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ORIGINAL PAPER

Gluconeogenic pathway does not display metabolic cold adaptation in liver of Antarctic notothenioid fish

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Abstract Antarctic notothenioid fish display specializations related to cope with their chronically cold environment, such as high triacylglycerol (TAG) content in tissues. The metabolic fate of glycerol, a product of TAG mobilization, has not been studied in Antarctic fish. To assess the importance of glycerol as a substrate for gluconeogenesis and to determine whether this pathway is metabolically cold adapted (MCA), key hepatic enzyme activities were measured in Antarctic (*Notothenia coriiceps*, *Gobionotothen gibberifrons*, and *Chionodraco rastrospinosus*) and non-Antarctic (*Dissostichus eleginoides*, *Patagonotothen ramsayi*, and *Eleginops maclovinus*) notothenioid fish. Fructose 1,6-biphosphatase (FBP), phosphoenolpyruvate carboxykinase (PEPCK), and glycerol kinase (GK) activities were similar in both groups at common temperatures (1, 6, 11, or 21 °C). In particular, thermal sensitivity for the reactions catalyzed by FBP and PEPCK was analogous between Antarctic and non-Antarctic species, reflected by similar values for Arrhenius energy of activation (E_a) and Q_{10}. Additionally, hepatic glycerol, glucose, and glycogen contents together with plasma glycerol and glucose concentrations were similar for all of the species studied. Our results do not support the concept of MCA in hepatic gluconeogenesis and may indicate that the use of glycerol as a precursor for glucose synthesis by this pathway is of low physiological importance in Antarctic fish.

Keywords Gluconeogenesis · Glycerol · TAG mobilization · Antarctic notothenioid fish · Metabolic cold adaptation

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Abbreviations

AAC	Antarctic circumpolar current
MYA	Million of years ago
AFGP	Antifreeze glycopeptides
TAG	Triacylglycerol
MCA	Metabolic cold adaptation
FBP	Fructose 1,6-biphosphatase
PEPCK	Phosphoenolpyruvate carboxykinase
GK	Glycerol kinase
E_a	Energy of activation

Introduction

The establishment of the Antarctic Circumpolar Current (ACC) is thought to have developed about 34 MYA, thermally isolating the waters surrounding Antarctica by preventing the intrusion of warm currents (Cristini et al.

2012). It is believed that an ancestral Antarctic notothenioid fish may have escaped this confinement approximately during the last 14 MYA and colonized more temperate waters north of the ACC (DeVries and Steffensen 2005). Since then, notothenioid fish have been subjected to geographical isolation and speciation (Eastman 1993), evolving in the extremely cold (−1.9 to 2 °C) and stable waters surrounding Antarctica, or in the waters adjacent to New Zealand, Australia, and South America which are 5–10 °C warmer (Johnston et al. 1998; Near et al. 2012).

Antarctic notothenioids have a number of biochemical and physiological specializations that are considered to be cold adaptations. The most well-known specialization includes the production of antifreeze glycopeptides compounds or AFGP (Chen et al. 1997). AFGP are present in all the Antarctic notothenioid fish in adult stage (Cheng and Detrich 2007) and are secreted to the intestinal lumen (O'Grady et al. 1982; Cheng et al. 2006; Evans et al. 2011). Other characteristics of Antarctic notothenioid fish are the elevated content of lipids they hold stored as triacylglyceride (TAG) (Lund and Sidell 1992), a high reliance in the use of fatty acids as metabolic fuel (Sidell 1991), and a large capacity for oxidative metabolism (Crockett and Sidell 1990; Kawall et al. 2002). Because the glycerol released from TAG mobilization is also a precursor for the synthesis of glucose through gluconeogenesis, all these specializations may have an impact on this metabolic pathway. Additionally, because the synthesis of AFGP requires amino acids (Peltier et al. 2010; Wojnar et al. 2011), it is possible that these compounds may be spared and become less important than glycerol as gluconeogenic precursor.

Gluconeogenesis is a primarily hepatic metabolic pathway that plays a fundamental role in vertebrates supplying glucose as a metabolic fuel to critical tissues such as the nervous system (Cahill 1986). The function of this pathway in maintaining glucose homeostasis is critical in fish because they have a limited capacity to store large quantities of hepatic glycogen and have limited access to dietary carbohydrate (Moon and Foster 1995). Glucose production is particularly well correlated with the activities of phosphoenolpyruvate carboxykinase and fructose 1,6-biphosphatase in fish, two enzymes regulating this metabolic pathway (Mommsen 1986). Additionally, glycerol kinase is considered a key enzyme when glycerol is used as a precursor for the synthesis of glucose (Newsholme and Taylor 1969; Savina and Wojtczak 1977).

