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ORIGINAL PAPER

Physical, chemical, and functional properties of neuronal membranes vary between species of Antarctic notothenioids differing in thermal tolerance

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Abstract

Disruption of neuronal function is likely to influence limits to thermal tolerance. We hypothesized that with acute warming the structure and function of neuronal membranes in the Antarctic notothenioid fish *Chaenocephalus aceratus* are more vulnerable to perturbation than membranes in the more thermotolerant notothenioid *Notothenia coriiceps*. Fluidity was quantified in synaptic membranes, mitochondrial membranes, and myelin from brains of both species of Antarctic fishes. Polar lipid compositions and cholesterol contents were analyzed in myelin; cholesterol was measured in synaptic membranes. Thermal profiles were determined for activities of two membrane-associated proteins, acetylcholinesterase (AChE) and Na^+/K^+-ATPase (NKA), from brains of animals maintained at ambient temperature or exposed to their critical thermal maxima (CT_{MAX}). Synaptic membranes of *C. aceratus* were consistently more fluid than those of *N. coriiceps* ($P < 0.0001$). Although the fluidities of both myelin and mitochondrial membranes were similar among species, sensitivity of myelin fluidity to in vitro warming was greater in *N. coriiceps* than in *C. aceratus* ($P < 0.001$), which can be explained by lower cholesterol contents in myelin of *N. coriiceps* ($P < 0.05$). Activities of both enzymes, AChE and NKA, declined upon CT_{MAX} exposure in *C. aceratus*, but not in *N. coriiceps*. We suggest that hyper-fluidization of synaptic membranes with warming in *C. aceratus* may explain the greater stenothermy in this species, and that thermal limits in notothenioids are more likely to be influenced by perturbations in synaptic membranes than in other membranes of the nervous system.

Keywords Antarctic fishes · Neuronal membranes · Membrane fluidity · Arrhenius break temperature · Cholesterol · Phospholipids

Abbreviations

AChE	Acetylcholine esterase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
CNPase	Cyclic nucleotide phosphodiesterase
CT_{MAX}	Critical thermal maximum
DPH	1,6-diphenyl-1,3,5-hexatriene
ePC	Plasmalogen PC
LDH	Lactate dehydrogenase
NKA	Sodium-potassium ATPase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PK	Pyruvate kinase
PS	Phosphatidylserine
SDH	Succinate dehydrogenase
UI	Unsaturation index

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Introduction

Members of the perciform suborder Notothenioidei account for approximately 90% of the fish biomass in the continental shelf waters surrounding Antarctica (Eastman and Eakin 2000). This group of teleosts likely represents the

most stenothermal assemblage of vertebrate ectotherms (Somero and DeVries 1967), although variation in thermal tolerance is present among notothenioid species (Beers and Sidell 2011; Bilyk and DeVries 2011). In comparison to red-blooded notothenioids collected in the same region (i.e., the Western Antarctic Peninsula), the hemoglobinless icefishes exhibit a reduced thermal tolerance (Beers and Sidell 2011). The red-blooded *Notothenia coriiceps* has a critical thermal maximum (CT_{MAX}) of 17 °C, while the icefish *Chaenocephalus aceratus* has a CT_{MAX} of 14 °C (Beers and Sidell 2011). What physiological factor(s) account for differences in thermal tolerance among notothenioids is (are) not fully understood.

Several species of fishes exhibit behaviors that indicate neuronal failure with warming (Friedlander et al. 1976; Bondar and Roots 1977; Ern et al. 2015). In vitro experiments with notothenioids indicate that brain metabolism fails at temperatures approaching the organisms' upper thermal limits, possibly due to fluidization of biological membranes (Somero and DeVries 1967). In addition, it has been demonstrated that synaptic transmission is impaired with warming in several Antarctic notothenioids (Macdonald et al. 1988). Because several neuronal processes are influenced by physical properties of biological membranes, membrane stability and integrity are likely to play important roles in setting thermal limits. In addition, the activities of synaptic membrane-associated enzymes, such as acetylcholinesterase (AChE) and Na^+/K^+-ATPase (NKA), are highly sensitive to membrane properties, including fluidity and lipid composition (Yeagle et al. 1988; Spinedi et al. 1993; Crockett and Hazel 1997; Chen et al. 1998; Yoneda et al. 2014). Enzyme-substrate affinity can also be impacted by membrane properties; for instance, acetylcholine receptor function is mediated by both lipid composition and fluidity (Fong and McNamee 1986).

The central nervous system of notothenioids is largely similar to that of other coastal perciform fishes, with a segmented brain that connects to a rostral spinal cord and ten cranial nerves (Eastman 1993). However, the synaptic membranes of Antarctic fishes have been shown to display greater fluidity relative to temperate ectotherms and mammals, an adaptation involving an increase in unsaturation of membrane phospholipids to maintain physical constancy of membrane properties even at very cold temperatures (Logue et al. 2000). This trend has also been shown to differentiate species of notothenioids that reside in non-Antarctic and Antarctic regions (Logue et al. 2000). While extreme membrane unsaturation allows for optimal function at subzero temperatures, this phospholipid composition is likely to be unsuitable at elevated temperatures. In temperate fishes exposed to thermal variation over an acclimation or acclimatization time course, homeoviscous adaptation has been demonstrated in neuronal membranes (Roots 1968; Cossins and Friedlander 1977; Cossins 1977; Cossins and Prosser 1982), indicating that lipid restructuring is often necessary for adjusting to more long-term thermal changes. However, loss of membrane integrity in neuronal membranes is likely to limit thermal tolerance with more acute warming.

