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Antifreeze Proteins in Pelagic Fishes from Marquerite Bay (Western Antarctica)

by

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

The Southern Ocean is home to two major types of fishes: endemics in the suborder Notothenioidii 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 (AFGP's) 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 AFGP's. The oceanic fish

families analyzed: Myctophidae (*Electrona* and *Gymnoscopelus*), Zoarcidae

(*Melanostigma*), Gempylidae (*Paradiplospinus*), Paralepididae (*Notolepsis*), 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 (*Vomeridens*). The

absence of AFGP's in the blood of Antarctic oceanic species makes a strong case for

temperature exclusion of oceanic fishes in the coastal Antarctic.

Introduction

The Southern Ocean is home to two major types of fishes: endemics in the suborder Notothenioidii 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, DeWitt 1968, DeWitt 1970, DeWitt 1971, Ekau 1990, Hubold 1991, Clarke & Johnston 1996, Greely et al. 1999). The notothenioidii are primarily benthic as adults with the exception of the Antarctic silverfish, *Pleuragramma antarcticum*, and the toothfish, *Dissostichus mawsoni*, which are both found in the coastal pelagic zone (Andriashev 1965, DeWitt 1965, DeWitt 1968, Andriashev 1970, DeWitt 1971, Hubold 1991, Eastman 1993, Clarke & Johnston 1996, Eastman 1997,). The coastal benthos is dominated by the notothenioids with a few other families (e.g. Rajidae, Muraenolepidae, Zoarcidae, Liparidae, and Bothidae) represented (Andriashev 1965, DeWitt 1965, DeWitt 1968, Andriashev 1970, DeWitt 1971, Hubold 1991, Eastman 1993, Clarke & Johnston 1996, Eastman 1997,).

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 & Torres 2008). This difference in faunal

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representation may be a result of the warmer profile found in the water column. Because of its position relative to the Antarctic Circumpolar Current, warm Circumpolar Deep Water (CDW) (2°C) episodically intrudes onto the shelf, making the intermediate depths (200-400 m) warmer here than in the remainder of the coastal Antarctic (Dinniman and Klinck 2004, Donnelly & Torres 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 Notothenioidii 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, without which they would freeze (Devries and Wohlschlag 1969, Devries 1970, Devries & Lin 1977, Devries 1986, Devries & Cheng 2005, Cziko et al. 2006,). Like most vertebrates, fishes have an internal osmotic pressure (OP) of 300-500 mOsm, about 1/3 to ½ 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 should freeze long before the seawater surrounding them. The antifreeze which the notothenioids possess prevents ice crystals from propagating in their blood and other body fluids (Devries and Wohlschlag 1969, Devries 1970, Devries & Lin 1977, Devries 1986), thereby allowing them to survive in the ice-laden waters that would be rapidly lethal to most fishes.

Antifreezes are fairly small molecules (2,600-33,700 daltons), that can be either peptides or glycoproteins. Antifreeze proteins (AFP) were the first to be discovered (Devries and Wohlschlag 1969). There are at least 8 different size classes of glycoproteins with 1 being the largest (33,700 daltons) and 8 being the smallest (2,600 daltons). All of the AFGP's consist of an alanine/proline-alanine-threonine backbone with threonine O-glycosylated by a disaccharide (Devries and Cheng 2005); The two smallest size classes are normally the most abundant in the body fluids. The same antifreeze glycoproteins are found not only in the notothenioids, but also in several northern Arctic gadids. Antifreeze peptides (AFP) were first identified in the winter flounder, *Pseudopleuronectes americanus* and can be found in Artic, northern temperate fish, and two Antarctic zoarcid fishes (Devries and Cheng 2005). Three more types of antifreeze peptides have been found since their initial discovery. These antifreeze peptides differ in protein sequence and secondary and tertiary structures, where as the AFGP only differ in the number of Ala/Pro-Ala-Thr repeats (Devries and Cheng 2005).

Both types of antifreeze (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 the small seed crystal of ice, no growth is observed until the non-equilibrium freezing point or the thermal hysteresis freezing point is reached. At that point growth occurs as a rapid burst of spicules from the ice crystal. The spicules grow in the non-preferred axis of growth or c-axis. The concentration of AFGP also affects the shape of ice crystal growth, with samples containing more AFGP having smaller spicules and those with lower AFGP

concentrations having more of a starburst/firework pattern of growth (personal

observation).

