antarctic coastal system, but can be found in the warmer waters of the WAP shelf, suggests that temperature may be playing an important role in their distribution (DeWitt, 1970; Eastman, 1993, 1997; Clarke and Johnston, 1996; Donnelly et al., 2003; Donnelly and Torres, 2008). To investigate this possibility we examined 9 Antarctic fish species, including 5 oceanic species, to determine if antifreeze was present in their blood.

2. Methods

Fishes for the study were captured during the fall process cruise of the Southern Ocean Global Ocean Ecosystems Dynamics Program conducted in the vicinity of Marguerite Bay (SO GLOBEC III—April 2002–May 2002, ARSV Lawrence M. Gould), Antarctic Peninsula (Hofmann et al., 2004). Fishes were collected using either a 10 m^2 MOCNESS net system or a 2.25 m^2 Tucker trawl, both with closed cod-ends to minimize damage to specimens. Trawls were conducted from the surface to 1000 m. Immediately upon recovery the cod-ends were emptied and fishes were extracted and placed on ice. Blood was collected by wicking it up from the caudal vein into a heparinized capillary tube. The blood was then frozen and maintained at −80 °C for the remainder of the cruise (a maximum of 5 weeks). After the cruise, samples were shipped to St. Petersburg, FL, on dry ice where they were again stored at −80 °C until analyzed. Samples were taken from the following species: *P. antarcticum* ($N=20$), *Electrona antarctica* ($N=10$), *Gymnoscopelus nicholsi* ($N=5$), *G. braueri* ($N=5$), *Melanostigma gelatinosum* ($N=2$), *Paradiplospinus gracilis* ($N=1$), *Notolepis coatsi* ($N=1$), *Vomeridens infuscipinis* ($N=1$), and *Bathylagus antarcticus* ($N=1$). All of the fishes analyzed were adults.

Blood samples were analyzed using either a Clifton or an Otago nanoliter osmometer. Before analysis each sample was thawed and approximately 5 μl of blood was drawn up into each of the two 10 μl capillary tubes. One end of each tube was sealed using a Bunsen burner and both tubes were centrifuged for 1–2 min at 9000 rpm in an Eppendorf micro-centrifuge. After centrifugation, both tubes were removed, and the pellet, which contained red blood cells and other material, was discarded. A sample of the supernatant was then drawn up into the sample loader: a hand-drawn glass needle filled with Cargille Type A immersion oil. A small drop of sample was placed into the center of all 6 wells in the sample holder, which had been filled with Cargille Type B heavy immersion oil. Each drop was approximately half the diameter of one of the six 500 μm diameter sample wells, and as close to the center as possible. The entire sample holder was placed on a small (4 × 6 cm) Peltier apparatus that allowed fine regulation of sample temperature, but was small enough to fit easily on the stage of a dissecting microscope. The sample holder was then covered with a glass cover slip and cooled until frozen using the osmometer's freezing cycle. A trickle of dry nitrogen was blown across the cover slip to prevent condensation. Sample wells were viewed at a magnification of 70 × to determine the melting point and freezing point for each sample.

The freezing cycle of the Otago nanoliter osmometer lowered the sample's temperature to −20 °C. To determine the melting point of a single sample, the temperature was initially raised to −2 °C. From this point the temperature was slowly raised until a single ice crystal, spanning approximately 1/4 of the diameter of the blood drop, remained and was stable. This was recorded as the melting point for that sample. After determining the melting point,

the temperature was lowered approximately .01 °C every 15 s until the ice crystal was observed to grow uncontrollably. This was recorded as the freezing point of the sample. The difference (if any) between the melting point and freezing point was recorded as the thermal hysteresis. After the initial reading, the six samples in the sample holder were again put through the machine's freezing cycle and a different well chosen for analysis. This sequence was repeated until all of the wells were analyzed. An average melting point, freezing point, and thermal hysteresis were calculated for each fish as well as an overall average melting point, freezing point, and thermal hysteresis for each species. The thermal hystereses among all species were then analyzed for significant differences using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test to determine homogeneous groups. Only species with $N > 1$ were used in the ANOVA.

3. Results

The oceanic fish families analyzed: Myctophidae (*Electrona* and *Gymnoscopelus*), Zoarcidae (*Melanostigma*), Gempylidae (*Paradiplospinus*), Paralepididae (*Notolepis*), and Bathylagidae (*Bathylagus*) showed no antifreeze activity. The small hysteresis (Table 1) that occurred was a result of lag between the readout and the actual temperature at the sample holder. A thermal hysteresis of the same magnitude was observed during calibrations with deionized water. In contrast, the nototheniid (*Pleuragramma*) and bathydraconid (*Vomeridens*), two species from Antarctic endemic families, did show unequivocal antifreeze activity. Analysis of variance showed a significant ($df=37$, $F=32.5$, $p<0.0001$) difference between the nominal hysteresis observed in oceanic fishes and the more pronounced difference between melting and freezing point in the endemics. The difference was corroborated by a Duncan multiple range test, where the oceanics grouped together leaving *Pleuragramma* alone in its own group ($P<0.0001$).

