Comparative Biochemistry and Physiology, Part A 185 (2015) 43–50

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Variation in blood serum antifreeze activity of Antarctic *Trematomus* fishes across habitat temperature and depth

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ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form 5 March 2015
Accepted 5 March 2015
Available online 12 March 2015

Keywords:
Antifreeze glycoprotein
Ecological physiology
Thermal hysteresis

ABSTRACT

High latitude waters in the Southern Ocean can be near their freezing point and remain ice-covered throughout the year whereas lower latitude Southern Ocean waters have seasonal ice coverage and comparatively large (6 °C) annual temperature changes. The genus *Trematomus* (suborder Notothenioidei) is regarded primarily as a high latitude group because of its abundance there, they also inhabit the warmer regions in smaller numbers. Freeze avoidance in the notothenioids is linked to the presence of two antifreeze proteins (AFPs); the antifreeze glycoproteins (AFGPs) and antifreeze potentiating protein (AFPP), both of which adsorb to internal ice crystals inhibiting growth. Both high and low latitude trematomids possess sufficient AFP to lower their blood freezing point below that of seawater (−1.9 °C). We investigated the contributions of AFGPs and AFPP to the blood freezing point depression to determine how they varied with depth, water temperature, and the presence of ice. High latitude trematomids had lower blood freezing points than those inhabiting lower latitude waters indicating differences in their freeze avoidance capacities. Lower freezing points were associated with higher levels of antifreeze activity due to higher levels of both AFGP and AFPP. Populations of *Trematomus hansoni* and *Trematomus bernacchii* from shallow depths appear more freeze avoidant than populations inhabiting deep, ice-free water based on their lower freezing points and higher antifreeze activities. Gel electrophoresis of the trichloroacetic acid-soluble AFGPs indicates that only high molecular weight isoforms, which contribute more to AFGP activity, vary across species as well as between individuals of a species.

1. Introduction

In the high latitudes of McMurdo Sound seawater reaches its freezing point of −1.9 °C year-round except for an approximate 1 °C temperature rise for about 2 weeks in mid-summer (Hunt et al., 2003) and is ice-covered for most of the year. McMurdo Sound is bordered on the south by the Ross Ice Shelf which is the source of cold water that flows into the Sound by freezing seawater generated at the underside of the ice shelf at depth (400–500 m) flowing northward into McMurdo Sound (Foldvik and Kvinge, 1977; Orsi and Wiederwohl, 2009). When advected towards the surface it becomes supercooled and spontaneously nucleates forming "clouds" of minute ice crystals to depths as deep as 30 m. These crystals appear to attach to benthic substrates and grow into large platelets (15 cm diameter) forming aggregations referred to as anchor ice (Dayton et al., 1969, 1970). The anchor ice masses, if sufficiently large, can break away from the bottom substrate and float to the underside of the sea ice adding to the sub-ice platelet layer which also appears to originate from the growth of similar small crystals. The platelet aggregations constitute a habitat for algae, invertebrates, and even cryopelagic fishes (Dayton et al., 1970).

In the low latitudes such as the Western Antarctic Peninsula (WAP) and Scotia Arc, freezing temperatures and ice cover occur only during the winter months (Dinniman and Klinck, 2004) but in most locations the warm Circumpolar Deep Water (CDW) or modified CDW is present below 100 m (Garcia et al., 2002). Temperatures of these water masses can vary between −0.5 °C and +1.5 °C depending upon the location (Jacobs et al., 1996) so fishes inhabiting the WAP deep water can be as much as 3° warmer than both the WAP winter-caught, shallow-water fishes, and all fishes in McMurdo Sound. Because of these temperature differences between the WAP and the southwestern Ross Sea we use latitude as a proxy for habitat temperature.