The antifreezes of the 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. (Andriashev 1965, DeWitt 1965, DeWitt

1968, DeWitt 1970, DeWitt 1971, Ekau 1990, Hubold 1991, Eastman 1993, Clarke &

Johnston 1996, Eastman 1997, Greely et al. 1999, Donnelly et. al 2003, Donnelly &

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.

Methods and Materials

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 (Fig 1), (SO GLOBEC III April 2002- May 2002, ARSV Lawrence M.

Gould) Antarctic Peninsula (Hofmann et al. 2004). Fishes were collected using either a

$10m^2$ MOCNESS net system or a 2.25 m^2 Tucker trawl, both with closed codends, to

minimize damage to the specimens. Trawls were conducted from the surface to 1000m.

Immediately upon recovery the cod ends were emptied and fish were extracted and

placed on ice. Blood was collected by wicking it up into a heparinized capillary tube from

the caudal vein. The blood was then frozen at -80°C and shipped to St. Petersburg, FL

where it was stored in a -80°C freezer until analysis. Samples were taken from the caudal

veins of *Pleuragramma 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 fish that had blood removed, as well as

representatives of the species caught, were also frozen whole and shipped to Saint

Petersburg, FL. All of the fishes analyzed were adults. Additional blood samples were

wicked out of the pericardial cavity of the frozen fish analyzed *Melanostigma*

gelatinosum (N=1), *Vomeridens infuscipinis* (N=1), and *Bathylagus antarcticus* (N=1).

Figure 1: GLOBEC III cruise track and study area

a)

b)
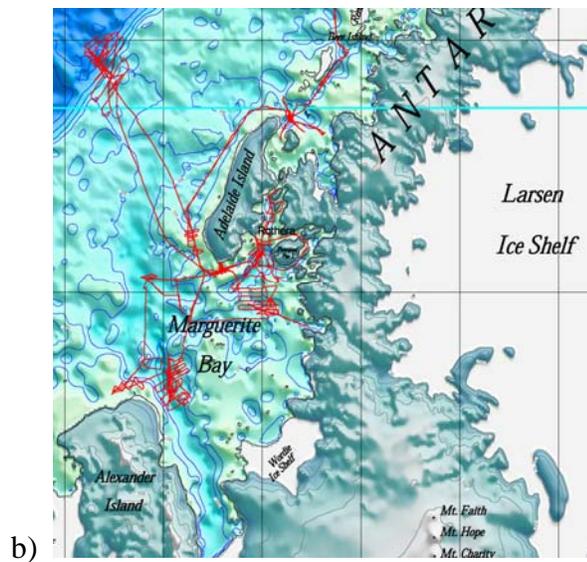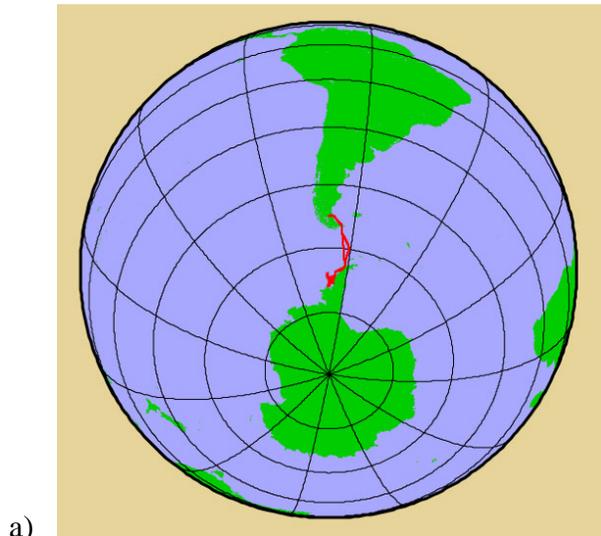
a) Global view of cruise track (red line) for GLOBEC III cruise from Chile to Western

Antarctic Peninsula.

b) Study area cruise track (red line) showing Antarctic circle (neon blue line).