How the physical and chemical properties of neuronal membranes vary in different Antarctic notothenioid species has not yet been explored. In particular, these questions have not been examined for membranes beyond the synaptic membranes, or within the context of known differences in thermal tolerance limits. We hypothesized that species differences in thermal tolerance might be explained by physical (i.e., fluidity), chemical (i.e., lipid composition), and/or functional (i.e., thermal sensitivities of integral proteins) attributes of neuronal membranes. To this end, we measured fluidity in three types of neuronal membranes (synaptic membranes, myelin, and mitochondria) in two species of notothenioids, the more thermotolerant red-blooded *N. coriiceps* and the hemoglobinless *C. aceratus*. We also quantified activities of two membrane-associated proteins (NKA and AChE) in brains from animals held at ambient temperature or subjected to their CT_{MAX}. In addition, activities at 5 °C of cytoplasmic enzymes, pyruvate kinase (PK), and lactate dehydrogenase (LDH) were also measured in animals from ambient and CT_{MAX} groups to help distinguish effects of membrane lipids on activities of membrane-bound enzymes. Our primary objectives were (1) to elucidate factors related to neuronal membrane structure and function that may explain thermal boundaries in *N. coriiceps* and *C. aceratus*, and (2) to determine which membrane type might be most responsible for setting thermal tolerance limits. Our results reveal species differences in synaptic membrane fluidity as well as dissimilarities among the responses to temperature variation in myelin fluidity and activities of integral proteins. Together, these findings shed light on the underpinnings of thermal tolerance associated with acute warming in Antarctic notothenioids.

Materials and methods

Animal and tissue collection

Adult specimens (800–2000 g) of *N. coriiceps* and *C. aceratus* were collected in the Western Antarctic Peninsula region during the austral fall of 2015, using otter trawls deployed from the ARSV *Laurence M. Gould* in Dallmann Bay (64°10'S, 62°35′W) and off the southwestern shore of Low Island (63°24'S, 62°10′W). Additionally, *N. coriiceps* were captured at these sites using baited pots. Animals were held in circulating seawater tanks on the vessel before being transferred to circulating seawater tanks at Palmer Station, Antarctica, where they were kept at ambient temperatures

(0 ± 1 °C) for a maximum of three weeks before tissue collection. *N. coriiceps* were fed to satiation with ~ 10 g fish muscle every other day. The icefish, which maintains a lower metabolic rate than other notothenioids (La Mesa et al. 2004), did not feed in captivity during this study. Animal work from this field season was approved by Ohio University's Institutional Animal Care and Use Committee (14-L-004).

In addition, *C. aceratus* and *N. coriiceps* were captured in nearby sites during the austral winters of 2005 (Dallmann Bay) and 2011 (southwestern shore of Low Island and south of Brabant Island) as described previously (Urschel and O'Brien 2008; Devor et al. 2016). These animals were maintained using the same protocol described above, but tissues were collected after a shorter, several-day recovery period. Tissues from these animals were used for the determination of the apparent Michaelis constant (K_m) for acetylthiocholine iodide of AChE. All animal work in the earlier field seasons was approved by the University of Alaska Fairbanks' Institutional Animal Care and Use Committee (134774-2).

Animals were euthanized by a blunt blow to the head followed by severing the spinal cord. Membrane preparations were carried out on freshly extracted brain tissue in animals held at ambient temperature. Brain tissue was excised and flash frozen in liquid nitrogen for enzymatic activity measurements. A subset of individuals from each species was selected for CT_{MAX} experiments; this involved exposing animals to an acute thermal ramping treatment as described (Beers and Sidell 2011). In brief, animals were transferred to a 700 L experimental tank and temperature was increased at a rate of 3.6 °C h^{-1} to the point of CT_{MAX}, which was characterized as the temperature at which loss of righting ability occurred. Immediately following loss of righting, the animal was removed from the tank and sacrificed.

Membrane preparations and marker enzyme analyses

Myelin, synaptic membranes, and mitochondria were fractionated from brain tissue as described (Dunkley et al. 2008), with modifications (see supplementary material). Tissues were pooled as needed; generally, two animals were required per membrane preparation. Membrane pellets were collected and resuspended in 250 µl storage buffer (25 mM Tris, pH = 7.4 at 25 °C) and stored at − 80 °C.

Enrichments of each membrane fraction were determined by measuring the protein-specific activities of marker enzymes: cyclic nucleotide phosphodiesterase (CNPase) for myelin, acetylcholinesterase (AChE) for synaptic membranes, and succinate dehydrogenase (SDH) for mitochondria (see supplementary material). All marker assays were conducted at ~ 23 °C and adapted to a microplate reader.