The term metabolic cold adaptation (MCA) has been introduced to designate diverse physiological processes that enable an organism living at cold temperatures to have a greater active metabolic rate than an organism of similar ecotype from a warmer environment acutely exposed or acclimated to the same low temperatures. The hypothesis of MCA shows mixed support in aquatic animals and has been subjected to controversy (Holeton 1974; Peck and Conway 2000; Steffensen 2002). A recent and extensive data analysis shows that high-latitude fish species have higher standard metabolic rates, mitochondrial respiration rates, and aerobic enzyme activities than low-latitude species, suggesting that metabolic compensation in these species is present but incomplete (White et al. 2012). When the catalytic rate for an enzyme is measured at a common temperature, orthologs from more cold-adapted species have higher values (Hochachka and Somero 2002). A higher catalytic rate in cold-adapted species is possible by increasing intracellular concentration for the enzyme, and/or by reducing its energy barrier (Somero 1991; Hochachka and Somero 2002), so that an enzyme from a cold-adapted species has a lower energy of activation (E_a) for the reaction catalyzed, which reflects an adjustment to the low-temperature environment in which these enzymes function (Low et al. 1973; Feller and Gerday 1997; Lonhienne et al. 2000). A number of studies have shown that both adaptive mechanisms may be occurring for various enzymes in Antarctic notothenioid fish when compared with species that live in warmer waters. These MCA enzymes included citrate synthase, cytochrome oxidase, carnitine palmitoyltransferase and 3-hydroxyacyl-CoA dehydrogenase in cardiac and skeletal muscles (Crockett and Sidell 1990), citrate synthase in brain (Kawall et al. 2002) and white muscle (White et al. 2012), and lactate dehydrogenase in skeletal muscle (Fields and Somero 1998). Therefore, it will be important to investigate if other enzymes have accumulated adaptive changes during the evolution of Antarctic notothenioids fish, particularly those involved in hepatic gluconeogenesis, to ensure sustained glucose production in chilled conditions. For this purpose, we measured FBP, PEPCK, and GK maximal enzymatic activities in the liver of Antarctic (*Gobionotothen gibberifrons*, *Notothenia coriiceps*, and *Chionodraco rastrospinosus*) and non-Antarctic notothenioid species (*Patagonotothen ramsayi*, *Eleginops maclovinus*, and *Dissostichus eleginoides*) at several temperatures (1, 6, 11, and 21 °C). In addition, we determined the levels of metabolites involved in hepatic gluconeogenesis and carbohydrate metabolism in the plasma of these fish.

Materials and methods

Chemicals

Substrates, cofactors, and enzymes were purchased from Sigma Chemical (St. Louis, Missouri), ICN Pharmaceuticals (Costa Mesa, California), and Boehringer Mannheim (Darmstadt, Germany). All other chemicals were from various commercial sources and were reagent grade.

Experimental animals

All fish studied belong to the order Notothenioidei. Antarctic fish (*G. gibberifrons*, *N. coriiceps*, and *C. rastrospinosus*) were captured from depths of 98–215 m from ARSV Laurence M. Gould at sites near Low Island (63° 25′ S, 62° 10′ W) and Dallman Bay in the vicinity of Astrolabe Needle (64° 10′ S, 62° 35′ W) off the Antarctic Peninsula (Fig. 1). In addition, some individuals of *N. coriiceps* were captured off the pier at Palmer Station, Antarctica (64° 46′ S, 64° 03′ W). The non-Antarctic fish *P. ramsayi* and *D. eleginoides* were caught from depths between 140 and 176 m from R/V Oca Balda (INIDEP, Argentina) at sites in the Atlantic Ocean waters (47° 47′ S, 61° 27′ W and 48° 48′ S, 62° 21′ W, respectively). *E. maclovinus* was caught in Chilean coastal water (Pacific Ocean) near La Boca and La Matanza (33° 58′ S, 71° 56′ W and 34° 20′ S, 72° 06′ W, respectively). Except for *E. maclovinus* where gill nets or hook and line were employed and some individuals of *N. coriiceps* for which hook and line were employed, all the fish were caught with otter trawls. Data on size and others morphometric parameters of the fish used in this study are included in Table 1. Animals were maintained at ambient sea temperatures (1 °C for *G. gibberifrons*, *C. rastrospinosus*, and *N. coriiceps*; 6 °C for *D. eleginoides* and *P. ramsayi*; and 11 °C for *E. maclovinus*) in running seawater tanks on the ships and/or the laboratory (Palmer Station, United States, and Las Cruces experimental station, Catholic Pontifical University of Chile) for at least 48 h before sampling. During this period, the animals were not fed.

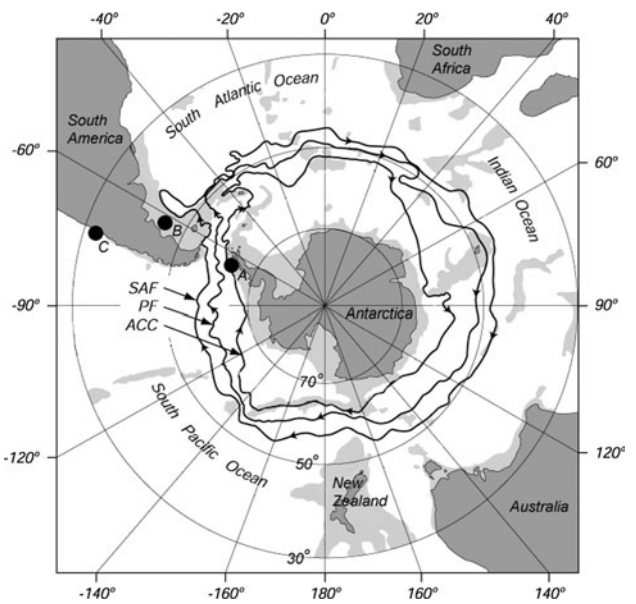

Fig. 1 Map of the sampling sites for Antarctic and non-Antarctic notothenioid fish species. *Black dots* indicate the sampling locations for the different species as described in "Materials and methods". (*A*) *N. coriiceps, G. gibberifrons, and C. rastrospinosus* (Antarctic); (*B*) *D. eleginoides, P. ramsayi* (non-Antarctic); and (*C*) *E. maclovinus* (non-Antarctic). *PF* polar front, *ACC* Antarctic convergence current, *SAF* Sub-Antarctic front

Sample collection

The fish were removed quickly from the holding tanks with a dip net and stunned by a sharp blow to the head. Prior to killing the animal, blood was obtained from the caudal peduncle. Plasma samples were prepared by centrifugation of blood at 10,000×*g* for 5 min at 4 °C. The supernatant was placed in cryogenic tubes and frozen at once in liquid nitrogen. After sampling the blood, fish were killed by severing the spinal cord posterior to the cranium. The liver was immediately dissected from the animal and placed in liquid nitrogen. The total time elapsed from killing the fish to freezing the liver samples was in the range of 1–2 min for all individuals. The frozen samples were transported on dry ice to our laboratory at University of Maine and stored at −80 °C for subsequent analyses.

