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Freezing resistance of antifreeze-deficient larval Antarctic fish

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Summary

Antarctic notothenioids, along with many other polar marine fishes, have evolved biological antifreeze proteins (AFPs) to survive in their icy environments. The larvae of Antarctic notothenioid fish hatch into the same frigid environment inhabited by the adults, suggesting that they must also be protected by sufficient AFPs, but this has never been verified. We have determined the contribution of AFPs to the freezing resistance of the larvae of three species: *Gymnodraco acuticeps, Pagothenia borchgrevinki* and *Pleuragramma antarcticum*. Of the three, only *P. borchgrevinki* larvae are protected by high, adult levels of AFPs. Hatchling *G. acuticeps* and *P. antarcticum* have drastically inadequate AFP concentrations to avoid freezing at the ambient seawater temperature (–1.91°C). We raised *G. acuticeps* larvae and measured the AFP levels in their blood for ~5 months post hatching. Larval serum freezing point was –1.34±0.04°C at the time of hatch; it began to decrease only after 30 days post hatch (d.p.h.), and finally reached the adult value (–2.61±0.03°C) by 147 d.p.h. Additionally, AFP concentrations in their intestinal fluids were very low at hatching, and did not increase with age throughout a sampling period of 84 d.p.h.

Surviving in a freezing environment without adequate AFP protection suggests that other mechanisms of larval freezing resistance exist. Accordingly, we found that *G. acuticeps* hatchlings survived to –3.6±0.1°C while in contact with external ice, but only survived to –1.5±0.0°C when ice was artificially introduced into their tissues. *P. antarcticum* larvae were similarly resistant to organismal freezing. The gills of all three species were found to be underdeveloped at the time of hatch, minimizing the risk of ice introduction through these delicate structures. Thus, an intact integument, underdeveloped gill structures and other physical barriers to ice propagation may contribute significantly to the freezing resistance and survival of these larval fishes in the icy conditions of the Southern Ocean.

Key words: Notothenioidei, Bathydraconidae, Nototheniidae, antifreeze glycoprotein, antifreeze potentiating protein, development, gills, mitochondrial NADH dehydrogenase subunit 2, temperature logging.

Introduction

The marine environments of McMurdo Sound and Terra Nova Bay (Ross Sea, Antarctica) are among the coldest and iciest in the world, due to their extremely high latitudes and proximity to large floating masses of ice (the Ross Ice shelf and the Drygalski ice tongue, respectively). The seawater in these areas is commonly at its freezing point (FP, –1.91°C), with only rare excursions above –1.0°C (Buffoni et al., 2002; Hunt et al., 2003), and the top 10–30 m of the water column in McMurdo Sound is often slightly supercooled (Hunt et al., 2003; Lewis and Perkin, 1985). Consequently, ice growth is observed on the underside of the sea ice, on fishing lines, and on the sea floor to these depths between September and January (Dayton, 1989; Dayton et al., 1969; Hunt et al., 2003), indicating the presence of minute ice crystals in the water column (Littlepage, 1965).

Despite the prevalence of ice and freezing conditions, many members of the marine teleost suborder Notothenioidei inhabit, spawn and develop in the near-shore waters of the Antarctic continental shelf (Eastman, 2005; Kock and Kellermann, 1991). These fish have blood and other body fluids that are hypo-osmotic to seawater, with colligative FPs that are approximately 1°C higher than the coldest environmental temperatures (DeVries, 1971). It is generally assumed that teleosts inhabiting the surface waters of the polar seas cannot avoid freezing by supercooling because of ice crystals in the water column (DeVries and Lin, 1977; Gordon et al., 1962; Scholander et al., 1957). Therefore, that certain fishes survive contact with ice at temperatures below the colligative FP of their fluids (freezing resistance) is attributed to a combination of physical barriers to ice propagation and antifreeze proteins (AFPs; DeVries and Cheng, 2005).

In adult Antarctic notothenioids freezing resistance is conferred by the expression of high concentrations of antifreeze glycoproteins (AFGPs; DeVries, 1988), and an antifreeze potentiating protein (AFPP; Jin, 2003) in their extracellular fluids. These AFPs inhibit the growth of ice crystals that may enter their bodies and, in conjunction with ions and other osmolytes, depress the FP of blood and intestinal fluid to as much as 1°C below the coldest environmental temperatures, thereby preventing freezing and death (DeVries and Cheng, 1992; Raymond and DeVries, 1977). AFPs interact specifically with ice, creating a difference between the temperature at which ice melts (melting point, MP; equal to the colligative FP) and grows (non-equilibrium FP) in a solution. This effect is termed thermal hysteresis (TH=MP–FP), the magnitude of which is directly related to the concentration and type of AFP (DeVries, 1988).

The role of AFPs in the freezing resistance of adult notothenioids and other polar teleosts has been extensively described (DeVries and Cheng, 2005; Fletcher et al., 2001; Fletcher et al., 1986; Raymond and DeVries, 1977), but their role in the survival of the early life stages has not been previously reported. The ready-to-hatch eggs and hatchling larvae of the naked dragonfish *Gymnodraco acuticeps* Boulenger 1902, the bald notothen *Pagothenia borchgrevinki* (Boulenger 1902) and the Antarctic silverfish *Pleuragramma antarcticum* Boulenger 1902, were discovered during field work in Antarctica, enabling studies of their freezing resistance during development. In the present study, we determined the contribution of AFPs to the FP depression of eggs and body fluids of the newly hatched larvae relative to those of the adults, and followed AFP expression in the blood and intestinal fluid of *G. acuticeps* larvae during a 5-month period following hatching. Additionally, we assessed the organismal freezing resistance of the larvae and the relative contributions of AFPs and physical barriers, such as the chorion, skin and the gill epithelium, to larval survival in their freezing environments.

Materials and methods

Temperature logging in McMurdo Sound

Seawater temperature data have been recorded continuously since January 2000 using a SBE-39 (Sea-Bird Electronics Inc., Bellevue, Washington, USA) temperature and pressure logger (recording interval of 15 min) positioned at 40 m depth, 50 m from the McMurdo Station saltwater intake jetty (77°51.033′S, 166°39.759′E). Temperature measurements have an initial accuracy of ±0.002°C and maximum drift of 0.0002°C per month. The instruments are recovered at least once per year for data download and replacement of the batteries. Identical instruments are also deployed in McMurdo Sound at Cape Armitage (77°14.564′S, 166°21.315′E), Cape Bird (77°14.558’ S, 166°21.409′E) and New Harbor (77°34.572′S, 163°31.651′E).

Collection and rearing of fish larvae

The locations of the different fish egg sampling sites are depicted in Fig. 1. In early to mid-September of 2002 and 2003 divers collected ready-to-hatch *Gymnodraco acuticeps* Boulenger 1902 eggs from rocks at depths of 15–35 m near the saltwater intake jetty of McMurdo Station. Spawning occurs in mid-October, with larvae hatching in early September of the following year (Evans et al., 2005). Eggs were transferred to tanks with flow-through seawater (–1.3°C to –1.6°C) at McMurdo Station, where all viable larvae hatched within 24 h. In 2002 the yolk-sac larvae were left unfed for the first week following hatching, after which they were fed regularly with powdered fish food and fresh plankton collected from McMurdo Sound. To enhance larval survival, in 2003 the larvae were fed fresh plankton immediately after hatching and regularly for the duration of the sampling period. A description of spawning behavior and larval development is provided in Evans et al. (2005).