Membrane physical and chemical properties

Membrane fluidity was quantified by fluorescence depolarization as described (Crockett and Hazel 1995) (see supplementary material). Change in polarization (excitation = 356 nm, emission = 430 nm) was measured between 2 and 40 °C using a PerkinElmer LS-50B spectrophotometer. Temperatures were elevated at 2 °C intervals (for myelin) and 5 °C intervals (for synaptic membranes and mitochondria) at a rate of ~ 0.3 °C min^{-1}.

Lipids were extracted from myelin as described (Bligh and Dyer 1959) (see supplementary material). Extracts were sent to the Kansas Lipidomics Research Center for phospholipid analysis, and a diacyl polar lipid profile dataset was generated by quadrupole mass spectrometry using an Applied Biosystems 4000 QTRAP mass spectrometer as described (Xiao et al. 2010). Relative abundances of the major phospholipid classes were compared between species. The unsaturation index (UI) was calculated as described (Grim et al. 2010). Polar lipid compositions were not analyzed in synaptic membranes due to lack of sufficient material.

Cholesterol was quantified in myelin and synaptic membranes using a Cayman assay kit and normalized to total phospholipid content, which was measured as hydrolyzed inorganic phosphate in membranes as described (Rouser et al. 1970) (see supplementary material).

Membrane functional properties

Tissues were homogenized in ten volumes of reaction buffer (100 mM potassium phosphate buffer, pH = 8.0 at 25 °C) on ice using a Wheaton Tenbroeck ground glass homogenizer. AChE activity was determined as described for the marker assays but was scaled to a final assay volume of 1 ml (see supplementary material). Partitioning of AChE activity between membrane and cytosolic fractions was also determined (see supplementary material). Activity rates were reported as total units present in the volume of each fraction.

Apparent K_m values for acetylthiocholine iodide of AChE were determined in brains of *C. aceratus* and *N. coriiceps* (see supplementary material). Concentrations of 13, 9.4, 6.3, 4.7, 3.1, and 2.3 mM substrate (acetylthiocholine iodide) were used. Maximal reaction velocities were determined and apparent K_m values were calculated using the Lineweaver–Burke equation.

NKA activity was measured in a pyruvate kinase (PK)/lactate dehydrogenase (LDH) coupled reaction as described (Crockett 1999) (see supplementary material). Samples were measured at 5 °C intervals ranging from 5 to 45 °C. Reaction rates in the presence and absence of ouabain were compared with the former subtracted from the latter to obtain NKA activities.

Activities of cytoplasmic enzymes

Activities of cytoplasmic enzymes were measured as described for PK (Bergmeyer et al. 1974) and LDH (Torres and Somero 1988) with slight modifications (see supplementary material). Assays were conducted at 5 °C.

Statistical analyses

All statistical analyses were performed in SPSS Statistics except when otherwise noted. Membrane fluidity and AChE and NKA activity curves were analyzed using an analysis of covariance (ANCOVA). Tukey's post hoc tests were performed as necessary. For enzyme activity curves, Arrhenius break points (ABTs) were determined for each sample using a two-phase linear regression model to minimize residual sum of squares (Yeager and Ultsch 1989). ABTs were compared among groups by a two-way analysis of variance (ANOVA). Points after the discontinuity were excluded from the ANCOVA. Discontinuities were compared between NKA and AChE by an unpaired two-tailed *t* test. PK and LDH activities were compared among groups by a two-way ANOVA.

Polar lipid class abundances, unsaturation indices, unsaturation distribution, and cholesterol-to-phospholipid ratios were analyzed using unpaired two-tailed *t* tests to compare lipid profiles between species. Analyses of polar lipids and unsaturation distributions were adjusted for the Bonferroni correction to account for multiple *t* tests, with a minimum *P* value of 0.0042. Apparent K_m values were compared by two-tailed *t* tests in Microsoft Excel. All assumptions were tested before performing statistical analyses. Although the fluidity measurements for the synaptic membranes displayed heterogeneity of variance, all other assumptions were met and sample sizes were equivalent between these groups.

Results

Cell fractionation revealed three distinct membrane types

Fraction 2 (between 3% and 10% Percoll layers) was enriched approximately 3.5-fold with myelin for *C. aceratus* and *N. coriiceps* (Table S1, supplementary material). Fraction 4 (between 15% and 23% Percoll layers) was enriched 2.9- and 3.9-fold with synaptic membranes for *C. aceratus* and *N. coriiceps*, respectively. Fraction 5 (below the 23% Percoll layer) was enriched 5.4- and 4.3-fold with mitochondria for *C. aceratus* and *N. coriiceps*, respectively.