Despite the presence of ice and freezing temperatures marine life is abundant in these waters (Kock, 1992). The endemic fish fauna of this region survives in these ice-laden waters due to their capacity for freeze avoidance associated with high levels of blood antifreeze proteins

Abbreviations: ACC, Antarctic circumpolar current; AFGP, antifreeze glycoprotein; AFP, antifreeze protein; AFPP, antifreeze potentiating protein; CDW, circumpolar deep water; PAGE, polyacrylamide gel electrophoresis; PCA, principal component analysis; WAP, Western Antarctic Peninsula.

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http://dx.doi.org/10.1016/j.cbpa.2015.03.006
1095-6433/

(AFPs) which inhibit the growth of ice crystals that enter the body (DeVries and Cheng, 2005; Praebel et al., 2009). Were it not for the AFPs, environmental ice that enters the fish through either ingestion or lesions in the skin or gills would propagate resulting in freezing death because like other fish blood salts and small organic molecules depress the freezing point to only −1.0 °C (DeVries and Cheng, 2005; Jin and DeVries, 2006; Praebel et al., 2009). In polar fishes AFPs bind to and inhibit the growth of internalized ice. With the Antarctic notothenioid fishes antifreeze glycoproteins (AFGPs) account for the majority of freeze protection and exist in a number of size isoforms that contribute differentially to the overall antifreeze activity. A small antifreeze potentiating protein (AFPP), which by itself has little antifreeze activity, substantially enhances the activity of the larger AFGP size isoforms (Jin, 2003; Jin and DeVries, 2006). The AFGPs and AFPP are present in differing amounts and total antifreeze activity is dependent on the total concentration and also the proportions of the large and small size isoforms of AFGP (Jin and DeVries, 2006) and the potentiation of the large isoforms by AFPP. The evolution of AFPs and their high circulating concentrations were one of the adaptations that led to the radiation of the notothenioids into niches left vacant when the late Eocene fish fauna disappeared as the Southern Ocean waters cooled to their freezing point (Eastman, 2005). One taxon, the trematomids, became the predominant genus in the high latitude regions (DeVries and Cheng, 2005; Eastman, 2005).

The largest group of Antarctic fishes is the suborder Notothenioidei and accounts for >90% of the Antarctic fish biomass (Eastman, 2005). Within this suborder the largest of the 6 families, the family Nototheniidae, is made up of 49 species while the next largest families, Artedidraconidae and Channichthyidae have 25 and 16 species respectively (Eastman, 2005). Of the nototheniids, the genus *Trematomus* is a recent radiation (Lautredou et al., 2012). It is made up of 14 species common in the shelf waters around the Antarctic continent. They inhabit both high and low latitude regions and are found at depths ranging from just under the ice, such as the cryopelagic *Pagothenia borchgrevinki*, to benthic species found at depths greater than 500 m, such as *Trematomus loennbergii*, and depths in between (Eastman, 1993). The habitat depths of some species range from the sub-tidal waters down to the deep shelf break waters (1500 m). Though circum-Antarctic, the *Trematomus* genus is considered a high latitude radiation due to their predominance in the ichthyofaunal biomass of the Ross Sea (Eastman, 2005).

With habitat temperature differences and variable ice coverage it is expected that the trematomid blood freezing points and associated AFGP and AFPP activities will vary. The freeze avoidance of 10 common species of *Trematomus* was investigated by determining their blood freezing points (a proxy for organismal freezing point (DeVries et al., 1982)) and the contribution of the AFGP and AFPP activity to their freezing points between their habitat temperatures and as a function of depth at a constant temperature. We hypothesized that freezing points would be lowest in fishes collected from the year-round freezing waters of the high latitudes of the southwestern Ross Sea with correspondingly high AFP activities of both the AFGPs and AFPP, while populations from the Western Antarctic Peninsula which inhabit warmer waters would exhibit higher freezing points with lower AFP activities. We also hypothesized that shallow water populations would have significantly different freezing points than deep water populations in response to the exposure to ice crystals in their environment even though their habitat temperatures were essentially the same.