Eggs and newly hatched larvae of *Pleuragramma antarcticum* Boulenger 1902 were obtained in mid-November

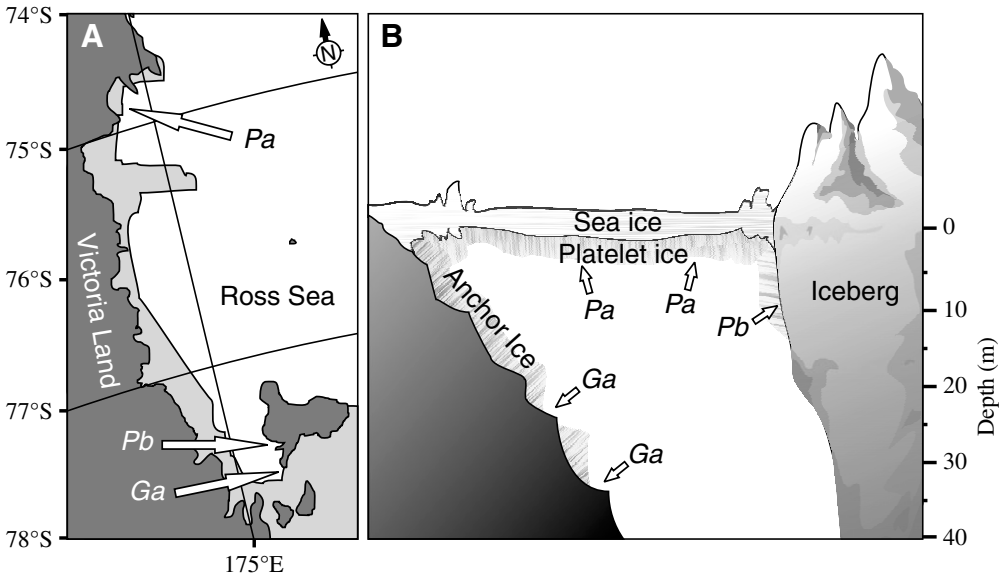

Fig. 1. Geographic location (A) and schematic of the collection sites (B) of the fish eggs. *P. antarcticum* (*Pa*) eggs were collected from the platelet ice in Terra Nova Bay. *G. acuticeps* (*Ga*) eggs were found on the shallow bottom near McMurdo Station on Ross Island during several seasons. *P. borchgrevinki* (*Pb*) eggs were found in a crevice in the side of an iceberg near Cape Evans, Ross Island. All eggs were collected from below sea ice cover.

2002 as a generous gift from Dr Marino Vacchi and colleagues participating in the XVIII Italian Antarctic Expedition at Terra Nova Bay (Vacchi et al., 2003). Large numbers of eggs and larvae were found encased within and floating among platelet ice dislodged from the underside of the sea ice while drilling fishing holes over 25–452 m of water near 74°41′S, 164°05′E. Both eggs and larvae were flown to McMurdo Station, where the majority of the eggs hatched during the following week. The delicate larvae survived for approximately 2 weeks in tanks of ice-free, continuously flowing seawater and in containers of seawater kept near –2°C in a cold room.

A small number of ready-to-hatch eggs of *Pagothenia borchgrevinki* (Boulenger 1902) were collected from a mass of eggs found within a crevice on the side of a grounded iceberg near Cape Evans (77°38.1′S, 166°24.8′E), Ross Island in late October 1997. Hatching began upon their introduction into aquaria at McMurdo Station, and all of the hatched larvae were used in studies shortly thereafter. Eggs of all three species were collected from beneath sea ice cover.

Sampling of egg and larval fluids

The contents of newly spawned *G. acuticeps* eggs were obtained with a 23-gauge needle from eggs that had been gently blotted to remove the seawater on their surface. For each sample the contents of 10 eggs were collected into the same syringe and then expelled into a 1.5 ml tube for centrifugation (10 000 ***g***) to sediment debris. The perivitelline fluid was included in this whole egg homogenate, as it was not possible to remove the chorion without disrupting the membranes surrounding the early stage embryo. Perivitelline fluid was obtained from ready-to-hatch eggs of *G. acuticeps* and *P. antarcticum* by first blotting them gently to remove seawater, then submerging them in cold mineral oil to prevent evaporation, and finally puncturing the chorion and sampling the fluid with a pulled glass micropipette.

Collection of fluids from *G. acuticeps* larvae, anesthetized with 0.1% (w/v) tricaine methanesulfonate (MS-222, Sigma Chemical Co., St Louis, Missouri, USA), for freezing point (FP) analyses was accomplished under cold mineral oil using a pulled glass micropipette connected to a mineral oil-filled micrometer syringe. Blood was collected directly from the bulbus arteriosus by inserting the micropipette through the isthmus on the ventral side. Blood sampling in both 2002 and 2003 included 4–23 individuals for each age group, and continued until 147 d.p.h. Intestinal fluid was collected from partially dissected larva, midway between the pyloric sphincter and the anus; sampling was carried out until 84 d.p.h. in 2002 only, and included 4–7 individuals for each age group. Yolk was sampled from the center of the yolk mass of intact hatchling larvae with a pulled glass micropipette. All fluid samples, generally 0.5–2.0 μl, were drawn into the micropipette, expelled into the cold mineral oil, and then collected into a 5 μl microcapillary tube. The tube was sealed at both ends with sealing putty and centrifuged at 6000 ***g*** for 5 min to sediment blood cells or tissue debris.

Larval homogenates were obtained before and after removal of the yolk by gently blotting the larvae to remove seawater, and then simultaneously passing several through a 1 ml syringe fitted with a 27-gauge needle. Homogenates were centrifuged to sediment tissue debris, leaving a clear supernatant that was analyzed promptly. Individual serum and intestinal fluid samples could not be obtained from hatchlings of either *P. antarcticum* (because of their small size) or *P. borchgrevinki* (larvae were frozen and stored at –80°C at the time of sampling).

Collection and sampling of adult fish

Divers collected adult *G. acuticeps* (50–250 g) with hand nets from 15 to 35 m depth near the salt-water intake jetty of McMurdo Station, between September and January of 2002–2004. *P. borchgrevinki* (18–25 cm total length) were caught with hand lines and plastic lures through holes drilled in the sea ice at various locations in McMurdo Sound. The fish were held in tanks of flowing seawater at –1.3°C to –1.6°C and were fed periodically with juvenile fish (for *G. acuticeps*) and macroplankton (for *P. borchgrevinki*), collected locally. Blood was obtained with a 23-gauge needle from the caudal vein of fish anesthetized with 0.1% (w/v) MS-222, and was allowed to clot for several hours at 4°C. Serum was recovered after centrifugation (10 000 ***g***). Intestinal fluid was obtained from dissected fish immediately following sacrifice using a 23-gauge needle. Fluids that were not be analyzed promptly were frozen in liquid nitrogen and stored at –80°C.

Serum and intestinal fluid samples were obtained from adult specimens of *P. antarcticum* (50–70 g) collected from midwater trawls during Antarctic research cruises between 1996 and 2003, and from specimens found in very good condition in the stomachs of recently caught and dissected *Dissostichus mawsoni* (the Antarctic toothfish) from McMurdo Sound. *P. antarcticum* blood and intestinal fluid were collected immediately after capture, and processed as for *G. acuticeps*.

Determination of the MP, FP and TH of fish fluids

Melting point (MP), freezing point (FP) and thermal hysteresis (TH) of the sampled fluids were determined using a Clifton nanoliter osmometer (Clifton Technical Physics. Hartford, NY, USA), calibrated with distilled-deionized water (0 mOsm) and a 1000 mOsm standard (Opti-Mole, Wescor Inc., Logan, UT, USA). Approximately 10 nl of each sample was suspended in the sample holder in heavy immersion oil (Type B, Cargille Labs Inc., Cedar Grove, NJ, USA) using a pulled glass micropipette connected to a mineral oil-filled micrometer syringe. While under observation through a microscope at 320× magnification, the samples were quickly frozen at –40°C and then slowly warmed; the temperature at which the last ice crystal melted within the sample was taken as the MP. The sample was frozen and then melted back to a single ice crystal of 10–20 μm in diameter, which was then cooled at 0.05–0.2°C min^{-1} until the onset of unrestricted growth at the FP. TH was calculated from TH=MP–FP. Each sample was loaded into and analyzed separately in 2–6 wells of the sample holder; the values obtained from all wells

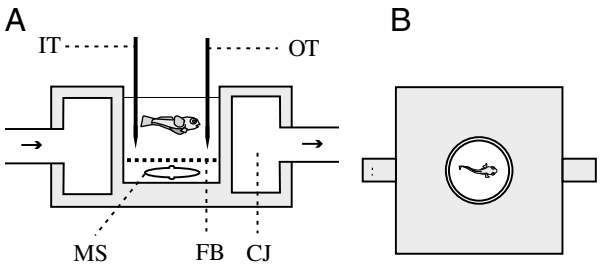

Fig. 2. Cooling chamber used to determine the freezing resistance of *G. acuticeps* larvae. External ice was applied with a frozen toothpick; internal ice was introduced by touching the caudal peduncle with a cold needle. (A) Cross-section showing the placement of the inlet (IT) and outlet (OT) thermocouples, magnetic stirring bar (MS), false bottom (FB) and the cooling jacket (CJ) through which coolant from a refrigerated bath was circulated. (B) Top view. The larva is approximately 12 mm long.

containing the same sample were averaged to give a single data point. MP and FP readings from the osmometer were converted from osmotic concentration to temperature using 1000 mOsm=1.858°C of FP depression.