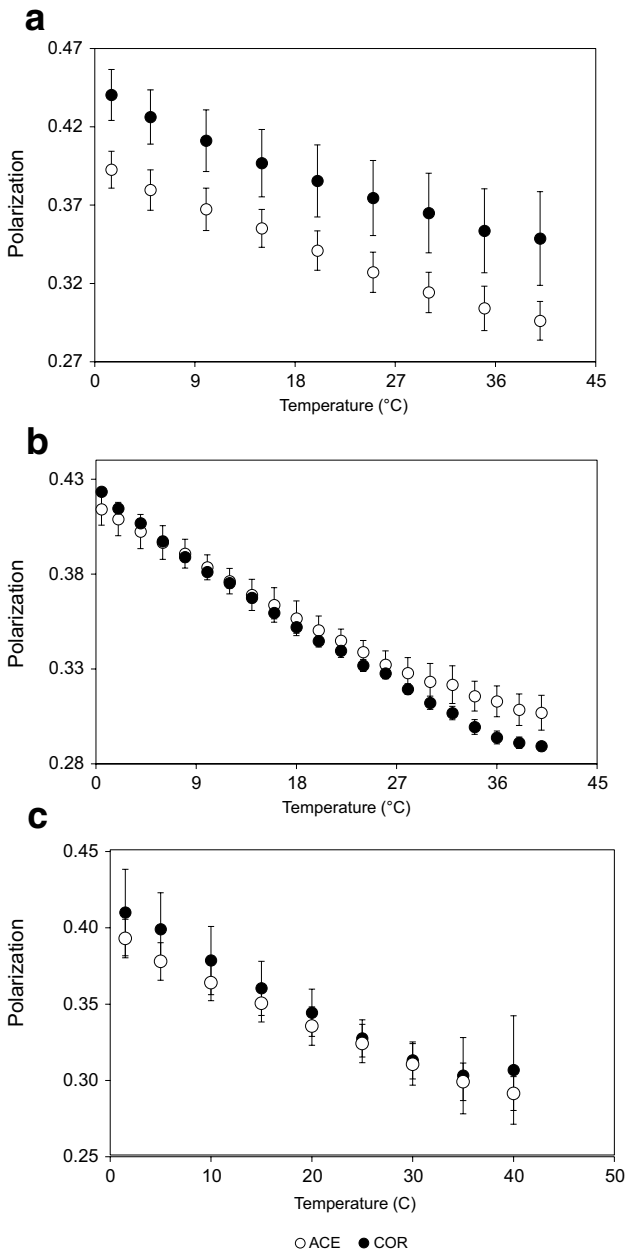

Fig. 1 Membrane fluidity for *C. aceratus* (ACE, open circles) and *N. coriiceps* (COR, closed circles) ($N=8$) in synaptic membranes (**a**), myelin (**b**) and mitochondria (**c**). All animals in this group were held at ambient temperatures (~0 °C) before membrane preparation. Polarization values for synaptic membranes from *C. aceratus* were consistently lower (i.e., greater fluidity) than those of *N. coriiceps* ($P<0.0001$). Variation in polarization with in vitro temperature change was approximately 1.3-fold greater in myelin from *N. coriiceps* relative to myelin from *C. aceratus* ($P<0.001$). Error bars represent ± s.d.

Fluidity of synaptic membranes was greatest in the less thermotolerant species

No significant discontinuities in slope were present in

membrane fluidity measurements of synaptic membranes, myelin, or mitochondria (Fig. 1a–c). Polarization values of synaptic membranes from *C. aceratus* were consistently lower than those of *N. coriiceps* ($P < 0.0001$), indicating a greater degree of fluidity in synaptic membranes from the icefish compared with membranes from the red-blooded species (Fig. 1a). In contrast, the absolute fluidities of both myelin and mitochondria did not differ significantly between species (Fig. 1b–c). However, myelin fluidity of *N. coriiceps* was significantly more influenced by thermal variation in vitro (i.e., greater thermal sensitivity) than in *C. aceratus*. Specifically, the change in polarization with temperature was approximately 1.3-fold greater in myelin from *N. coriiceps* than in myelin from *C. aceratus* ($P < 0.001$).

Lipid profiles of myelin showed variation between species

The most abundant polar lipid classes in myelin from both notothenioid species were phosphatidylcholine (PC), representing more than 50% of total phospholipids, and phosphatidylethanolamine (PE), representing approximately 25% (Table 1). Plasmalogen PC (ePC) and phosphatidylserine (PS) each represented 5–7.5% of the total phospholipid content.

The relative abundance of PC (expressed as mol%) in myelin was significantly greater in *N. coriiceps* than in *C. aceratus* ($P < 0.001$). Additionally, the ratio of PC to PE was significantly greater in *N. coriiceps* ($P < 0.01$). Myelin from *C. aceratus* contained a significantly greater unsaturation index (UI) than myelin from *N. coriiceps* ($P < 0.01$) (Table 2). Specifically, myelin from *C. aceratus* contained a greater proportion of highly unsaturated fatty acids (11–12 double bonds per pair of acyl chains), while myelin from *N. coriiceps* included a greater proportion of mono- and di-unsaturated fatty acids (Table 2). Cholesterol-to-phospholipid ratios in myelin were approximately twofold greater in *C. aceratus* than in *N. coriiceps* ($P < 0.05$), whereas cholesterol contents in synaptic membranes did not differ significantly between species (Fig. 2).