Homogenate preparation

Frozen liver samples were homogenized at a ratio 9:1 (10 % w/v) in ice-cold homogenization buffer containing 40 mM Hepes (pH = 7.26 at 25 °C), 2 mM dithiothreitol, and a tablet of protease inhibitor cocktail (Boehringer Mannheim complete® mini) per 7 ml buffer, using a ground glass homogenizer held on ice. Phosphoenolpyruvate carboxykinase enzyme activity determination was performed on crude homogenates after sonication at 35 % maximal power in two 15 s bursts, with a 15 s cooling interval between them (Artek-Sonic 300 Dismembrator). For the other enzyme activities and metabolite determinations, the homogenate was centrifuged at 12,400×*g* for 10 min at 4 °C (IEC Micromax), and the supernatants were used. The aqueous phase was drawn from beneath lipid layers when they were present on top of the centrifuged samples. Protein was assayed in homogenates as detailed by Bradford (1976), using bovine serum albumin as standard. All the metabolite and enzyme activity determinations were performed in triplicate.

Metabolite concentration determinations

Homogenate and plasma supernatants were deproteinized by the addition of ice-cooled 6 % perchloric acid in a ratio of 1:3 (supernatant/perchloric acid), kept on ice, and mixed repeatedly over a 10-min period, followed by centrifugation at 10,000×*g* for 15 min at 4 °C. The supernatant was neutralized with ice-cold 5 mM K_2CO_3 and centrifuged at 10,000×*g* for 5 min at 4 °C. This remaining supernatant was used for subsequent metabolite determination.

Table 1 Size and morphology data of non-Antarctic and Antarctic notothenioid fishes

Character	Non-Antarctic species			Antarctic species		
	E. maclovinus (3)	*P. ramsayi* (8)	*D. eleginoides* (5)	*N. coriiceps* (8)	*C. rastrospinosus* (8)	*G. gibberifrons* (8)
Total length (L_T, cm)	36.7 ± 8.6	27.5 ± 1.3	62.0 ± 5.6	37.1 ± 3.8	40.2 ± 0.5	35.1 ± 0.8
Body weight (W, g)	292 ± 119	226 ± 13	1,303 ± 300	629 ± 66	557 ± 24	480 ± 33
Liver weight (W_L, g)	6.4 ± 0.7	4.1 ± 0.1	22.2 ± 1.2	15.1 ± 0.2	15.0 ± 0.1	7.7 ± 0.1
Hepatosomatic index (HIS, %)	2.2 ± 0.6	1.8 ± 0.5	1.7 ± 0.4	2.4 ± 0.3	2.7 ± 0.2	1.6 ± 0.2
Liver proteins (mg/g tissue)	163.0 ± 6.1	165.5 ± 2.9	159.2 ± 5.9	159.9 ± 3.7	153.4 ± 3.5	163.9 ± 4.1

HIS computed from $100 \times W_L \times W^{-1}$. Values are given as mean ± SE with sample size between parentheses

Glycerol determination

Glycerol concentration was estimated in samples utilizing a kit (UV-method 148270, Boehringer Mannheim). The assay conditions were as follows: 0.32 mg × ml^{-1} NADH, 1 mg × ml^{-1} ATP, 0.5 mg × ml^{-1} phosphoenolpyruvate-CHA, SO_4Mg, 3 U × ml^{-1} pyruvate kinase, 2.75 U × ml^{-1} lactate dehydrogenase, and 0.42 U × ml^{-1} glycerol kinase. By this method, the amount of NADH oxidized by a series of coupled reactions is stoichiometric to the amount of glycerol in the sample. NAD^+ formation is determined by the extent of decrease in light absorption at 340 nm.

Glucose determination

Glucose concentration was determined in samples utilizing a glucose assay kit (GAHK-20, Sigma Chemical) with the following assay conditions: 1.5 mM NAD, 1.0 mM ATP, 1 U × ml^{-1} hexokinase, and 1 U × ml^{-1} glucose 6-phosphate dehydrogenase. The amount of NAD^+ reduced is proportional to the concentration of glucose in the sample. NADH formation is determined by measuring the increase in absorbance at 340 nm.

Glycogen determination

Liver glycogen levels were assessed using the Keppler and Decker method (1974) in which the glucose liberated after glycogen breakdown catalyzed by amyloglucosidase was quantified (after subtracting free levels of glucose in liver). The conditions were as follows: 200 mM buffer acetate (pH = 4.8 at 20 °C) and 9.23 U × ml^{-1} amyloglucosidase (omitted from control) were incubated with 100 µl of supernatant for 120 min at 40 °C (total volume 1.1 ml). The reaction was stopped with the addition of 1 ml 3.64 % perchloric acid and centrifuged for 5 min at 10,000×*g*. An aliquot of the supernatant was used for glucose quantification as described earlier.