Freezing resistance of larval G. acuticeps

The freezing resistance of newly hatched (0–3 d.p.h.) *G. acuticeps* larvae was investigated using single live specimens placed in a 15% (v/v) glycerol/seawater solution (3438 mOsm, determined using a Wescor vapor pressure osmometer; calculated FP –6.3°C) in a 15 ml chamber in a brass cooling block (Fig. 2). Coolant from a refrigerated bath circulated through the cooling block to lower the temperature of the larva by ~0.2°C min^{-1} while in contact with external ice, or with artificially introduced internal ice. The glycerol/seawater solution allowed cooling of the larva to below the FP of seawater without the introduction of ice from the solution. The temperature of the solution was monitored with two needle probes attached to a scanning thermocouple thermometer (Digisense 92800-00, Cole-Parmer, Vernon Hills, IL, USA), which was calibrated at 0°C with an accuracy of 0.1% of the reading. The thermocouple probes were placed 0.25 cm from the wall of the chamber, near both the coolant inlet and outlet of the cooling block. A miniature magnetic stirring bar was placed under a false bottom in the chamber to aid in mixing the solution. The inlet thermocouple occasionally read 0.1–0.2°C colder than the thermocouple placed near the outlet, thus the temperature of the solution was taken as the average of the two.

Initially, a single larva was cooled from –1.9°C to –5.1°C over a period of 22 min to determine if the larvae contained endogenous nucleators that would interfere with the experiment. Next, in order to determine the maximum effect of submersion in the glycerol/seawater solution on the osmolality of the blood, the integuments of two larvae were perforated by gripping near their caudal peduncles with forceps during transfer to the cooling chamber. These larvae remained in the solution at –2.3°C for 10 min, after which they were rinsed briefly with seawater, and blotted dry for sampling under mineral oil for blood from the bulbus arteriosus. The serum osmolality was determined using the Clifton nanoliter osmometer as described above.

To determine the freezing resistance of newly hatched *G. acuticeps* while in contact with external ice, five larvae were individually introduced into the cooling chamber. These larvae were touched with a small piece of ice on the end of a wooden toothpick at 0.2–0.3°C intervals while cooling from –1.9°C, until ice was observed propagating through the trunk and head of the fish with the help of a stereo-microscope (10× magnification). The larval fish were completely frozen within 3 s of the onset of ice growth at the organismal FP.

The cooling chamber was also used to determine the freezing resistance in the presence of introduced (internal) ice. In this experiment, cooling began at approximately –1.2°C (±0.1°C), just below the MP of the blood (–1.08±0.03°C) as determined using the Clifton nanoliter osmometer. Ice was introduced into each larva only once, at the starting temperature, by cooling a 25-gauge needle with a freeze-spray can (Envi-ro-tech 1672 Freezer, Techspray, LP, Amarillo, TX, USA), and then immediately touching the skin of the larva near the caudal peduncle. This small patch of skin remained frozen as the chamber was cooled. The organismal FP of the fish was apparent, as ice quickly and visibly spread from the caudal peduncle to the head. *P. antarcticum* and *P. borchgrevinki* larvae were not available for study at the time of this experiment.

Freezing resistance of larval P. antarcticum

To determine the freezing resistance of intact *P. antarcticum* larvae while in contact with external ice, a single live hatchling or ready-to-hatch egg was placed in drop of seawater on a solid 6 mm×6 mm aluminum slide mounted on the thermoelectrically controlled cooling module of the Clifton nanoliter osmometer, and observed (30× magnification) while controlling the temperature (Fig. 3). For larvae, the temperature was set at –2.0°C (0.1°C below the FP of the seawater), with the larva and seawater, but no ice, present on the slide. A 23-gauge needle was then cooled in liquid nitrogen and applied to the surface of the drop of water, causing a small cluster of ice crystals to form in the seawater that surrounded the larva or egg. The temperature of the slide was lowered at 0.2°C min^{-1}, causing the ice crystals to grow and surround the larva. At the organismal FP ice visibly propagated throughout the fish, freezing them completely within 1–2 s of the onset of crystal growth, and resulting in fish that were considerably more opaque than unfrozen specimens. The frozen larvae were slowly warmed after freezing, with the temperature at which the last ice crystal within the intact yolk melted taken as the *in situ* MP. Ready-to-hatch eggs were placed on the cooling module as for the larvae, and held at –9.6°C for 30 min while surrounded by ice, and observed for freezing. At the end of the 30 min period the chorion was pierced with a needle. *G. acuticeps* larvae and eggs were considerably larger than hatchling *P. antarcticum* and could not be accommodated on

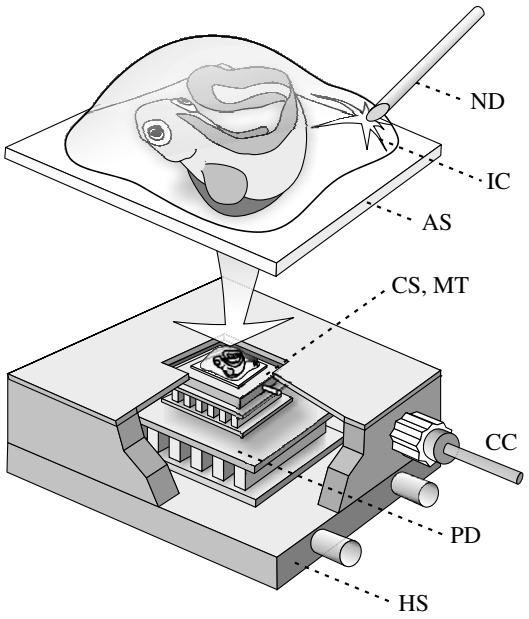

Fig. 3. Method used to determine the freezing resistance of *P. antarcticum* larvae. An individual larva was placed in a drop of seawater on a solid 6 mm×6 mm aluminum slide (AS), which was mounted on the thermoelectric cooling module (CS) of the Clifton nanoliter osmometer. A needle (ND) was cooled in liquid nitrogen and used to initiate ice crystal (IC) growth in the seawater surrounding the larva. Cooling is accomplished with stacked Peltier devices (PD) mounted on a water-cooled brass heat sink (HS). The stage temperature is controlled *via* a negative-feedback mechanism using a micro-thermistor (MT) mounted within the stage, and a separate temperature control module, connected to the cooling module by a cable (CC).

the cooling module, thus no measurements were made for this species.

In a separate experiment, approximately 50 hatched and ready-to-hatch eggs of *P. antarcticum* were placed in 500 ml glass beakers of seawater in a constant temperature room set to –5°C. At this temperature the seawater quickly reached its FP, and ice crystals formed on all sides of the container. Larvae were observed periodically to ascertain whether they displayed behavioral avoidance of ice in freezing seawater, and if organismal freezing occurred upon contact with ice. After several hours, a centimeter-thick slab of ice encasing the positively buoyant eggs was removed from the surface and melted in seawater at –1°C, and the larvae within the eggs were checked for movement to indicate survival.

Gill morphology

Whole-mounts of gill tissues were prepared from 1 d.p.h. larvae of *P. antarcticum* and *P. borchgrevinki*, and from 1 and 70 d.p.h. larvae of *G. acuticeps*, by dissecting fixed larvae immersed in notothenioid PBS (86 mmol l^{-1} Na_2HPO_4, 12 mmol l^{-1} NaH_2PO_4, pH 7.6, adjusted to 450 mOsm with NaCl). Gill tissue was transferred to cavity slides in a small amount of the buffered solution, mounted under a coverslip, and viewed and photographed using a Leica DMRE microscope (Leica Microsystems AG, Wetzlar, Germany) and DC500 digital camera (Leica Camera AG, Solms, Germany).