Blood samples were analyzed using either a Clifton nanoliter osmometer or an Otago nanoliter osmometer. Before analysis each sample was thawed and approximately 5µl of blood was drawn up into each of two 10 µl capillary tubes. One end of each tube was sealed using a Bunsen burner and both tubes were spun down at 9000 RPM's in an Eppendorf micro-centrifuge. The *Pleuragramma antarcticum* samples were spun down for 1 minute and all other samples were spun down for 2 minutes because of the large concentration of tissue in the blood samples. Both tubes were removed and the bottom portion, which contains red blood cells and other material, was discarded. A sample 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 then placed into the center of all 6 wells in the sample holder, which had been filled with Cargille Type B immersion oil and was located in the peltier apparatus that allowed regulation of sample temperature. Each drop was approximately half the diameter of the well and as close to the center as possible. A small amount of dry nitrogen was blown across the sample holder to prevent condensation in the sample area. The sample compartment was covered with a glass cover slip and frozen solid using the machine's freezing cycle. A microscope was used to view the sample wells at 70x and a melting point and freezing point were determined for each sample.

The freezing cycle of the Otago nanoliter osmometer lowered the sample's temperature to -20°C. In order to determine the melting point of a single sample, the temperature was manually raised to -2°C. From this point the temperature was slowly

raised until a single ice crystal, spanning approximately ¼ of the diameter of the blood

drop, remained and was stable. This point was recorded as the melting point for that

sample. From here the temperature was lowered approximately .01 °C every 15 seconds

until the ice crystal was seen to grow uncontrollably. This point 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. The six samples were again put

through the machine's freezing cycle and a different well chosen for analysis. This was

repeated until all of the wells were analyzed. An average melting point, freezing point,

and thermal hysteresis for that fish was then calculated as well as an overall average

melting point, freezing point and thermal hysteresis for all the fish analyzed from that

species. The average thermal hysteresis for each fish analyzed was then entered into

STATISTICA and an ANOVA was preformed. A Duncan Multiple Range Test was then

preformed to determine if groups were homologous.

Results

Table 1: Mean melting point, freezing point and thermal hysteresis of fish sampled

Fish family	Fish species	N	n	SL (mm)	MP (-°C)[a]	FP (-°C)[a]	Hysteresis	Osmotic Pressure of blood (mOms) [b]
Nototheniidae								
	Pleuragramma antarcticum	20	3-4	157	0.71 ± 0.05	1.16 ± 0.07	0.45 ± 0.05	382
Myctophidae								
	Gymnoscopelus nicholsi	5	2	155	0.75 ± 0.08	0.82 ± 0.08	0.07 ± 0.01	404
	Gymnoscopelus braueri	5	2	133	0.84 ± 0.10	0.90 ± 0.10	0.06 ± 0.01	452
	Electrona antarctica	10	2	94	0.77 ± 0.08	0.84 ± 0.08	0.06 ± 0.01	414
Zoarcidae								
	Melanostigma gelatinosum	2	3	160	0.56 ± 0.01	0.63 ± 0.01	0.07 ± 0.01	301
	Melanostigma gelatinosum (Whole fish)	1	4	172	1.04 ± 0.01	1.14 ± 0.07	.10 ± 0.02	560
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
	Vomeridens infuscipinis (Whole fish)	1	6	156	0.98 ± 0.26	1.64 ± 0.18	0.80 ± 0.17	527
Bathylagidae								
	Bathylagus antarcticus	1	2	158	0.84 ± 0.01	0.91 ± 0.01	0.07 ± 0.02	452
	Bathylagus antarcticus (Whole fish)	1	1	142	0.79	0.85	0.06	425