Enzyme activity assays

All enzymes were assayed in freshly prepared samples. Activities were measured using a Perkin-Elmer Lambda 40 UV–VIS spectrophotometer. Assay temperature was maintained at 1, 6, 11, or 21 °C (±0.1 °C) with a Neslab RTE-111 temperature-regulated water bath circulating a mixture of ethanol and distilled water (1:1). Reaction rates of enzymes were determined by increase or decrease in absorbance at 340 nm. Cuvettes were preincubated at each specific temperature. A low stream of nitrogen gas in the spectrophotometer chamber prevented water condensation on the cuvettes. Between 5 and 20 µl of supernatants were added to the cuvettes with a pre-established volume (final volume 1 ml) to give a linear rate of change in absorbance over the duration of the assay. Substrate was omitted in controls, and background activity was subtracted from that measured in the presence of substrate. Enzymatic analyses were all carried out with substrate and cofactor concentrations yielding maximum reaction velocities, with the reaction mixtures and homogenate dilution established in preliminary tests to render the highest activity possible. The reactions were started by the addition of substrate. Enzymatic activities were measured utilizing imidazole buffers adjusted to a baseline pH 7.37 at 25 °C and were allowed to follow their intrinsic pH/temperature relationship ($\Delta pKa/^oC = -0.017$), which parallels that of physiological fluids (Somero 1981). All activities were expressed in units (µmol substrate converted to product × min^{-1}) per gram wet weight of tissue. The specific conditions for enzyme assays, expressed as final concentrations, were as follows.

Fructose 1,6-biphosphatase (FBP; EC 3.1.3.11)

This enzyme activity was measured according to the procedure described by Mommsen et al. (1980), using 50 mM imidazole, 6 mM $MgCl_2$, 0.4 mM NADP, 2 U × ml^{-1} phosphoglucose isomerase, 2 U × ml^{-1} glucose

6-phosphate dehydrogenase, 0.1 mM fructose 1,6-biphosphate, and an AMP trapping system composed of 0.5 mM phosphoenolpyruvate, 0.05 mM ATP, 5 U × ml^{-1} myokinase, and 10 U × ml^{-1} pyruvate kinase. The reduction of $NADP^+$ was followed for 40 min.

Phosphoenolpyruvate carboxykinase (PEPCK; EC 3.1.3.11)

The assay used to measure the activity of this enzyme was described by Petrescu et al. (1979) with some modifications. In this assay, the medium contained 50 mM imidazole, 1 mM $MnCl_2$, 0.15 mM NADH, 1.6 mM deoxy-guanosine diphosphate, 8 U × ml^{-1} malate dehydrogenase, 5 mM phosphoenolpyruvate, and 160 mM $NaHCO_3$ (saturated with CO_2 and omitted from control). Oxidation of NADH was followed for 15 min.

Glycerol kinase (GK; EC 2.7.1.30)

The activity of this enzyme was measured according to the assay described by Bublitz and Wieland (1962). The reaction mixture included 50 mM imidazole, 1.8 mM $MgCl_2$, 4.1 mM ATP, 0.49 mM NAD, 17 U × ml^{-1} glycerol 3-phosphate dehydrogenase, and 6 mM glycerol. The reduction of NAD^+ by glycerol 3-phosphate dehydrogenase was monitored for 30 min.

Measurements of maximum enzyme activity at different temperatures were used to calculate the energy of activation (E_a) for the reaction according to the Arrhenius equation, where the slope of the plot (ln [maximal enzyme activity] vs. 1/Temperature [°K]) can be related to E_a by the equation:

$$E_a = -\text{slope} \times R,$$

where R represents the universal gas constant (8.31441 J $mol^{-1}°K^{-1}$) (Feller and Gerday 1997).

Q_{10} values for each enzymatic reaction were calculated according to the equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

where R_1 and R_2 represents the activities measured at temperature T_1 and T_2, respectively ($T_1 < T_2$).

Statistical analysis

Comparisons for metabolite concentrations and enzymatic activities between species were performed by using one-way analysis of variance (ANOVA). When the assumption of normality or homoscedasticity was not met, Kruskal–Wallis analysis of variance on ranks was substituted for ANOVA. To determine whether E_a and Q_{10} values were different among orthologs, statistically significant differences within each species were determined using Tukey–Kramer multiple comparisons tests ($\alpha < 0.05$). Values reported are means ± standard errors (SE) with sample size between parentheses.

Results

Metabolite content in plasma

Considering the high reliance on fatty acids as metabolic fuels by aerobic muscle of Antarctic notothenioid species, we anticipated finding an increased mobilization of TAG and therefore glycerol content in plasma of these fish when compared with non-Antarctic notothenioid species. However, no significant differences regarding glycerol content in plasma were found between Antarctic and non-Antarctic species (Table 2). Also, a clear distinction between Antarctic and non-Antarctic fish cannot be established regarding glucose concentration in plasma, although the non-Antarctic species *E. maclovinus* had significantly higher values of glucose than the other species examined. Hyperglycemia in individuals of this species may be related to the stress produced by the capture method utilized, as has been shown for gillnetted perch (*Perca fluviatilis*) and striped bass (*Morone saxatilis*) (Haux et al. 1985; Hopkins and Cech 1992).

Metabolite content in liver

Except for differences found between *C. rastrospinosus* and *P. ramsayi*, we measured similar glycerol concentration in the livers of both Antarctic and non-Antarctic fish (Table 2). The livers of *E. maclovinus* and *P. ramsayi* had significantly higher levels of glucose than the other species examined, reflecting a similar pattern of glucose levels in plasma from Antarctic and non-Antarctic fish. Glycogen contents in the livers of Antarctic and non-Antarctic fish had generally similar values for all the species studied [177–275 μmol glycosyl units × (g wet weight tissue)$^{-1}$].