Identification of fish larvae

The eggs collected near the saltwater intake jetty of McMurdo Station were identified as those of *G. acuticeps* by the presence of nest-guarding adults, and the eventual development of adult morphological features in the aquarium-reared larvae (Evans et al., 2005). The identities of the other two newly hatched larvae were tentatively designated as *P. antarcticum* (Terra Nova Bay) and *P. borchgrevinki* (Cape Evans iceberg) based on morphological characteristics, but verification awaited DNA sequence analysis. Their identity was confirmed by phylogenetic analysis using the DNA sequence of the 1047 nt mitochondrial NADH dehydrogenase subunit 2 gene (mtND2). DNA was obtained from twelve pooled *P. antarcticum* larvae and from two single *P. borchgrevinki* larvae, as well as from a variety of adults of these and other species of the family Nototheniidae, using a standard lysis procedure and either guanidine thiocyanate (1 mol l^{-1} with 0.025 mol l^{-1} Tris-Cl, pH 7.5) for protein precipitation, or the standard phenol–chloroform extraction method (Sambrook and Russell, 2001). The forward and reverse primers used for both PCR and direct DNA sequencing were 5′-CTACCTGAAGAGATCAAAAC-3′ and 5′-CGCGTTTAGCTGTTAACTAA-3′, respectively. Conditions for polymerase chain reaction (PCR) followed those presented in Cheng et al. (2003). DNA sequencing was performed using ABI Inc. (Foster City, CA, USA) BigDye v3.0 chemistry and standard reaction conditions. The mtND2 DNA sequence data obtained in this study (17 adult and 3 larval sequences; GenBank accession nos. DQ184487–DQ184506) were used in conjunction with previously published sequences (AY256561–AY256570), resulting in sequences from a total of 27 individuals of known identity representing 13 species of the family Nototheniidae. These sequences were used to construct a distance-based (nucleotide differences) neighbor-joining phylogenetic tree (1000 bootstrap pseudoreplicates, transversions and transitions equally weighted), implemented in MEGA 3.1 (Kumar and Tamura, 2004). The positions of the larval fish with respect to the other taxa (adults of known identity) on the phylogenetic tree were used to confirm their identity.

Results

Long-term temperature logging

Nearly 5 years (Jan. 2000–Sept. 2004) of high-resolution temperature logging near a *G. acuticeps* spawning site reveals that the eggs and larvae are exposed to freezing temperatures for a considerable portion of each year. For clarity, only data from years 2002–2004 are presented here (Fig. 4). The seawater temperature is cold and stable (approx. –1.91°C) between June and January, with a small amount of inter-annual variation in the timing, extent and duration of the summer

warming events. The average temperature at this site is approximately –1.8°C, with excursions above –1.0°C totaling less than 23 days in this 5 year period. Deeper water (to at least 400 m) seems to be perennially cold and stable (approx. –1.91°C) with the warming events occurring in the top 100–200 m only. Representative vertical salinity and temperature (CTD) profiles are presented in Hunt et al. (2003). The surface seawater in the Ross Sea is likely to remain at its FP for approximately 4 months after the hatching of *G. acuticeps,* and perhaps as much as 2 months after the hatching of both *P. antarcticum* and *P. borchgrevinki.* Simultaneous temperature logging at other locations in McMurdo Sound (P. A. Cziko, A. L. DeVries et al., unpublished data) as well as in Terra Nova Bay (Buffoni et al., 2002) reveal nearly identical annual temperature records, with variation primarily in the extent of the warming events. The annual maximum temperatures were between 0.5°C and –1.7°C at all locations in this study during the observation period.

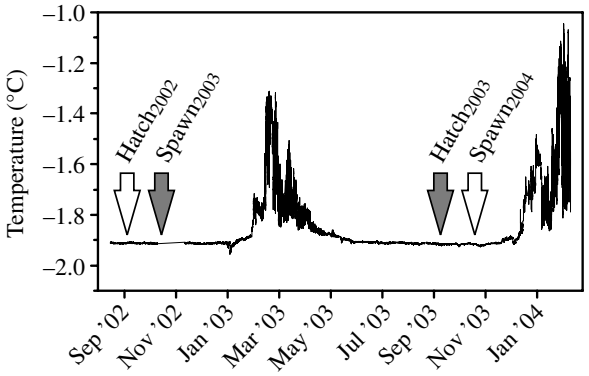

Fig. 4. Seawater temperatures at McMurdo Station. Seawater temperatures were recorded from August 2002 to March 2004 at 40 m depth near a spawning site of *G. acuticeps* at the McMurdo Station. Arrows indicate approximate hatching and spawning dates for the yearly cohorts. *G. acuticeps* spawns in mid-November and embryonic development is protracted across c. 10 months. Hatching is in early September, near the start of the austral spring. The seawater is within 0.05°C of its freezing point (–1.91°C) between June and December, and shows little variation in temperature with depth during this period.

MP, FP and TH of egg and larval fish fluids

The MP, FP and TH values for all egg and larval fluids are presented in Table 1. Newly spawned eggs of *G. acuticeps* contain a significant amount of AFPs, indicated by a TH value of 1.02±0.05°C for the whole egg homogenate. The perivitelline fluid isolated from ready-to-hatch eggs of both *G. acuticeps* and *P. antarcticum* is essentially iso-osmotic to seawater (1025 mOsm), and the perivitelline fluid from the eggs of *G. acuticeps* contains small amounts of AFPs, with a TH of 0.06±0.01°C.

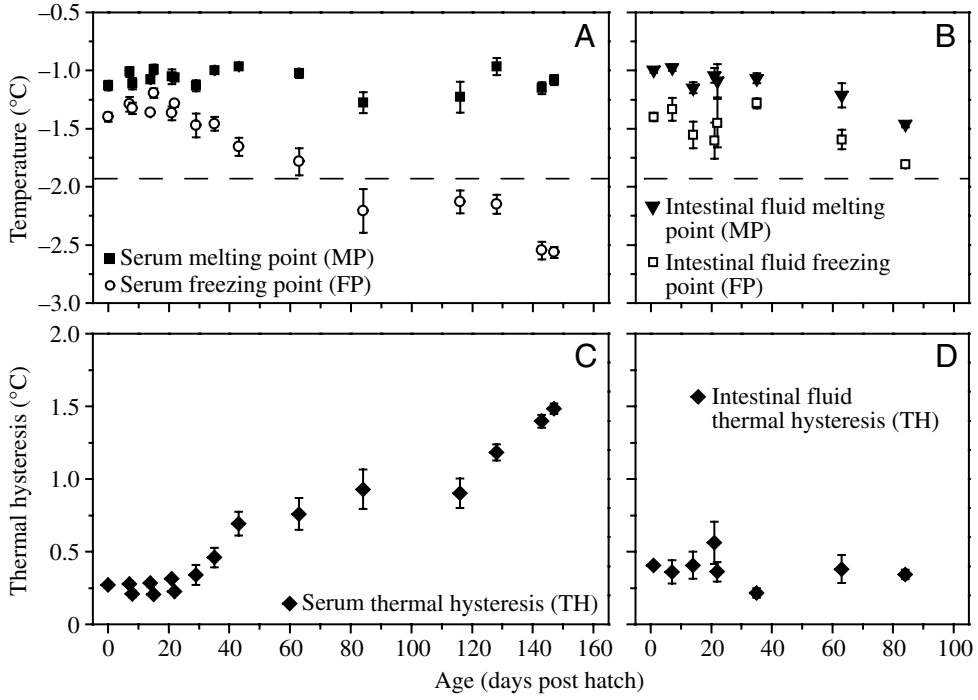

Fig. 5. Developmental trends in the TH of body fluids of *G. acuticeps* larvae. (A) Serum MP (squares) and FP (circles) of larvae collected and reared in 2002 and 2003. (B) Intestinal fluid MP (triangles) and FP (squares) of the 2002 cohort only. Intestinal fluid MP is partially dependent on feeding status, which may account for the decrease in FP in the oldest larvae. (C) Serum TH did not increase significantly from 0 to 30 d.p.h. For larvae ⩾30 d.p.h., TH was positively correlated with age, increasing by 0.008°C per day (linear regression, r^2=0.77). (D) Intestinal fluid TH; TH did not change significantly throughout the intestinal fluid sampling period (84 d.p.h.). For *G. acuticeps*, the yolk-sac is completely absorbed by about 15 d.p.h. The broken line (A,B) indicates the seawater temperature at the time of hatch (–1.91°C). Values are means ± s.e.m. (serum samples, *N*=132; intestinal fluid samples, *N*=75).