Patterns of ice crystal growth observed in the samples correlated well with their level of hysteresis (Table 1) and with literature descriptions of ice crystal growth in blood with and without antifreezes (DeVries, 1986). As soon as the solidly frozen samples began to melt, a difference was observed between the crystal structure in *Pleuragramma* and *Vomeridens* when compared to those of the other fish species. Many straight lines could be observed in the ice crystals of *Pleuragramma* and *Vomeridens*, and when the sample had melted down to a single stable crystal it appeared square, rectangular, or trapezoidal in shape, with clear facets present. In contrast, samples from the other fishes contained almost no faceting and the solid piece of ice within the well broke into many small spheres while it was melting, resulting in a final ice crystal either spherical or oblong in shape. Ice growth in *Pleuragramma* samples took place at the edges (*a*-axis) of the seed crystal disk, producing a starburst pattern with many spikes emanating from the central crystal in all directions. The ice growth pattern for *Vomeridens infuscipinnis* was also planar in nature, but the pattern exhibited was hexagonal bipyramidal, with the ice crystal growing from either end (*c*-axis growth) to form a long spike. The ice growth pattern for all other species was spherical in nature with the ice crystals growing equally from all sides of the seed disk.

Interestingly, with the exception of the zoarcid *M. gelatinosum*, the oceanic species uniformly exhibited a slightly higher blood osmotic pressure than the two notothenioids (means: 446 vs. 369 m Osm), though the difference was not enough to provide any physiological refuge from cold temperature.

4. Discussion

Based on the lack of a hysteresis seen in the oceanic midwater fishes (*E. antarctica*, *G. nicholsi*, *G. braueri*, *M. gelatinosum*, *P. gracilis*, *N. coatsi*, and *B. antarcticus*) and their pattern of ice crystal growth, it can be concluded that they do not contain AFGPs or AFPs. The lack of antifreeze activity in oceanic species suggests that they would rapidly freeze in the presence of ice at the −2 °C temperatures typical of the Ross and Weddell Sea shelves. However, on the WAP shelf, where temperatures are typically well above 0 °C at intermediate depths (200–400 m, Klinck et al., 2004), the oceanic fishes are easily able to survive. The low levels of antifreeze in Pleuragramma most likely only protect them from limited contact with ice, which may be why the majority of the silverfish population is found below 100 m of depth (Lancraft et al., 2004; Donnelly and Torres, 2008). The hysteresis observed in Marguerite Bay fish is smaller (mean=0.45 °C) than that found in fish captured from McMurdo Sound (mean=0.91 °C—Cziko et al. (2006)), which could be a result of warmer temperatures in the WAP. Jin and Devries (2006) found that some Antarctic fishes are able to adjust their AFGP levels based on temperature exposure. With more CDW entering Marguerite Bay Pleuragramma may not need as much AFGP for protection because of the warmer layer (2 °C) at intermediate depths, which does not exist in McMurdo Sound.

Table 1
Mean (± 95% confidence interval) melting point, freezing point, and thermal hysteresis of fishes sampled. N=# of fish sampled, n=# of trials per fish, and SL is the mean standard length of fish sampled for that species. Blood osmotic pressure (OP) was calculated from the mean of the melting point readings.

Family *Species*	*N*	*n*	SL (mm) (range)	MP (°C)	FP (°C)	Hysteresis	Blood OP (m Osm)
Nototheniidae							
Pleuragramma antarcticum	20	2–3	157 (133–190)	0.71 ± 0.05	1.16 ± 0.07	0.45 ± 0.05	382
Myctophidae							
Gymnoscopelus nicholsi	5	2	155 (140–165)	0.75 ± 0.08	0.82 ± 0.08	0.07 ± 0.01	404
Gymnoscopelus braueri	5	2	133 (95–151)	0.84 ± 0.10	0.90 ± 0.10	0.06 ± 0.01	452
Electrona antarctica	10	2	94 (87–100)	0.77 ± 0.08	0.84 ± 0.08	0.06 ± 0.01	414
Zoarcidae							
Melanostigma gelatinosum	2	3	160 (155–160)	0.56 ± ± 0.01	0.63 ± 0.01	0.07 ± 0.01	301
Gempylidae							
Paradiplospinus gracilis	1	2	360	0.88 ± 0.02	0.95 ± 0.01	0.07 ± 0.01	474
Paralepididae							
Notolepis coatsi	1	3	178	0.89 ± 0.15	0.96 ± 0.15	0.07 ± 0.02	479
Bathydraconidae							
Vomeridens infuscipinis	1	3	156	0.66 ± 0.13	1.29 ± 0.25	0.62 ± 0.12	355
Bathylagidae							
Bathylagus antarcticus	1	2	158	0.84 ± 0.01	0.91 ± 0.01	0.07 ± 0.02	452

Our results agree substantially with those of Wohrmann (1995, 1996, 1998) and Wohrmann and Haselbeck (1997) who also found strong evidence of antifreezes in Pleuragramma, though the techniques employed were very different.