N = # of fish sampled, n = # of trials run per fish, SL is the average standard length of fish sampled for that species; a: mean ± 95% confidence interval; b: Osmotic pressure was calculated from the melting point readings.
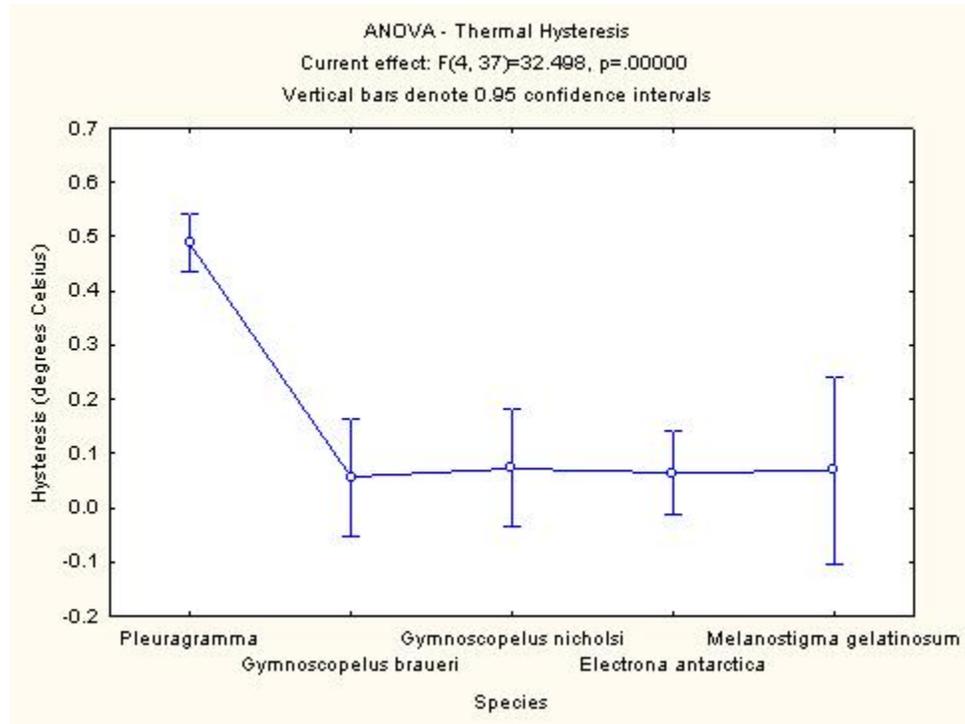
Figure 2 : ANOVA

ANOVA - Thermal Hysteresis
Current effect: $F_{(4, 37)}=32.498$, p=.00000
Vertical bars denote 0.95 confidence intervals

Figure 3: Duncan Multiple Range Test – Homogenous Groups

	Species	Hysteresis	1	2
2	Gymnoscopelus braueri	0.055660	****	
4	Electrona antarctica	0.063500	****	
5	Melanostigma gelatinosum	0.068315	****	
3	Gymnoscopelus nicholsi	0.073000	****	
1	Pleuragramma	0.488292		****

The ice growth patterns seen in the samples correlated with the level of hysteresis they exhibited. As soon as the solidly frozen samples began to melt, a difference was seen between the ice structure of *Pleuragramma* and *Vomeridens* when compared to that of the other fish species. Many straight lines could be observed in the ice crystal 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 comparison, samples from the other fish contained almost no faceting. The solid piece of ice within the well broke into many small spheres while it was melting with the final ice crystal being spherical or oblong in shape. Differences were also seen in the growth of the crystals. Ice growth for the *Pleuragramma* samples was from the edges (a-axis) of the seed crystal disc, which resembled a starburst pattern, with many spikes emanating from the central crystal in all directions. The ice growth pattern for *Vomeridens infuscipinis* 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 disc. The samples run from the frozen fish exhibited a higher osmotic pressure, but no other difference was seen.

Discussion and Conclusions

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 that occurred was likely a result of lag between the readout and the

actual temperature at the sample holder. A thermal hysteresis of the same magnitude was

also seen during some of the calibrations that were preformed using deionized water,

which should exhibit no hysteresis at all. Even if this hysteresis was not also seen in the

deionized water the amount of antifreeze that would cause a hysteresis this small would

offer no protection to the fish in such an ice laden environment as Antarctica. The

Nototheniidae (*Pleuragramma*) and Bathydraconid (*Vomeridens*), two species from

predominantly Antarctic families, did show antifreeze activity. The ANOVA preformed

clearly shows that there is a significant difference between the nominal hysteresis seen in

the oceanic and that seen in the endemics (Figure 2). This can also be seen in the Duncan