The mean serum MP for larval *G. acuticeps* for the entire sampling period (0–147 d.p.h.) was –1.08±0.01°C, which is not significantly different from the MP of adult *G. acuticeps* serum (–1.06±0.01°C; Student's *t*-test, P=0.25). However, the serum FP of *G. acuticeps* hatchling larvae was approximately 1.3°C higher than that of the adults and as much as 0.5°C higher than the FP of local seawater. The mean TH of serum for <30 d.p.h. larvae was only 0.25±0.02°C, with no significant change during this period (linear regression, slope not significantly different from zero, P=0.98; Fig. 5). After 30 d.p.h., TH increased steadily with age by approximately 0.008°C per day (linear regression, larvae ⩾29 d.p.h., r^2=0.77), with the FP of the serum dropping below that of seawater (–1.91°C) between 63 and 84 d.p.h. The larval serum AFPs finally reached adult levels by 147 d.p.h., almost 5 months after hatching, with a TH of 1.48±0.03°C depressing the FP to –2.56±0.05°C at this time.

The mean MP, FP and TH values of the intestinal fluid of

Table 1. *Melting point (MP), freezing point (FP) and thermal hysteresis (TH) of various fluids*

	MP (°C)	FP (°C)	TH (°C)	*N*
G. acuticeps				
Adults				
Serum	–1.06±0.01	–2.61±0.03	1.55±0.04	7
Intestinal fluid	–1.28±0.04	–3.42±0.11	2.15±0.15	4
All larvae				
Serum (0–30 d.p.h.)[a]	–1.08±0.01[b]	–1.34±0.04	0.25±0.02	24
Intestinal fluid (0–85 d.p.h.)[c]	–1.10±0.02	–1.45±0.03	0.38±0.02	75
Hatchling larvae (0–1 d.p.h.)				
Whole larvae homogenate	–1.04±0.00	–1.48±0.03	0.44±0.03	2[d]
Larval homogenate, no yolk	–1.12	–1.36	0.24	1[d]
Yolk	–1.31±0.14	–1.75±0.16	0.44±0.01	2[e]
Ready-to-hatch eggs				
Perivitelline fluid	–1.91±0.01	–1.97±0.01	0.06±0.01	14
Newly spawned eggs				
Whole egg homogenate	–1.29±0.00	–2.31±0.04	1.02±0.05	5
P. antarcticum				
Adults				
Serum	–0.94±0.04	–1.84±0.14	0.91±0.14	4
Intestinal fluid	–1.15±0.10	–2.63±0.24	1.48±0.02	3
Hatchling larvae (0–1 d.p.h.)				
Whole larvae homogenate	–0.88±0.09	–0.99±0.08	0.11±0.02	5[f]
Larval homogenate, no yolk	–0.76±0.02	–0.82±0.04	0.06±0.02	5[e]
Yolk	–0.72±0.03	–0.90±0.04	0.17±0.02	5
Ready-to-hatch eggs				
Perivitelline fluid	–1.88±0.01	–1.88±0.01	–	6
P. borchgrevinki				
Adults				
Serum	–1.01±0.02	–3.24±0.04	2.23±0.03	3
Intestinal fluid	–1.39±0.04	–3.52±0.28	2.12±0.27	4
Hatchling larvae (0–1 d.p.h.)				
Whole larvae homogenate	–1.45±0.00	–2.63±0.30	1.18±0.30	2[g]

The seawater temperature at hatching was approximately –1.91°C, for all species.
Values are means ± s.e.m., where applicable.
[a]FP and TH did not change significantly from 0 to 30 d.p.h., but increased after 30 d.p.h.
[b]MP value includes all larvae (0–147 d.p.h., N=132). MP values did not change significantly throughout the entire sampling period.
[c]MP and FP decreased throughout the sampling period, but TH did not change significantly.
[d]50 larvae per sample, combined for homogenization.
[e]Combined yolks of 17 and 30 larvae per sample.
[f]Ten individuals per sample, combined for homogenization.
[g]Three larvae per sample, combined for homogenization.

larval *G. acuticeps* were –1.10±0.02°C, –1.45±0.03°C and 0.38±0.02°C, respectively, for samples collected during the sampling period (0–84 d.p.h., Fig. 5A,C). Although the MP and FP of intestinal fluid were observed to decrease slightly with age (Fig. 5B), intestinal fluid TH did not increase (linear regression, slope not significantly different from zero, P=0.54; Fig. 5D).

Since individual serum and intestinal fluid samples could not be obtained from either *P. antarcticum* or *P. borchgrevinki* larvae, the total AFP content of these larvae was assessed by comparing the MP, FP and TH values of larval homogenates. The results indicate that hatchling *P. antarcticum* larvae possess a lower concentration of AFPs in their body fluids than do hatchling *G. acuticeps* (Table 1). The MP value for the whole larval homogenate of hatchling *P. borchgrevinki* (–1.45±0.00°C) is lower than for both *G. acuticeps* and *P. antarcticum*, and its considerably lower FP (–2.63±0.30°C) is a result of a much greater TH (1.18±0.30°C).

MP, FP and TH of adult fish fluids

The MP, FP and TH values for the adult fish sera and intestinal fluids are presented in Table 1. The mean MPs for the sera are broadly similar for adults of all species, with a value of approximately –1.0°C. Due to the high TH in adult serum, FP values are below the FP of local seawater (–1.91°C) for both the shallow benthic *G. acuticeps* (–2.61±0.03°C) and the cryopelagic *P. borchgrevinki* (–3.24±0.04°C). The serum of adults of the pelagic *P. antarcticum* exhibits a slightly smaller TH value, and has a FP that is significantly higher than that of the other two species (–1.84±0.14°C, Tukey's LSD P<0.01=0.14°C). The intestinal fluid FP values are below the FP of local seawater for all three species.

Freezing resistance of G. acuticeps larvae

In the absence of ice, a hatchling *G. acuticeps* larva could be cooled to –5.1 °C without freezing, indicating that the larvae are capable of significant supercooling. The larva remained active or responsive to disturbance during the entire procedure. Two larvae with their integuments compromised to allow the maximum possible dehydration, or diffusion of glycerol into their blood over a 10 min period, showed increases in serum osmolality of ⩽100 mOsm (<0.2°C FP depression). When ice was applied externally to the skin, hatchling larvae resisted freezing to –3.6±0.1°C. After the artificial introduction of ice into the body, *G. acuticeps* larvae were resistant to freezing only to –1.5±0°C (Table 2).

Freezing resistance of P. antarcticum larvae

The mean organismal FP of hatched *P. antarcticum* larvae in a drop of freezing seawater in the cooling module of the nanoliter osmometer was –2.75±0.11°C. The larvae froze almost immediately at the organismal FP, making it impossible to identify the precise origin of ice nucleation. All ready-to-hatch larvae remained alive and active with ice surrounding the outside of their chorions for 30 min at –9.6°C (Table 2). Freezing of the larvae was instantaneous once the chorions were breached at this temperature in the presence of ice.

Table 2. *Freezing points of G. acuticeps and P. antarcticum larvae with externally applied and internally introduced ice*

	Freezing point (°C)	*N*
G. acuticeps		
Hatchling larvae with:		
Externally applied ice	–3.6±0.1	5
Introduced ice	–1.5±0.0	5
P. antarcticum		
Larvae in intact chorion with:		
Externally applied ice	<–9.6	9
Hatchling larvae with:		
Externally applied ice	–2.7±0.1	9

Values are means ± s.e.m., where applicable.

Greater than 90% of larvae (N=50) that were placed in beakers of freezing seawater at –5°C survived the experiment. The swimming larvae did not avoid ice and repeatedly contacted the growing ice crystals on the sides of the container and on the surface without freezing. The small number of hatched larvae that did not survive had been trapped within interstices in the growing ice, and were completely surrounded by ice crystals before freezing. All of the larvae within eggs that had been completely encased in ice at the surface survived the treatment.