A remaining potential problem for the vertically migrating oceanic fishes is the presence of very cold winter water (WW; −1.5 to −1.8 °C) that results from winter surface cooling, forming a very cold mixed layer in the in the upper 100 m during the winter months. During spring and summer the surface water warms to form Antarctic surface water (AASW), which mixes with WW during the warmer seasons to raise the uppermost 50 m to temperatures above −1 °C (Smith et al., 1999). However, a cold intermediate layer of WW persists at depths of 60–80 m (Klinck et al., 2004) until the strong winds of fall mix the WW with the warmer waters above and below, removing the WW layer until the cold winter temperatures initiate WW formation at the surface to begin the process once again.

Though the other oceanic species treated in the study are rarely found above 100 m, *Electrona* has been shown to migrate into the upper 50 m in other regions of the Antarctic (Ainley et al., 1986; Torres and Somero, 1988) as well as occasionally in the waters of the WAP (Donnelly and Torres, 2008). Thus, a layer of winter water could potentially be a threat, particularly since its preferred prey, *E. superba,* is most abundant in the upper 50 m during much of the year in waters of the WAP shelf (Quetin et al., 1996; Lascara et al., 1999; Lancraft et al., 2004). Given that the persistent layer of WW is well beneath the surface, where the threat of nucleation by ice crystals would be absent, temporary undercooling of the fishes would almost certainly allow survival in most encounters with persistent WW. Further, the vertical distribution of winter krill shows a substantial fraction of the population below 50 m (Lascara et al., 1999; Ashjian et al., 2004), allowing oceanic fishes to avoid the upper 100 m in winter and still have access to their preferred prey. Oceanic fishes were virtually absent in the upper 100 m in our winter trawls (Donnelly and Torres, 2008)

The presence of oceanic species on the Western Antarctic Peninsula shelf, but not in waters of the Ross Sea or Weddell Sea shelves, suggests that temperature is indeed an important limiting factor in the distribution of oceanic fishes in Antarctic waters, a surmise that has been tacitly assumed to be true, but up to now, never definitively tested (cf. Moline et al., 2008; Clarke et al., in press). There is an abrupt transition from oceanic fauna to Antarctic endemics at the shelf break in the Ross Sea (DeWitt, 1970; Donnelly et al., 2003), the best studied of the Antarctic coastal regions with respect to fish distributions. Other than differences in the water column temperature there are few obvious differences that would stop oceanic species from residing in the shelf waters of the Ross Sea. For example, the shelf depths in the Ross Sea and Marguerite Bay are similar enough so that depth would not be limiting. Dinniman et al. (2003) modeled the circulation in the Ross Sea and found that warm CDW does periodically intrude onto the shelf of the Ross Sea, which could introduce oceanic species onto the Ross Sea shelf as similar intrusions apparently do on the WAP.

One other item of interest to consider is the possible competition between *E. antarctica* and *P. antarcticum,* the two dominant fishes in the oceanic and coastal ecosystems, respectively. Both fish have similar diets: copepods in their early life history and krill later in life (Hubold, 1985; Lancraft et al., 2004), and they are both vertical migrators with pelagic eggs. The main differences between them occur in their rate of growth and age at first reproduction. *E. antarctica* lives for 4 years and reproduces in its last year (Greely et al., 1999). It is long lived and slow growing in comparison to other myctophids, but not when compared to the nototheniid *P. antarcticum. Pleuragramma* lives for 21 years and reproduces in its ninth year of life (Hubold and Tomo, 1989; Reisenbichler, 1993). Without the presence of antifreeze in *P. antarcticum*, *E. antarctica* might be able to out-compete it in the coastal regions of the WAP. In fact, this competition may already be occurring because *P. antarcticum* is apparently becoming scarcer in the mid-regions of the Antarctic Peninsula (Ducklow et al., 2007) as the peninsula warms and ice cover are reduced. With the continued warming trend (Smith and Stammerjohn, 2001, Ducklow et al., 2007), *P. antarcticum* may have a hard time competing in the peninsular region of Antarctica.

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