Enzymatic activities in liver

Surprisingly, FBP specific activity measured at 1 °C in the livers of Antarctic and non-Antarctic fish was, in general, similar for all species (Fig. 2a), with the exception of *C. rastrospinosus*, in which a lower activity for this enzyme was found than in the rest of the species, excluding *E. maclovinus*. Similar results were found when FBP activity measured at 1, 6, 11, and 21 °C was expressed per g of wet weight tissue (Table 3). PEPCK activity in liver of the

Table 2 Metabolite concentrations in plasma and liver of non-Antarctic and Antarctic notothenioid fishes

	Non-Antarctic species			Antarctic species		
	E. maclovinus (3)	*P. ramsayi* (6)	*D. eleginoides* (5)	*N. coriiceps* (6)	*C. rastrospinosus* (8)	*G. gibberifrons* (6)
Plasma						
Glucose	5.41 ± 0.96	2.76 ± 0.32^{a}	0.90 ± 0.31^{b}	1.80 ± 0.19$^{a, b}$	1.15 ± 0.26^{b}	1.24 ± 0.22^{b}
Glycerol	0.19 ± 0.03	0.07 ± 0.01^{c}	0.10 ± 0.01^{c}	0.07 ± 0.01^{c}	0.05 ± 0.01^{c}	0.08 ± 0.01^{c}
Liver						
Glucose	24.76 ± 3.03	10.03 ± 1.42	1.86 ± 0.56^{a}	5.09 ± 0.90^{a}	2.32 ± 0.35^{a}	2.79 ± 0.53^{a}
Glycerol	1.04 ± 0.28$^{b, c}$	1.39 ± 0.14^{c}	0.89 ± 0.27$^{b, c}$	1.12 ± 0.23$^{b, c}$	0.85 ± 0.10^{b}	1.32 ± 0.22$^{b, c}$
Glycogen	176.89 ± 2.49^{d}	190.44 ± 12.00$^{d, e}$	186.16 ± 12.06$^{d, e}$	274.92 ± 27.44^{e}	181.74 ± 5.77^{d}	214.17 ± 6.44^{e}

Plasma glucose and glycerol concentrations are expressed in mM. Liver glucose and glycerol concentration are expressed as μmol × g wet tissue^{-1}, whereas glycogen concentration is expressed as μmol glycosyl units × g wet tissue^{-1}. Values are given as mean ± SE with sample size between parentheses. Similar letter denotes values that are not significantly different between species ($P < 0.05$)

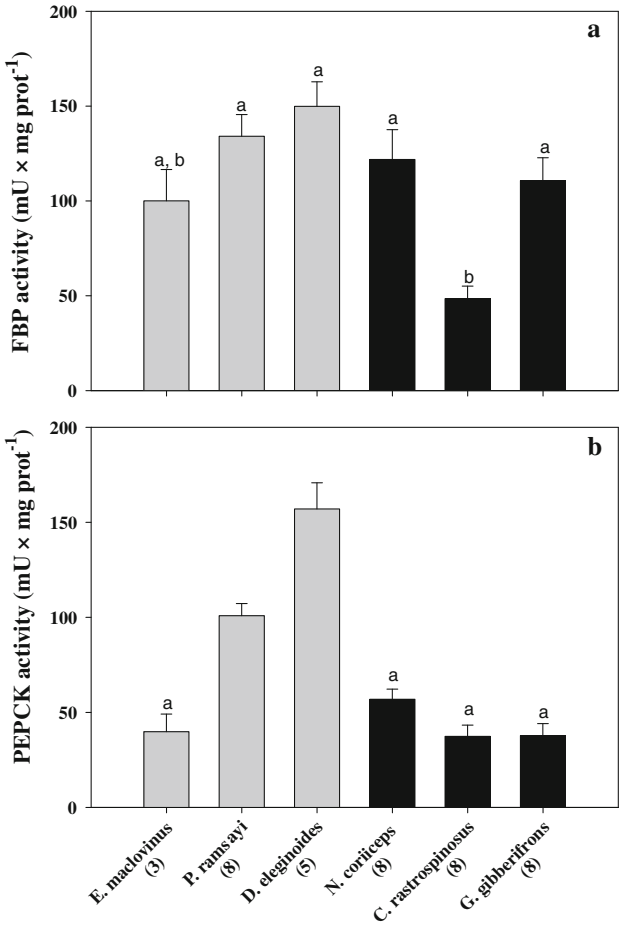

Fig. 2 Fructose 1,6-biphosphatase (**a**) and phosphoenolpyruvate carboxykinase (**b**) specific enzyme activities in liver of non-Antarctic (*gray bars*) and Antarctic (*black bars*) fish measured at 1 °C. Data are shown as mean ± SE with sample size between parentheses. *Similar letter* denotes values that are not significantly different between species ($P < 0.05$)

non-Antarctic fish *D. eleginoides* and *P. ramsayi* was significantly higher than the rest of the species when measured at 1 °C (Fig. 2b). However, PEPCK specific enzymatic activity in livers of *E. maclovinus*, *C. rastrospinosus*, *G. gibberifrons*, and *N. coriiceps* did not significantly differ from each other. A similar pattern was observed when PEPCK activity measured at 1, 6, 11, and 21 °C was expressed per gram of wet weight tissue (Table 4). No significant differences were found among GK activity in liver of Antarctic and non-Antarctic fish, which were remarkably low for all species (Table 5).