Gill morphology

Phase contrast images of the gills of *P. antarcticum* (Fig. 6A) and *P. borchgrevinki* (Fig. 6B) larvae showed absence of both filaments and lamellae at 1 d.p.h. The gills of 1 d.p.h. *G. acuticeps* possessed rudimentary filaments (Fig. 6C) with lamellae developing later, as illustrated by their presence in the gills of 70 d.p.h. *G. acuticeps* larvae (Fig. 6D).

Identification of P. antarcticum and P. borchgrevinki larvae

Phylogenetic analysis using a distance-based neighbor-joining tree constructed with the mtND2 gene DNA sequences recovered all multiple adult individuals representing the same species as monophyletic groups with 100% bootstrap support. The larvae discovered at Terra Nova Bay and Cape Evans grouped with the adults of *P. antarcticum* and *P. borchgrevinki*, respectively, with 100% bootstrap support, thus confirming species identification based on morphology (Fig. 7). A Bayesian analysis, implemented in MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using a sequence evolution model (GTR+I+Γ) for nucleotide substitution rates and invariant sites, and a Γ-distribution for among-site rate variation (J. A. A. Nylander, 2004: MrModeltest v2.2: Program distributed by the author, Uppsala University, Sweden; Posada and Crandall, 1998; Waddell and Steel, 1997), resulted in a topologically identical phylogenetic tree (results not shown), thereby supporting the use of the simpler neighbor-joining analysis for the confirmation of species identity. Intra-specific nucleotide

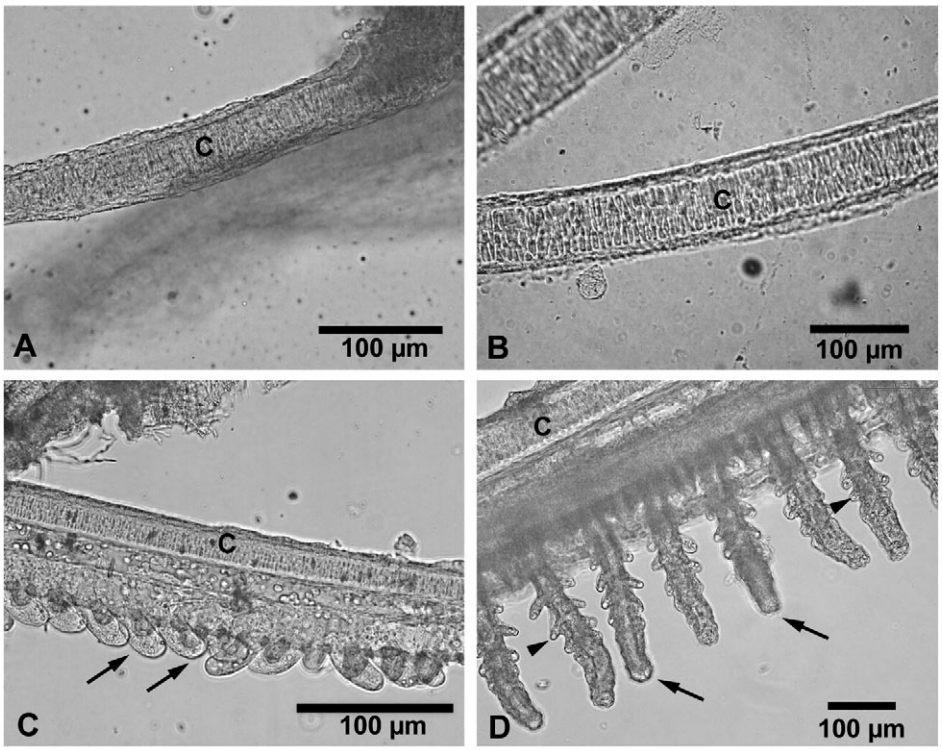

Fig. 6. Morphology of the gills of larval notothenioids. The gill arches of 1 d.p.h. *P. antarcticum* (A) and *P. borchgrevinki* (B) larvae were found to completely lack even rudimentary filaments. *G. acuticeps* larvae of the same age were found to possess developing gill filaments (C; arrows). Lamellae (arrowheads) form later in development, as illustrated by their presence in the gills of 70 d.p.h. *G. acuticeps* larvae (D). c, cartilaginous gill arch. Scale bars, 100 μm.

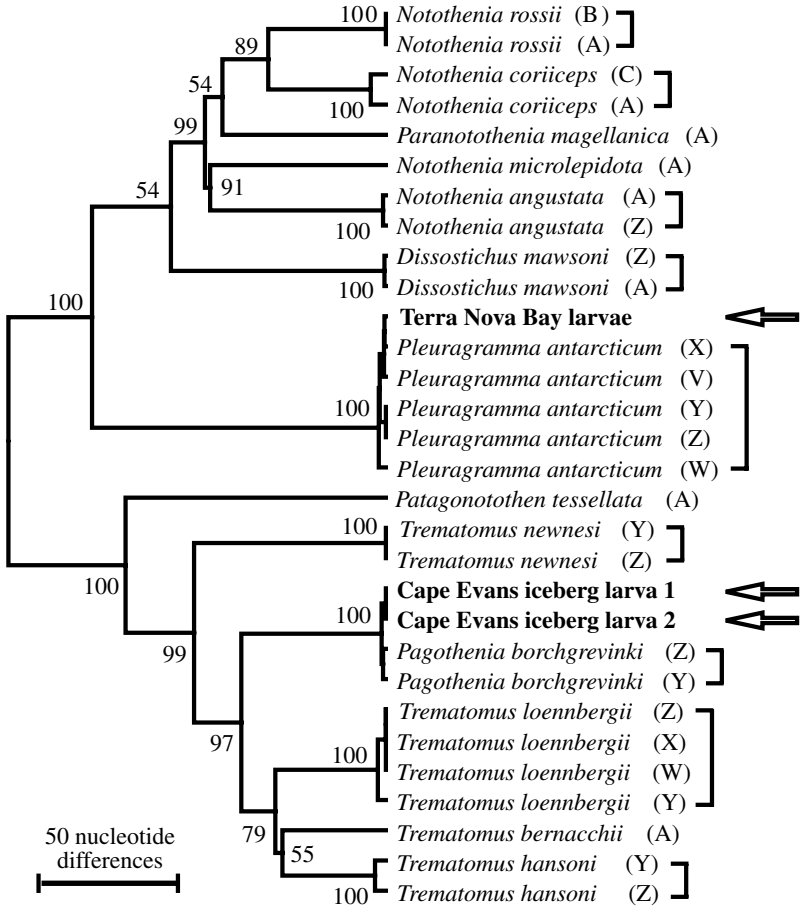

Fig. 7. Phylogenetic analysis for confirmation of larval identities. An unrooted consensus tree resulting from neighbor-joining analysis of the complete 1047 nt mtND2 gene sequence from adults of several species within the family Nototheniidae (Notothenioidei), and the larvae collected at Terra Nova Bay and Cape Evans. The positions of the larvae within the tree identified them as *Pleuragramma antarcticum* and *Pagothenia borchgrevinki* (arrows). Adult individuals of the same species are indicated by brackets. Bootstrap values (1000 pseudoreplicates) are presented, but within-species values have been omitted for clarity. The scale bar indicates the relationship between branch length and the number of nucleotide differences between individual gene sequences.

differences in the 1047 nt mtND2 gene sequence were greatest between two individuals of *N. coriiceps* (11 nt, GenBank accession nos. AY256563 and AY256564). The mean intra-specific difference for all species was 3.4±1.2 nt (N=9 species with multiple representative individuals). The mean inter-specific difference was 218.9±7.9 nt (N=13 species), with the most similar inter-specific sequences being from *Trematomus hansoni* (DQ184500 and DQ184501) and *Trematomus bernacchii* (AY2565690), and having 76 nt differences. Although the mtND2 sequence of the *P. antarcticum* larvae was obtained from pooled DNA isolated from multiple individuals, there were no ambiguous nucleotides observed in the gene sequence, suggesting that they are either of the same maternal origin or from closely related individuals. The mtND2 sequences obtained from the two *P. borchgrevinki* larvae were identical.