Table 1 Distribution of phospholipid classes in myelin

Phospholipid class distribution			
PL class	ACE	COR	Significance
	mol% (s.d.)	mol% (s.d.)	
PC	53 (1.6)	***57 (1.9)***	**
PE	26 (2.0)	23 (3.7)	
ePC	7.4 (1.0)	7.2 (0.57)	
PS	6.4 (0.48)	5.8 (3.1)	
PI	3.1 (0.20)	2.5 (0.65)	
ePE	1.8 (0.18)	1.8 (0.27)	
LPE	0.71 (0.27)	0.59 (0.20)	
PA	0.46 (0.12)	0.55 (1.6)	
LPC	0.37 (0.17)	0.45 (0.045)	
SM, DSM	0.35 (0.28)	0.29 (0.14)	
PG	0.22 (0.062)	0.16 (0.082)	
ePS	0.063 (0.019)	0.081 (0.053)	
PC:PE ratio	2.1 (0.19)	***2.5 (0.34)***	*

Phospholipid class distribution in myelin in *C. aceratus* (ACE) and *N. coriiceps* (COR) is reported as mol%. $N = 8$. All animals in this group were held at ambient temperatures (~0 °C) before membrane preparation

Bolded/italicized entries indicate significantly higher values. $*P < 0.0042$. $**P < 0.001$

PC phosphatidylcholine, *PE* phosphatidylethanolamine, *ePC* plasmalogen PC, *PS* phosphatidylserine, *PI* phosphatidylinositol, *ePE* plasmalogen PE, *LPE* lysoPE, *PA* phosphatidic acid, *LPC* lysoPC, *SM* sphingomyelin, *DSM* deoxy-sphingomyelin, *PG* phosphatidylglycerol, *ePS* plasmalogen PS

Table 2 Distribution of fatty acid unsaturation in myelin samples by number of double bonds

Fatty acid unsaturation distribution			
Double bonds	ACE	COR	Significance
	mol% (s.d.)	mol% (s.d.)	
0	***2.9 (0.16)***	2.1 (0.51)	*
1	20 (1.2)	21 (1.2)	
2	8.7 (3.1)	***16 (5.1)***	*
3	0.94 (0.24)	1.4 (0.28)	
4	1.6 (0.076)	1.7 (0.017)	
5	6.9 (0.53)	7.3 (0.88)	
6	38 (2.5)	34 (2.8)	
7	***10 (0.88)***	8.2 (1.2)	*
8	0.28 (0.019)	0.22 (0.096)	
9	0.11 (0.034)	***0.20 (0.045)***	**
10	0.30 (0.023)	***0.42 (0.045)***	***
11	***0.74 (0.12)***	0.55 (0.79)	*
12	8.3 (1.0)	6.5 (1.4)	
Total unsaturation index			
	ACE	COR	
	UI	UI	Significance
	493 (23)	450 (28)	*

Unsaturation distribution of diacyl polar lipids in myelin in *C. aceratus* (ACE) and *N. coriiceps* (COR) is reported as mol%

$N = 8$. All animals in this group were held at ambient temperatures (~0 °C) before membrane preparation

($N = 8$). Bolded/italicized entries indicate significantly higher values. $*P < 0.0042$. $** P < 0.001$. $***P < 0.0001$

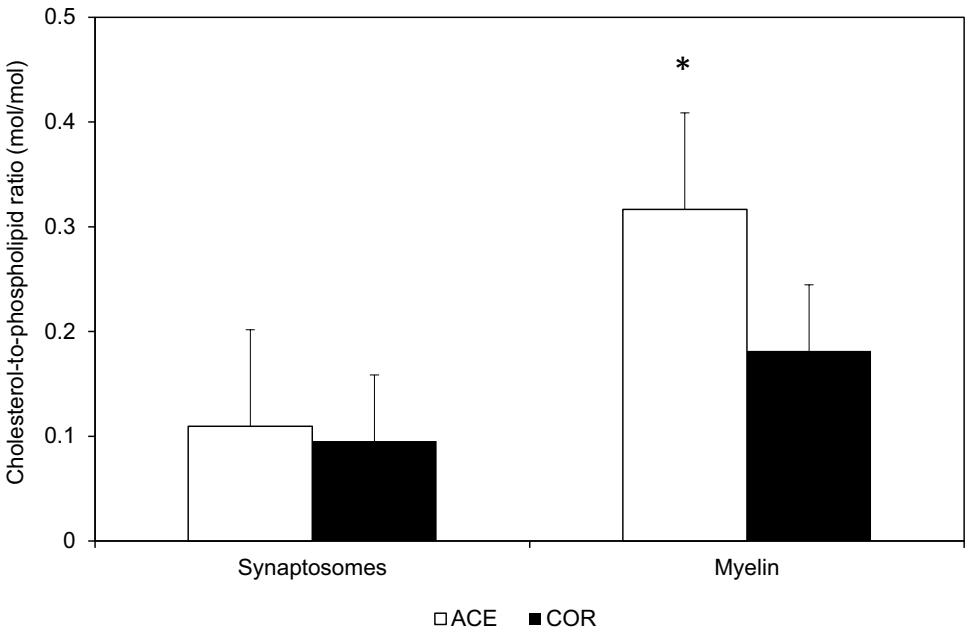

Fig. 2 Cholesterol-to-phospholipid ratios for *C. aceratus* (ACE, white bars) and *N. coriiceps* (COR, black bars) in synaptic membranes and myelin ($N=8$). One asterisk indicates $P<0.05$, indicating greater cholesterol contents in *C. aceratus*. All animals in this group were held at ambient temperatures (~0 °C) before membrane preparation. One asterisk indicates $P<0.05$. Error bars represent s.d.