Multiple Range Test where the oceanics grouped together and the *Pleuragramma* was

excluded (Figure 3). The blood from the frozen *Vomeridens* was extracted to use as a

standard to validate that the analysis procedure would work for blood drawn from frozen

fish. The only difference seen was an increase in osmotic pressure, which may result

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from cell lysis due to freezing, as additional osmotic solutes were released from cells which have stores of solutes that do not contribute to the osmotic activity of the cell. Once the procedure was validated other species of an appropriate length (>150mm SL) were analyzed. The lack of antifreeze activity in the oceanic families means that they will freeze and die when encountering ice crystals because of their supercooled state at the temperatures typical of the Ross and Weddell Sea shelves. However, on the WAP shelf where temperatures are typically above 0°C in the intermediate depths (200-400 m) the oceanic fishes, and other fishes lacking antifreeze, are better 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 meters of depth (Lancraft et al 2004). The hysteresis observed in Marguerite Bay fish is lower (ave = 0.45) than that found in fish captured from McMurdo Sound (ave = 0.91) (Cziko et al. 2006) which could be a result of warmer temperatures in the WAP. Jin and Devries (2006) found that some Antarctic fish are able to adjust their AFGP levels based on temperature exposure. With more CDW entering Marguerite Bay the *Pleuragramma* may not need as much AFGP for protection because of the warmer layer (2°C) which does not exist in McMurdo Sound. It is hard to compare the difference in hysteresis levels between this study and those conducted by Wohrmann (1995, 1996) because of the differences in techniques between the two studies.

AFGP presence was evident not only with the thermal hysteresis, but also in the nature of ice crystal growth and melting. Samples which contained AFGP (*Pleuragramma* and *Vomeridens*) showed a distinct ice-growth pattern. When ice grew from a single crystal during the freezing point determination, growth was very fast once the thermal threshold was reached. The pattern of growth was also unique. In the case of *Pleuragramma* the ice would grow in a star-burst pattern with spikes emanating from the central crystal at multiple angles. The growth of ice crystals in the blood of *Vomeridens* was similar, but in the form of a hexagonal bipyramide. It has been shown that this type of growth correlates with the presence of AFGP (Devries 1986) because the binding of the AFGP to the ice matrix constrains growth to a certain plane. Samples which did not contain AFPs (*Electrona antarctica, Gymnoscopelus nicholsi, G. braueri, Melanostigma gelatinosum, Paradiplospinus gracilis, Notolepis coatsi,* and *Bathylagus antarcticus*) melted down into spherical discs and ice growth occurred equally along all edges of the disc.

Based on the insignificant level of hysteresis seen in the midwater fish families (*Electrona antarctica, Gymnoscopelus nicholsi, G. braueri, Melanostigma gelatinosum, Paradiplospinus gracilis, Notolepis coatsi,* and *Bathylagus antarcticus*) and their pattern of ice crystal growth it can be concluded that they do not contain AFPs to protect them from freezing in the ice laden Antarctic waters. The presence of oceanic species on the Western Antarctic Peninsula shelf but not on the Ross Sea or Weddell Sea shelves suggests that temperature is indeed the limiting factor in the distribution of oceanic fishes

in Antarctic waters. There is an abrupt transition from oceanic fauna to Antarctic

endemics at the shelf break in the Ross Sea (Donnelly et al 2003, DeWitt 1970), 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

depth of the shelf in the Ross Sea and Marquerite Bay are similar enough that depth

would not be limiting. In 2003, Dinniman et al modeled the circulation in the Ross Sea

and found that warm CDW does in fact intrude on to the shelf of the Ross Sea

periodically which could in fact introduce oceanic species onto the Ross Sea shelf as on

the WAP. However, the fishes' mobility would allow access to the shelves of either

region without the CDW's intrusion, whereas the benthic fishes are unlikely to because of

their lack of mobility.

One other item of interest to consider is the possible competition between

Electrona antarctica and *Pleuragramma antarcticum,* the two dominant fish in the

oceanic realm 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 differences between them occur in

their growth and time of 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).

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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 disappearing from the mid-regions of the Antarctic Peninsula as the peninsula warms and the ice cover is reduced. With the continued warming trend (Smith and Stammerjohn 2001, Ducklow et al. 2007), *P. antarcticum* may have a hard time competing in this region of Antarctica.

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