Discussion

The accessibility and the relative ease of rearing larval *G. acuticeps* allowed us to study the production of AFPs throughout embryonic and larval development. At the time of spawning, a considerable amount of AFPs have been bestowed upon the eggs (which are hypo-osmotic to seawater) by the mother, as demonstrated by a TH of 1.02±0.05°C in the egg homogenate (Table 1). However, by the time of hatching the TH exhibited by whole larvae homogenates is less than half of that of the newly spawned eggs, and the FPs of hatchling G. *acuticeps* sera and intestinal fluids are more than 0.5°C higher than the ambient water temperature (–1.91°C, Table 1, Fig. 5). Paradoxically, larval *G. acuticeps* have been observed to seek out the iciest surface waters upon hatching (Evans et al., 2005). For aquarium-reared *G. acuticeps,* the yolk is completely absorbed by about 15 d.p.h. (Evans et al., 2005), but the serum showed no significant increase in TH until after 30 d.p.h. (Fig. 5A). After 30 d.p.h., serum TH increased slowly, by 0.008°C per day, and did not reach adult FP values until 147 d.p.h. (–2.56±0.05°C; Fig. 5B). The depression of the larval fish serum FP to below the FP of seawater (–1.91°C) finally occurs between 64 and 84 d.p.h., and it is likely that the larvae risk freezing should ice enter their blood or tissues until this time. Furthermore, *G. acuticeps* intestinal fluid AFP concentrations remain low until at least 84 d.p.h., and possibly much longer.

P. antarcticum also hatches into these icy waters with drastically inadequate amounts of AFPs for protection against freezing, despite the fact that their positively buoyant egg places them in contact with the surface ice upon hatching (Vacchi et al., 2003), and older larval stages are most abundant in the iciest top 50 m of the water column (Granata et al., 2002; Guglielmo et al., 1998; Hubold, 1984). A comparison of hatchling larval homogenates and yolk TH values between *G. acuticeps* and *P. antarcticum* shows that the latter (from which individual fluids could not be obtained) likely have serum and intestinal fluids with as little as 25% of the TH observed in the already AFP-deficient *G. acuticeps* fluids at the time of hatching (Table 1). Only the larvae of a third notothenioid, the cryopelagic *P. borchgrevinki,* possess the high, adult levels of AFPs at the time of hatching, indicating that the larvae of this species are in no more danger of freezing than the adults.

The low levels of AFPs in the body fluids of larval *G. acuticeps* and *P. antarcticum* contrast sharply with the high concentrations found in those of the adult stages of these and other notothenioids. Since high concentrations of AFPs have been considered essential to prevent the freezing and death of teleost fishes in ice-laden waters (DeVries and Cheng, 1992; DeVries and Cheng, 2005), the discovery of antifreeze-deficient larval stages which are nevertheless resistant to freezing begs an explanation, and demonstrates that multiple strategies of freezing resistance may exist for larval notothenioid fishes.

Freezing resistance of fish eggs

For eggs of both polar (Atlantic cod, *Gadus morhua* and capelin, *Mallotus villosus*) and temperate fish species (plaice, *Pleuronectes platessa* and rainbow trout, *Oncorhynchus mykiss*) the chorion acts as a significant barrier to ice propagation, allowing the intact eggs, which are hypo-osmotic to seawater, to survive while in contact with ice at temperatures far below any they would be exposed to in nature (Aarset and Jørgensen, 1988; Davenport et al., 1979; Harvey and Ashwood-Smith, 1982; Valerio et al., 1992a). Fully developed *P. antarcticum* larvae are similarly protected while in the egg, surviving both cooling to below –9°C in a frozen seawater droplet (Table 2) and encasement of the egg in ice in freezing seawater. In nature, some of the eggs become encased in the ice platelets beneath sea ice cover without any discernable damage. Preliminary experiments (not presented here) indicate a similar level of protection for *G. acuticeps* eggs, which are apt to contend with anchor ice growth on the shallow benthos of McMurdo Sound during their protracted 10 month development (Evans et al., 2005).

The ultrastructure of the chorion has been examined in a variety of notothenioids and is similar to the chorions of temperate species in both thickness and construction (Lonning, 1972; Riehl and Kock, 1989; Stehr and Hawkes, 1979). As in other teleosts, the surface of the notothenioid chorion is penetrated by pores (the radial canals, 0.3–0.7 μm in diameter at the surface; Stehr and Hawkes, 1979; White et al., 1996) and a single micropyle (5–15 μm in diameter at the surface narrowing to 2–4 μm at its internal aperture; Riehl and Kock, 1989; Stehr and Hawkes, 1979; White et al., 1996), allowing the passage of a single sperm into the egg (Coward et al., 2002; Yamamoto, 1951). Prior to fertilization, the teleost egg may be vulnerable to freezing as the oocyte plasma membrane is in direct contact with the unhardened chorion, which could be in contact with environmental ice. As in other biological tissues, the restriction of ice growth across the chorion's proteinaceous matrix may be due to a structurally caused FP depression (Bloch et al., 1963) in this multi-lamellar, acellular structure. However, both the radial canals and the micropyle openings are potentially large enough to allow for the entry of ice (Valerio

et al., 1992b), which might explain the high levels of AFPs found in the recently spawned eggs of *G. acuticeps* (Table 1). Once fertilized, the micropyle becomes occluded by the fertilization cone (White et al., 1996; Yamamoto, 1951), which prevents polyspermy, and is also likely to aid in preventing ice propagation into the egg. During egg activation events the vitelline membrane separates from the hardened chorion, filling the newly formed perivitelline space with a fluid that is iso-osmotic to seawater (Table 1). Consequently, with the osmotic concentration equal on either side of the chorion, the tendency for the growth of ice into the egg may be reduced, and additionally restricted by the helicoidal structure of the radial canals (Grierson and Neville, 1981). The small amount of AFPs in the perivitelline fluid of *G. acuticeps* eggs may add to the freezing resistance of these eggs, but their absence in perivitelline fluid of *P. antarcticum* eggs suggests they may be a result of excretion or leakage from the embryo, and it is unlikely that they are absolutely necessary to resist freezing.

Freezing resistance of larval fishes

Experiments with juvenile and adult teleosts from both temperate and polar regions consistently show that the organismal FPs of these fish, while in contact with external ice, are never more than a few tenths of a degree (°C) below the FP of their blood (Fletcher et al., 1988, 1986; Tien, 1995). For polar species, this attests to the need for AFPs, as seawater temperatures can often be lower than the colligative FP of teleost blood and ice is common in the surface waters of the polar seas. The freezing resistance of larval fish has been investigated previously in only two species: capelin *Mallotus villosus* (Davenport and Stene, 1986), and Atlantic cod *Gadus morhua* (Valerio et al., 1992a), neither of which possessed AFPs at the time of the experiments. Under laboratory conditions, *M. villosus* and *G. morhua* larvae freeze by –1.4°C in the presence of ice, which is ~0.5°C below the FPs of their body fluids. The larvae of both species risk exposure to ice and temperatures below their organismal FPs after hatching into the cold surface waters early in the Arctic spring, and Valerio et al. (1992a) states that '*in unusually cold years…the risk of [G. morhua] larval mortality directly attributable to freezing may be significant.*' Incidentally, the larvae of another teleost, *Zoarces viviparus*, the viviparous blenny that synthesizes a type III AFP, are well protected by AFPs at the time of parturition (Sørensen and Ramløv, 2001, 2002). Although their freezing resistance has not been investigated, because of their high AFP levels, the larvae of this species are unlikely to risk freezing in ice-laden waters.

In contrast to *M. villosus* and *G. morhua*, in the laboratory the Antarctic *P. antarcticum* do not freeze while in contact with ice to –2.75±0.11°C, despite low AFP levels (Tables 1, 2). Similarly, hatchlings of the Antarctic *G. acuticeps* are resistant to freezing while in contact with externally applied ice to –3.63±0.09°C, more than 2.3°C below the FPs of their body fluids and well below the FP of seawater (Table 2). For hatchling *G. acuticeps*, ice that was artificially introduced into the fish (by freezing a small area of the skin or caudal fin) led to freezing and death at –1.54±0.01°C, which is about 0.36°C higher than the ambient temperature of McMurdo Sound at the time of hatching. Hence, the organismal FP of the larval fish with internal ice corresponds with the FP of its serum as determined *in vitro* using the nanoliter osmometer (–1.34±0.04°C; Table 1), with the slight discrepancy possibly caused by the dehydrating effects of the glycerol/seawater solution. Similarly, ice crystal growth was observed within the yolks of frozen and then partially thawed *P. antarcticum* hatchlings at or near the FP of the extracted yolk (–0.90±0.04°C; Table 1), demonstrating that ice growth is not additionally restricted in these fluids *in vivo*. The correlation between the FP of the larval fish with introduced ice and that of the individual larval fluids and homogenates indicates that in their youngest stages, *G. acuticeps* and *P. antarcticum* larvae risk freezing only if ice enters their blood or tissues.