Thermal profiles of enzymes indicate differences in absolute activities between species and with exposure to CT_{MAX}

The ABT for AChE occurred at 22.7 °C (s.d.=1.8) and did not differ significantly between species or thermal treatment groups (Fig. 3a). For animals at ambient temperatures, *C. aceratus* displayed AChE activity that was significantly greater than that of *N. coriiceps* ($P<0.0001$). Additionally, AChE activity declined significantly in *C. aceratus* exposed to CT_{MAX} ($P<0.05$), while exposure to CT_{MAX} did not alter AChE activity in *N. coriiceps*. The apparent K_m values for acetylthiocholine iodide differed significantly between species ($P<0.05$) and with assay temperature ($P<0.01$), with a greater K_m in *C. aceratus* and at the higher assay temperature (Fig. 4).

The ABT for NKA occurred at 32.8 °C (s.d.=2.2), significantly greater than that of AChE ($P<0.0001$), and like AChE, did not differ significantly among species or thermal treatment groups (Fig. 3b). NKA activity was reduced significantly in *C. aceratus* following CT_{MAX} exposure ($P<0.001$). Species differences in NKA activity were present between animals exposed to CT_{MAX}, as *C. aceratus* displayed significantly lower NKA activity levels than those of *N. coriiceps* ($P<0.001$).

Enzymatic activities of PK expressed either per g wet weight (shown) or per mg protein (not shown) did not differ significantly among thermal treatment groups or between species (Fig. 5). LDH activities normalized in either fashion, as described for PK, also did not vary among species. However, for both species, LDH activities

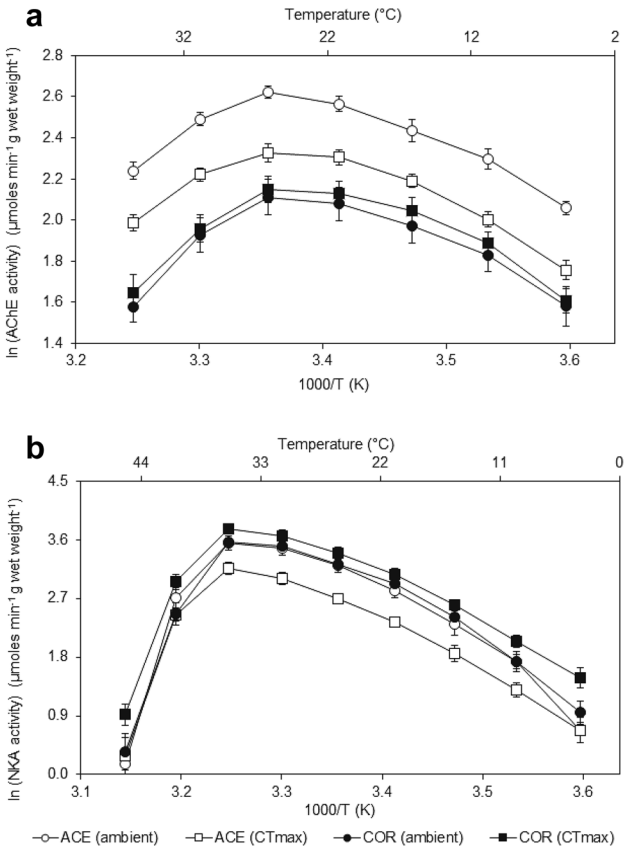

Fig. 3 Arrhenius plot of enzymatic activities of **a** acetylcholinesterase (AChE) and **b** Na^+/K^+ ATPase (NKA) for *C. aceratus* (ACE, open markers) and *N. coriiceps* (COR, closed markers) ($N=8$). Animals in this group were either held at ambient temperatures (~0 °C, circles) or exposed to CT_{MAX} (~14 or 17 °C, squares) before brain collection. Activity rates are reported as units per gram wet mass. Error bars represent ± s.d.

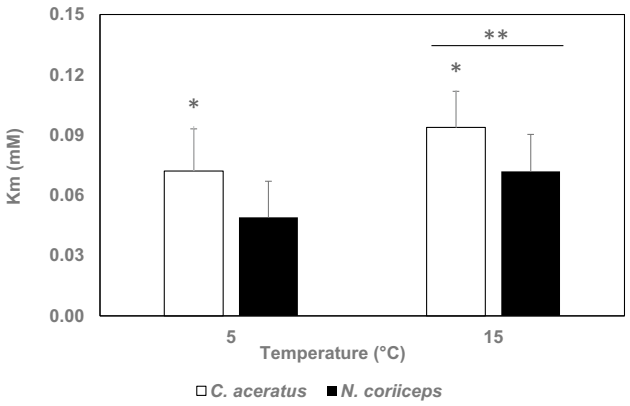

Fig. 4 Apparent K_m of acetylthiocholine iodide for AChE at two assay temperatures ($N=8$). The K_m is significantly different between species at both temperatures measured ($P<0.05$), which is indicated by the asterisks above the bars. Within each species, K_m is significantly greater at the higher assay temperature ($P<0.01$). Asterisks above line indicate significant differences between assay temperatures for both species ($P<0.01$). Error bars represent ± s.d.