Thermal sensitivity of enzymatic capacities in liver

The thermal sensitivity of gluconeogenesis (FBP and PEPCK) was not different when measured in livers of Antarctic and non-Antarctic notothenioid fish as reflected by the similar values for the apparent activation energies (E_a) and Q_{10} obtained over different portions of the experimental temperature range (Table 6). The only significant differences detected were E_a for the reaction catalyzed by FBP between the non-Antarctic species *P. ramsayi* and *E. maclovinus*, and for the Q_{10} value for the reaction catalyzed by PEPCK between *P. ramsayi* and *D. eleginoides*.

Discussion

Gluconeogenesis is crucial to glucose homeostasis, since fish require glucose for the metabolism of critical tissues (e.g., nervous system, gills, red blood cells, testes, and renal medulla) and to synthesize certain biological molecules (e.g., mucopolysaccharides, AFGP). This metabolic pathway, occurring principally in liver of fishes (Knox et al. 1980), is of great importance because they generally have limited access to dietary carbohydrates. A comparison of maximal enzyme activity and thermodynamic parameters between Antarctic species and fish from temperate zone can reveal patterns of evolutionary adaptation of polar

Table 3 Fructose 1,6-biphosphatase activity in liver of non-Antarctic and Antarctic notothenioid fishes measured at different temperatures

Fish species	Temperature (°C)			
	1	6	11	21
Non-Antarctic				
E. maclovinus (3)	0.608 ± 0.079[a, b]	0.773 ± 0.102[a, b]	1.254 ± 0.159[a, b]	2.988 ± 0.392[a, b]
P. ramsayi (8)	0.805 ± 0.058[a]	1.362 ± 0.093[c]	2.591 ± 0.155[c]	6.050 ± 0.351[c]
D. eleginoides (5)	0.909 ± 0.067[a]	1.490 ± 0.138[c]	2.527 ± 0.214[c]	6.004 ± 0.464[c]
Antarctic				
N. coriiceps (8)	0.752 ± 0.088[a]	1.082 ± 0.072[a, c]	1.559 ± 0.111[a]	3.283 ± 0.162[a]
C. rastrospinosus (6)	0.322 ± 0.041[b]	0.515 ± 0.083[b]	0.847 ± 0.158[b]	2.263 ± 0.384[b]
G. gibberifrons (8)	0.666 ± 0.063[a]	1.195 ± 0.110[a, c]	2.037 ± 0.183[a, c]	4.558 ± 0.378

Enzyme activity is expressed as U × g wet tissue^{-1}. Values are given as mean ± SE with sample size between parentheses. Similar letter denotes values that are not significantly different between species ($P < 0.05$)

Table 4 Phosphoenolpyruvate carboxykinase activity in liver of non-Antarctic and Antarctic notothenioid fishes measured at different temperatures

Fish species	Temperature (°C)			
	1	6	11	21
Non-Antarctic				
E. maclovinus (3)	0.243 ± 0.054[a]	0.608 ± 0.184[a, b]	1.203 ± 0.323[a]	3.831 ± 0.973[a, b]
P. ramsayi (8)	0.614 ± 0.036[b]	1.096 ± 0.113[b]	2.593 ± 0.393	5.173 ± 0.449[a]
D. eleginoides (5)	0.981 ± 0.054[b]	2.563 ± 0.202	4.854 ± 0.631	12.777 ± 0.555
Antarctic				
N. coriiceps (8)	0.356 ± 0.032[a]	0.732 ± 0.116[a, b]	1.454 ± 0.208[a]	4.326 ± 0.640[a, b]
C. rastrospinosus (6)	0.243 ± 0.038[a]	0.591 ± 0.085[a]	1.398 ± 0.254[a]	3.837 ± 0.496[a, b]
G. gibberifrons (8)	0.230 ± 0.038[a]	0.587 ± 0.033[a]	1.283 ± 0.037[a]	3.951 ± 0.078[b]

Enzyme activity is expressed as U × g wet tissue^{-1}. Values are given as mean ± SE with sample size between parentheses. Similar letter denotes values that are not significantly different between species ($P < 0.05$)

Table 5 Glycerol kinase activity in liver of non-Antarctic and Antarctic notothenioid fishes measured at 11 °C

	Non-Antarctic species			Antarctic species		
	E. maclovinus (3)	*P. ramsayi* (8)	*D. eleginoides* (5)	*N. coriiceps* (8)	*C. rastrospinosus* (8)	*G. gibberifrons* (8)
U × g wet tissue^{-1}	0.045 ± 0.024	0.043 ± 0.015	0.059 ± 0.018	0.045 ± 0.026	0.042 ± 0.030	0.056 ± 0.025
mU × mg prot^{-1}	7.3 ± 3.9	7.1 ± 2.5	9.7 ± 3.0	7.2 ± 4.2	6.3 ± 4.5	9.2 ± 4.1

Values, given as mean ± SE with sample size between parentheses, were not significantly different between species ($P < 0.05$)

organisms to cold temperature. This study was undertaken to clarify if the gluconeogenesis and glycerol conversion through this pathway were metabolically cold adapted in livers of Antarctic notothenioid fish (*G. gibberifrons*, *N. coriiceps*, and *C. rastrospinosus*) when compared to livers of non-Antarctic notothenioid fish (*P. ramsayi*, *E. maclovinus*, and *D. eleginoides*). For that purpose, we measured and compared maximal enzymatic activities of rate-limiting steps involved in hepatic gluconeogenesis between Antarctic and non-Antarctic species.