Environmental ice and freezing resistance

The survival of notothenioid fish larvae, despite substantial supercooling of their body fluids, points to the presence of a barrier to prevent environmental ice from entering the fish. Nevertheless, ice is commonly found concentrated in the spleens of adult notothenioids from McMurdo Sound (DeVries and Cheng, 1992; Tien, 1995), indicating that one or more routes exist for its entry into the fish. The most obvious routes of entry are by propagation across the surface epithelium of the integument, the cornea or the gills, with an increased likelihood in the event of damaged epithelial layers. Intact adult fish skin and corneal epithelium are effective at preventing ice propagation to 1.1°C and 0.7°C below the FP of the protected fluid, respectively, with improved effectiveness upon the addition of AFPs to the basal side (Turner et al., 1985; Valerio et al., 1992b). However, although membrane specializations may help fortify the skin of *G. acuticeps* against the propagation of ice (Eastman and Hikida, 1991), the protection offered by the skin likely depends predominantly on its physical condition, because damaged skin probably offers little resistance to the propagation of ice. Even though larval skin is delicate and only a few cells thick at the time of hatching (Rombough, 1988), it may be less likely to suffer from lesions and other damage than that of the adult stages, which has a much larger surface area and may be slow at healing at the cold environmental temperatures (da Silva et al., 2004). This is consistent with the organismal FPs of adult fish in the presence of external ice being only slightly below the FPs of their body fluids, while excised skin sections, which have been carefully selected for their integrity (Valerio et al., 1992b), and the skin of larval *G. acuticeps* and *P. antarcticum* (Table 2), offer more protection. Although the integument most likely plays a critical role in the freezing resistance of larval fishes, the mechanism may be more involved than it acting as a simple physical barrier to ice propagation. This is demonstrated by the AFP-lacking yolk-sac larvae of *G. morhua* freezing at –1.34±0.01°C (Valerio et al., 1992a), while the AFP-deficient *G. acuticeps* larvae resisted freezing to –3.6±0.1°C when ice was applied to the integument under very similar laboratory conditions (Table 1).

It is likely that the physical structure of the intact gill epithelium also offers some resistance to the propagation of ice. The fully developed gills in adult *G. acuticeps* possess a blood–water barrier in the lamellae of only a single cell layer of about 2.6 μm thick (42 times thinner than the mean thickness of the skin; Eastman and Hikida, 1991), and a large surface area (Fry, 1957), rendering them especially susceptible to damage. Damage to the gill epithelium is possible through lesions caused by parasitic infestation, disease, or by abrasion from passing ice crystals and mineralized planktonic organisms over the gills during ventilation, providing a route for ice to enter the fish. For adult notothenioids, ice propagation across the gill epithelium will be restricted by the high level of circulating AFPs, and ice growth will be arrested if ice traverses this barrier. Clearly, newly hatched *G. acuticeps* larvae possess insufficient circulating AFPs to arrest ice growth at the ambient temperature, so the exclusion of ice at the gills absolutely requires an intact gill epithelium. In many teleosts, the gills are only partially developed at the time of hatching, with the blood–water barrier being initially thicker than in the later stages (Rombough, 1988). The delayed development of the gills is also apparent in all three species of notothenioids investigated in this study, with hatchling *P. antarcticum* and *P. borchgrevinki* lacking both filaments and lamellae, and with only rudimentary gill filaments present in *G. acuticeps* at the time of hatching (Fig. 7A–C). Observations of *G. acuticeps* gills at a later date (70 d.p.h.; Fig. 7D), indicates that the development of gill lamellae proceeds slowly, and is apparently much slower than in a variety of temperate teleosts (El-Fiky and Wieser, 1988; Morgan, 1974; Phillips and Summerfelt, 1999; Wells and Pinder, 1996; Yamashita, 1978), but rather similar in developmental timing to the Arctic *G. morhua* (Von Herbing et al., 1996). The delayed gill development in these cold-water species may be a result of the high oxygen tensions in cold waters, or may be targeted specifically towards preventing damage to the delicate gill epithelium, thereby reducing susceptibility to freezing.

Another route for the entry of ice into the fish may be associated with eating and drinking in ice-laden waters, as this could introduce ice crystals associated with the water or food into the alimentary tract. Although *G. acuticeps* larvae begin feeding by 2 d.p.h. (P. A. Cziko, personal observations), the AFP concentration in the intestinal fluid is low at this time, and it remains paradoxically low until at least 84 d.p.h., when our measurements ceased (Fig. 5D). In adult notothenioids, the extremely high levels of AFPs in the intestinal fluid (Table 1) attest to the risk and importance of avoiding freezing in the intestine (O'Grady et al., 1982, 1983). Significantly, even the larval intestinal fluid TH was found to be slightly greater than that of the serum at the time of hatch (Student's *t*-test, P=0.002). With only a small amount of AFPs present in the intestinal fluid of larval *G. acuticeps,* the ingestion of an ice crystal should cause progressive freezing of the alimentary tract as the uptake of salts in the esophagus and intestine, and dilution in the stomach, renders the imbibed fluid iso-osmotic to the blood and hypo-osmotic to seawater (Parmelee and Renfro, 1983; Smith, 1930). Although the FP of the intestinal fluid appears to decrease throughout development, TH did not increase over this period (Fig. 5D), and the lower FP and MP values in the older larvae (Fig. 5B) are likely due to ingested seawater from the increased feeding activity of the growing larvae at the time of sampling.

It is difficult to imagine a mechanism that would physically exclude minute ice crystals from entering the gut of feeding or drinking larvae, thus it could be that ice crystal growth is somehow inhibited in the intestinal fluid *in vivo*. Yet, even if ice growth is inhibited, ice crystals that come into contact with or propagate across the intestinal epithelium, may provide a means for the transmission of environmental ice into the blood, and this would certainly result in freezing and death. It is evident that further investigations into the intestinal fluid-freezing resistance of larval fishes are warranted, as the mechanisms by which this fluid resists freezing remain unknown.

Conclusions

The pronounced discrepancies in AFP levels between the adult and larval *G. acuticeps* and *P. antarcticum* are perplexing, as the larvae may inhabit even colder, icier waters than the adults. In light of this study, it seems that the expression of high levels of extracellular AFPs may not be the only mechanism available for freezing resistance during the early life stages of teleost fish. Features restricted to larval fish, such as a small surface area, an initially undamaged integument and intestinal epithelium, and delayed development of the gills, may be absolute requirements to enable these larvae to survive in ice-laden waters without the high, adult levels of AFPs. Although low levels of AFPs may partially contribute to freezing resistance, it is likely that a combination of multiple mechanisms of freezing resistance have evolved in order to circumvent the need to express high concentrations of AFPs early on, since this would be energetically costly to the developing fish. Further investigations into the freezing resistance of these AFP-deficient larval stages may provide insights into the nature of internal ice and its acquisition from the environment, and perhaps help to clarify the interrelation between an icy environment and the expression patterns of AFPs in teleost fishes.

Additional remarks: identification of eggs and larvae

The accurate identification of eggs and larval fishes is important for increasing our knowledge of the Southern Ocean ichthyofaunal diversity. However, identification based on morphology may be subjective for eggs (Riehl and Ekau, 1990; Riehl and Kock, 1989), and difficult in very early life stages or in the case of damaged specimens. For the notothenioid family Nototheniidae we have demonstrated that identification using a simple molecular phylogenetic analysis is feasible, since DNA extraction, PCR and sequencing of the mtND2 gene is inexpensive and rapid, and the results are unambiguous (Fig. 7).

List of abbreviations

AFGP	antifreeze glycoprotein
AFP(s)	antifreeze protein(s)
AFPP	antifreeze potentiating protein
d.p.h.	days post hatch
FP	freezing point
MP	melting point
mtND2	mitochondrial NADH dehydrogenase subunit 2
nt	nucleotide
PCR	polymerase chain reaction
RT	room temperature
TH	thermal hysteresis

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