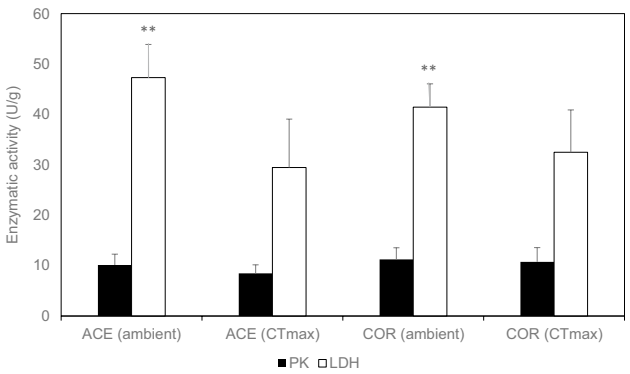

Fig. 5 Lactate dehydrogenase ($N=6$) and pyruvate kinase ($N=8$ for ACE, $N=7$ for COR) activity measurements of brain in *C. aceratus* (ACE) and *N. coriiceps* (COR). Measurements were performed at 5 °C. Activity rates are reported as units (μmol product/min) per gram wet mass. Two asterisks indicate a significant decrease in LDH activity at CT_{max} $P<0.01$. Error bars represent ± s.d.

were reduced 1.4-fold in animals exposed to their CT_{MAX} compared to ambient temperature ($P<0.01$) (Fig. 5).

Discussion

Species differences in synaptic membrane fluidity are associated with variation in thermal tolerance

We investigated properties of three types of neuronal membranes from two species of notothenioids that vary in thermal tolerance. Because the absolute fluidities varied between species only in synaptic membranes, disruption of physical properties in the synaptic junctions is likely to be of greater importance in governing limits to acute thermal stress rather than in either myelin or mitochondrial membranes. This interpretation is consistent with the observation that synaptic transmission is inhibited at elevated temperatures in several Antarctic notothenioids (Macdonald et al. 1988). We posit that in the icefish, *C. aceratus*, synaptic transmission is adversely affected at a lower temperature during thermal ramping than in *N. coriiceps*.

Greater sensitivity to thermal change of myelin from *N. coriiceps* may account for spasmodic behavior with acute warming

The absolute fluidity of myelin did not vary significantly between species, and this similarity in myelin fluidity can be explained, at least in part, by the polar lipid profiles of each species. While myelin in *C. aceratus* exhibited a higher UI, which should enhance fluidity (Hazel and Prosser 1974), myelin in *C. aceratus* also had a lower PC-to-PE ratio, which should impart an ordering effect (Fajardo et al. 2011). The fluidizing effect of a greater UI is likely to be countered by the lower PC-to-PE ratio, as well as the greater cholesterol contents, in myelin from *C. aceratus* compared with that of *N. coriiceps*.

In contrast with the similarity in myelin fluidity between species, the perturbation in fluidity with in vitro changes in temperature was greater in myelin from *N. coriiceps* than in *C. aceratus*. This finding can be explained by our observation that cholesterol-to-phospholipid ratios were significantly lower in myelin of *N. coriiceps*. Because cholesterol is known to stabilize membrane fluidity (van Meer et al. 2008), the higher cholesterol contents in myelin of the less thermotolerant *C. aceratus* do, at first glance, seem counterintuitive. However, the greater thermal dependence of myelin fluidity in the red-blooded species may play a role in some of the observed irregular animal behaviors during warming (Crockett and O'Brien, unpublished observations). For example, *N. coriiceps* displays spasms that suggest some disruption to neuronal function, even at temperatures well below the species' CT_{MAX} of 17 °C. Frequency of impulse conduction has been shown to increase with acute warming in both mammals and poikilotherms, but often becomes irregular beyond a critical temperature (Lele 1963; Prosser and Nelson 1981). Such irregular behaviors, however, were not observed in *C. aceratus*. Given our results pertaining to membrane fluidity, it seems possible that a breakdown in synaptic transmission may precede the destabilization of myelin in the icefish species, while in *N. coriiceps*, a disruption of myelin integrity could contribute to the irregular behaviors observed by our group at temperatures below CT_{MAX}.

Reduced activities of AChE and NKA in *C. aceratus* at CT_{MAX} may contribute to species differences in neuronal function upon warming

Total loss of function in AChE and NKA is unlikely to be a factor in setting limits to thermal tolerance, because both enzymes in notothenioids display robust enzymatic activity well beyond the species' upper thermal limits, as has been shown in other cases (Weinstein and Somero 1998; Pörtner et al. 2007). However, the reduction in enzymatic activity in brain tissues from *C. aceratus* exposed to CT_{MAX} may indicate that some function of these enzymes is compromised with warming, even if function is not fully impaired. In the teleost *Oncorhynchus mykiss* and in the bivalve *Mytilus* sp., various stressors, including heat, result in an elevation in AChE activity, a response which may enable these animals to cope with acute environmental changes (Dethloff et al. 1999; Pfeifer et al. 2005). In our study, however, AChE activity instead declined in brains of *C. aceratus* exposed to CT_{MAX}, a finding that may help account for the reduced thermal tolerance in this species. Inhibition of up to 40% of brain AChE activity can be lethal in fishes (Hogan 1970). Thus, it seems plausible that the reduced AChE activity upon CT_{MAX} exposure (approximately 12%) may incur functional consequences that ultimately result in a lower thermal tolerance in the icefish.