It has been proposed that environmental water temperatures can influence the rate at which the synthesis of glucose can proceed in fish (Moon 1988; Moon and Foster 1995). In particular, it has been shown that a decline in temperature can decrease the rate of gluconeogenesis when this pathway was assessed in hepatocytes of *Hemitripterus americanus* (Renaud and Moon 1980), *Anguilla anguilla* (Jankowsky et al. 1984), and *Oncorhynchus mykiss* (Seibert 1985). Therefore, it is possible to speculate that Antarctic notothenioid fish may display mechanisms that

Table 6 Thermal sensitivities for the reactions catalyzed by fructose 1.6-biphosphatase (FBP) and phosphoenolpyruvate carboxykinase (PEPCK) in liver of non-Antarctic and Antarctic notothenioid fishes

	Non-Antarctic species			Antarctic species		
	E. maclovinus (3)	*P. ramsayi* (8)	*D. eleginoides* (5)	*N. coriiceps* (8)	*C. rastrospinosus* (8)	*G. gibberifrons* (8)
FBP E_a	23.82 ± 2.37[a]	29.77 ± 1.31[b]	27.58 ± 1.54[a, b]	25.07 ± 2.20[a, b]	26.71 ± 2.89[a, b]	27.83 ± 1.75[a, b]
Q_{10} values						
1–6 °C	1.62 ± 1.30	2.86 ± 0.72	2.69 ± 1.24	2.07 ± 1.17	2.56 ± 1.27	3.22 ± 0.95
6–11 °C	2.63 ± 1.32	3.62 ± 0.68	2.88 ± 1.43	2.08 ± 0.67	2.70 ± 1.61	2.91 ± 0.92
11–21 °C	2.38 ± 1.35	2.34 ± 0.60	2.38 ± 1.35	2.11 ± 0.71	2.67 ± 1.87	2.24 ± 0.90
PEPCK E_a	39.44 ± 5.01	34.13 ± 2.03	36.03 ± 1.66	36.82 ± 2.44	39.55 ± 2.63	38.58 ± 2.27
Q_{10} values						
1–6 °C	6.27 ± 2.22[a, b]	3.19 ± 0.59[b]	6.83 ± 1.05[a]	4.23 ± 0.90[a, b]	5.92 ± 1.56[a, b]	6.51 ± 1.65[a, b]
6–11 °C	3.91 ± 3.03	5.60 ± 1.03	3.59 ± 1.29	3.94 ± 1.58	5.56 ± 0.76	4.78 ± 0.57
11–21 °C	3.18 ± 2.68	1.99 ± 1.52	2.63 ± 1.80	2.98 ± 1.43	2.74 ± 1.82	3.08 ± 0.29

Apparent Arrhenius activation energy (E_a) is given in kJ × mol^{-1} and was calculated from Arrhenius Plots as described in "Materials and methods" section. Q_{10} values were calculated as described in "Materials and methods" section. Values given as mean ± SE with sample size between parentheses. *Similar letter* denotes values that are not significantly different between species ($P < 0.05$)

allow them to overcome the reduction in the catalytic rate of enzymes involved in gluconeogenesis produced by low temperatures.

Phosphoenolpyruvate carboxykinase and FBP, two key enzymatic activities on the gluconeogenic pathway, have been reported in livers of a variety of vertebrates. Both enzymatic activities are correlated with glucose production and considered limiting the rate of gluconeogenesis in fish (Mommsen 1986). Additionally, the reaction catalyzed by GK is a rate-limiting step in the conversion of glycerol to glucose. If the hepatic enzymes PEPCK, FBP, and GK were metabolically cold adapted, a higher maximal enzymatic activity would be expected to occur in Antarctic fish with respect to non-Antarctic fish when measured at 1 °C. To our surprise, liver from Antarctic notothenioids fish shows comparable levels of activity than non-Antarctic notothenioids fish for all the enzymes assayed in this study.

The carbons included in the glycerol molecule feed into the gluconeogenic pathway through the triose level, thus bypassing the PEPCK rate-limiting step. However, PEPCK activity was included in this study to test if gluconeogenesis could display MCA from other metabolic precursors other than glycerol, such as amino acids and lactate. Our results suggest that this is not the case, although we should point out that PEPCK activity in this study was measured using total homogenates, in spite of the existence of cytosolic and mitochondrial forms of the enzyme (Suarez and Mommsen 1987). Additionally, because measurements of PEPCK activity did not contain an equivalent amount of Na^+ in the control, the values obtained by our study may represent an overestimation of the true activity in the livers of these fish. Therefore, it is possible that a potential increase in the activity of one of the forms of this enzyme may be overlooked by our approach. However, this seems unlikely because FBP, the other key gluconeogenic enzyme included in our study, did not display MCA. Future studies should be carried out to appraise gluconeogenesis in vivo (Bequette et al. 2006), allowing for a more complete assessment of this metabolic pathway in both groups of fish.

Hepatic GK activity was out of the limits of detection of the method used in this study when measured at 1 or 6 °C for all the species. Similarly, this enzyme was undetected in livers of *Myxine glutinosa* when measured at 10 °C (Foster and Moon 1986) or displayed a very low activity in liver of *Perca flavescens* [0.030 μmol × min^{-1} × (g wet weight tissue)$^{-1}$] at 15 °C (Foster and Moon 1991). The activity of this enzyme measured in liver of Antarctic and non-Antarctic fish was similarly low [0.043–0.059 μmol × min^{-1} × (g wet weight tissue)$^{-1}$] when measured at 11 °C. This low activity in liver of notothenioid fish may indicate that glycerol as a gluconeogenic precursor may be of little physiological importance in these groups of fish, although we cannot discard the conversion of this molecule to glucose in other tissues such as the kidney or the skeletal muscle. Lactate conversion to glucose through this pathway has been shown to be important in skeletal muscle of several vertebrates after exhaustive exercise (Fournier and Guderley 1992; Gleeson 1996), including fish (Milligan and Girard 1993). However, tissues other than the liver seem to have a less important role in this metabolic pathway when studied in several species of fish including *O. mykiss*, *G. morhua*, or *P. platessa* (Knox et al. 1980).