2. Materials and methods

2.1. Collection and sampling

Ten of the 14 species of the genus *Trematomus* were collected from the high latitude McMurdo Sound and Terra Nova Bay (southwestern Ross Sea) and from the lower latitude waters of the Western Antarctic Peninsula (WAP). These included *Trematomus hansoni*, *Trematomus bernacchii*, *Trematomus nicolai*, *Trematomus pennellii*, *Trematomus newnesi*, *T. loennbergii*, *Trematomus scotti*, *Trematomus tokarevi*, *Trematomus eulepidotus* and *P. borchgrevinki*. While *P. borchgrevinki* is sometimes separated into the genus *Pagothenia*, mitochondrial and nuclear sequence analyses suggest that *Pagothenia* and *Cryothenia* are more closely related to the *Trematomus* genus than as an outgroup to the *Trematomus* clade and if included in the genus *Trematomus* this genus would be a monophyletic clade (Ritchie et al., 1996; Kuhn and Near, 2009). For the purpose of this study they will be included with the trematomids.

Table 1 lists the locations, latitudes, depths, and water temperatures obtained by loggers with an accuracy of 0.01 °C, where each *Trematomus* species was collected. Shallow water individuals were collected by hook and line through the ice. Specimens of *P. borchgrevinki* were caught just under the platelet ice layer. Specimens of *T. newnesi* were caught just under the ice or a few meters off the bottom in 15 m of water while the remaining species were caught on the bottom at depths ranging from 20 to 30 m. Deep water individuals were caught via baited trap or by bottom trawl deployed from research vessel between 200 and 500 m depth (Table 1). Fish were transported to the laboratory where they were kept in aquaria with running seawater at temperatures between −1.2 °C and −1.5 °C. For all individuals blood was collected by caudal vein puncture with a 23 gauge needle. Fish collected in 2010 were anesthetized prior to blood sampling with MS-222 (tricaine methanesulfonate) at a concentration of 80 mg/l for 5 to 10 min. Blood was allowed to clot for 1 h at +4 °C, centrifuged for 10 min at 16,000 *g* and the serum stored at −80 °C until analyzed. Fish were classified as being either from low or high latitudes depending on whether they were collected from the Western Antarctic Peninsula region including the South Shetland Islands or from the southwestern Ross Sea region including McMurdo Sound and Terra Nova Bay. They were classified by depth with a collection depth of between 4 and 30 m being considered as “shallow-caught” and depths greater than 150 m as “deep-caught.”

All fishes were collected and sampled in accordance with the University of Illinois institutional animal care and use approval.

2.2. Determination of melting and freezing points

Blood serum freezing and melting points were determined using a Clifton nanoliter osmometer (Clifton Technical Physics) following the protocol of Cziko et al. (2006). The instrument was calibrated with distilled water (0 mOsm) and a 1000 mOsm standard (Opti-mole, Wescor Inc.). Ten nanoliter volumes of sample were suspended in heavy microscope immersion oil in each of the 6 wells in the Clifton nanoliter sample holder. The samples were cooled until frozen and then slowly warmed until a single ice crystal (approximately 10 μm diameter) slowly melted while observed at 200× magnification. This temperature was taken as the melting point or equilibrium freezing point.

Following determination of the melting point a 10 μm single ice crystal was slowly cooled at a rate of about 0.2 °C per minute until sudden rapid growth was observed and this value was taken as the serum freezing point. Serum freezing point was used as a proxy for organismal freezing point (DeVries et al., 1982). Melting and freezing point determinations for each individual were repeated twice per sample well in three separate wells. The thermal hysteresis which is the difference between the melting point and the non-equilibrium freezing point (temperature of ice growth) of the blood serum represents the total antifreeze activity. The averaged hysteresis value from the replicates from an individual fish was taken to be the hysteresis value for that particular individual. The individuals' hysteresis values were averaged together to determine the hysteresis value for a given species.