The elevated K_m at the higher assay temperature suggests a potential loss of enzymatic affinity for acetylcholine with warming, or at the very least, the requirement for a higher substrate concentration to reach 1/2 V_{max}. The trend of an increase in K_m with temperature is a well-known phenomenon as demonstrated previously in several fishes, including the notothenioid *Trematomus borchgrevinki* (Baldwin 1971), and in other enzymes, such as LDH, across a large range of taxonomic groups (Yancey and Somero 1978). The data herein suggest that in the Antarctic notothenioids, the interaction between AChE with substrate may be somewhat compromised at elevated temperatures, possibly as a result of alterations in membrane fluidity (Fong and McNamee 1986). Thus, it is quite possible that increased K_m may contribute to loss of AChE function at elevated assay temperatures. The K_m values cannot, however, explain species differences in AChE activity at ambient temperatures, as AChE activity was greater in *C. aceratus*. Thus, it is likely that either the concentration of AChE, or the catalytic efficiency of this enzyme (i.e., k_{cat}) is greater in *C. aceratus* than in *N. coriiceps*.

Because enzymatic activities of AChE (and NKA) in both species displayed ABTs that were unmatched by any indication of an abrupt change in membrane fluidity, it is unlikely that a membrane phase transition is responsible for the decline of activity. AChE, which degrades acetylcholine and is essential for the termination of synaptic transmission, occurs in both membrane-associated and cytoplasmic forms (Sáez-Valero et al. 1993). Our data show that in fact ~ 75% of total brain AChE activity co-localizes with a membranous fraction (Fig. S1, supplementary material), indicating that the majority of total brain AChE activity reflects the integral membrane protein in these animals. Enhancement of AChE activity has been shown to correspond with an augmentation of bilayer movement up to a fluidity threshold, beyond which enzymatic function is compromised (Chen et al. 1998). Greater activity of AChE in brains of *C. aceratus*, among animals held at ambient temperature, may, therefore, reflect the greater fluidity in synaptic membranes from the icefish.

NKA activity is dependent on the physical state of the membrane (Barnett and Palazzotto 1974). Yet despite greater fluidity in the synaptic membranes of *C. aceratus*, NKA activity was similar for the two species. However, NKA is also influenced significantly by cholesterol levels in the membrane (Cornelius et al. 2015). Consequently, the similar content of synaptic membrane cholesterol would appear to be at least one plausible explanation for comparable NKA activities among species.

To help distinguish the effect of the membrane environment on these integral membrane proteins, we measured the activities of two cytoplasmic enzymes, LDH and PK. The depression in LDH activity observed in both species upon CT_{MAX} exposure likely reflects a change in metabolic function with acute warming, possibly indicative of a response to heat stress. While increased LDH activity has been reported as a marker of various stressors in fish (Philip et al. 1995; Lawrence DeKoning et al. 2004; Rao 2006a), a reduction in LDH activity has been implicated as a general stress marker in several cases (Shakoori et al. 1996; Rao 2006b). At this time, we are unable to explain fully the function and impacts of depressed LDH activity upon acute warming in these species. It is possible, however, that the depressed activities in three (NKA, AChE, LDH) of the four enzymes measured in this study reflect an alteration in the intrinsic properties of these enzymes upon acute warming in *C. aceratus*.

Conclusion

We have found that synaptic membranes of *C. aceratus* were significantly more fluid than those of *N. coriiceps*, while no other neuronal membranes displayed differences in fluidity between species. We suggest that if indeed limits to thermal tolerance are related to neuronal membrane perturbation, it is the synaptic membranes, rather than either myelin or mitochondrial membranes, that represent a contributing factor. Further, reduced activity of synaptic enzymes (AChE and NKA) observed with acute warming (i.e., at CT_{MAX}) may contribute to loss of some physiological function in *C. aceratus*. With the waters surrounding the western Antarctic

Peninsula continuing to warm rapidly, the capacities of notothenioids to ensure membrane constancy will be key to their survival. Red-blooded notothenioids, including *N. coriiceps*, have demonstrated a capacity to increase thermal limits with warm acclimation (Bilyk and DeVries 2011), and these species may indeed be capable of undergoing at least modest degrees of membrane remodeling (Malekar et al. 2018). It has been known for several decades that temperate fishes have the capacity to alter lipid constituents of their neuronal membranes to achieve at least some compensation for thermal change (Roots 1968; Cossins and Friedlander 1977; Cossins 1977). Yet it is still largely unknown how the neuronal membranes of Antarctic notothenioids are likely to respond to the formidable challenges of rising temperatures, something that we are addressing in our ongoing work.

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Data Availability The datasets generated during and/or analyzed during the current study will be made available in Dryad Digital Repository upon acceptance for publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institutions at which the studies were conducted.

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