Metabolic cold adaptation has been shown to be present at several physiological levels in polar fish, including standard metabolic rate, mitochondrial respiration, and

citrate synthase activity (White et al. 2012). An increased enzymatic activity in tissues of cold-adapted organisms could be due to higher intracellular concentration, improved catalytic efficiency, or a combination of these two types of adaptations (Somero 1991; Hochachka and Somero 2002). A possible strategy for cold-adapted enzymes is to decrease the E_a value in order to reduce the temperature dependence of the activity (Lonhienne et al. 2000), producing a more "efficient" enzyme rather than increasing its concentration. In fact, a decrease of E_a has the effect of increasing maximal enzymatic activity, and this strategy may occur during the adaptation of enzymes to low temperatures (Feller and Gerday 1997). However, our results suggest that enzyme concentration and efficiency are not altered in liver of Antarctic fishes, as shown by similar specific activities and E_a values for enzymes involved in gluconeogenesis and glycerol use in both groups of notothenioid fishes. These findings contrast with compensatory increases in catalytic activity described in several tissues of Antarctic notothenioid when compared to warm-temperate species. For example, higher concentration of aerobic enzymes, such as citrate synthase, has been related to the extremely dense populations of mitochondria in oxidative tissues of Antarctic fishes when compared to homologous tissues of warm-temperate fish (Londraville and Sidell 1990; O'Brien and Sidell 2000). An increase in catalytic efficiency for the enzyme LDH in brain and skeletal muscle of several Antarctic notothenioids fishes accounts for most of the differences in activity when compared to warm-temperate fishes (Fields and Somero 1998; Kawall et al. 2002). Furthermore, E_a values obtained for the reaction catalyzed by LDH in Antarctic notothenioids fish were lower than those for warm-temperate fish (Fields et al. 2001), suggesting that concentrations for this enzyme do not differ markedly between fishes. Therefore, further studies will be needed to explore the possible presence of both adaptive mechanisms of MCA by comparing both groups of non-Antarctic and Antarctic notothenioid fish (Coppes Petricorena and Somero 2007), particularly of enzymes implicated in energy homeostasis.

Large quantities of TAG and a high reliance on fatty acids as metabolic fuel in skeletal muscle of Antarctic fish (Sidell 1991; Lund and Sidell 1992) imply an increased release of glycerol to plasma from this tissue, because the conversion of this metabolite to glycogen in muscle may be considered minor, as GK activity is negligible in skeletal muscle of several vertebrates, including fish (Newsholme and Taylor 1969). However, our results show that glycerol plasma concentration is similar between Antarctic and non-Antarctic fish and comparable to what has been measured in plasma of other teleosts, except for *Osmerus mordax*, in which antifreeze properties for that compound have been recognized (Driedzic et al. 1998). It seems possible that intramuscular lipolysis may occur to a similar extent in Antarctic and non-Antarctic notothenioid fish (Sidell and Hazel 2002), which may explain the similarity in plasma glycerol levels measured in these fish by our study. Glycerol can be directed through the TCA cycle to obtain energy instead of being used as gluconeogenic precursor. However, studies measuring metabolic turnover rates of glycerol and free fatty acids in *O. mykiss* indicate that the contribution of both products of lipolysis is well in excess of oxidative fuel requirements (Bernard et al. 1999). Therefore, the metabolic fate of glycerol released from intramuscular TAG mobilization is unclear, and further research is needed to investigate this, particularly in notothenioid fish.

When evaluating MCA, it is important to compare not only species that are phylogenetically related (Coppes Petricorena and Somero 2007), but also fish from a similar ecotype (Crockett and Sidell 1990). We included in this study benthic/pelagic sluggish notothenioid species, with the exception of the demersal active fish *D. eleginoides* (McKenna 1991; Xavier et al. 2002). Because variations between enzymatic activity and metabolic rates among species are being attributed to a disparity in the level of locomotory activity (Somero and Childress 1980), the higher values for enzymatic activities and metabolites observed in *D. eleginoides* with respect to the other species in our study may be related to such lifestyle difference. Additionally, differences in enzyme activity may be explained by the larger size of *D. eleginoides* individuals included in our study, because gluconeogenic enzyme activities may scale positively with fish body mass, as has been shown for some enzyme activities involved in glycolysis (Somero and Childress 1980).

Conclusions

The data presented in this comparative study on maximal enzymatic activities and thermal sensitivities of key enzymes involved in hepatic gluconeogenesis do not support the concept of MCA in Antarctic notothenioid species. Lack of MCA in this metabolic pathway may indicate that insufficient time has passed for evolutionary divergence in gluconeogenesis between these two closely related groups of notothenioids. In support of this idea, a comparative study on mitochondrial rDNA sequences suggested a recent divergence between Antarctic and non-Antarctic notothenioid fish (1.7–7 MYA), much later than the formation of the AAC (Stankovic et al. 2002). However, we cannot completely discard the possibility that a reduction in the metabolic costs of Antarctic notothenioid fish could place little evolutionary pressure on gluconeogenesis, which may explain the lack of MCA in this pathway when compared

with their non-Antarctic counterparts. Antarctic notothenioid fish did not display any hallmarks of an increased use of glycerol as a gluconeogenic precursor. Nevertheless, the importance of glycerol remains unclear in Antarctic notothenioid fish. Additional in vivo experiments utilizing infusion of radiolabeled compounds to analyze glycerol kinetics may help resolve this issue.

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