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Benzo(*a*)pyrene metabolism and EROD and GST biotransformation activity in the liver of red- and white-blooded Antarctic fish

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KEYWORDS

Ethoxyresorufin-*O*-deethylase, glutathione-*S*-transferase, BaP, benzo(*a*)pyrene metabolites, xenobiotic, ecotoxicology, anthropogenic pollution, biotransformation, metabolism, *Oncorhynchus mykiss, Notothenia rossii, Gobionotothen gibberifrons, Chaenocephalus aceratus, Champsocephalus gunnari*

ABSTRACT

Climate change and anthropogenic pollution are of increasing concern in remote areas such as Antarctica. The evolutionary adaptation of Antarctic notothenioid fish to the cold and stable Southern Ocean led to a low plasticity of their physiological functions, what may limit their capacity to deal with altered temperature regimes and pollution in the Antarctic environment. Using a biochemical approach, we aimed to assess the hepatic biotransformation capacities of Antarctic fish species by determining (i) the activities of ethoxyresorufin-*O*-deethylase (EROD) and glutathione-*S*-transferase (GST), and (ii) the metabolic clearance of benzo(*a*)pyrene by hepatic S9 supernatants. In addition, we determined the thermal sensitivity of the xenobiotic biotransformation enzymes.

We investigated the xenobiotic metabolism of the red-blooded *Gobionotothen gibberifrons* and *Notothenia rossii*, the hemoglobin-less *Chaenocephalus aceratus* and *Champsocephalus gunnari*, and the rainbow trout *Oncorhynchus mykiss* as a reference. Our results revealed similar metabolic enzyme activities and metabolic clearance rates between red- and white-blooded Antarctic fish, but significantly lower rates in comparison to rainbow trout. Therefore, bioaccumulation factors for metabolizable lipophilic contaminants may be higher in Antarctic than in temperate fish. Likewise, the thermal adaptive capacities and flexibilities of the EROD and GST activities in Antarctic fish were significantly lower than in rainbow trout. As a consequence, increasing water temperatures in the Southern Ocean will additionally compromise the already low detoxification capacities of Antarctic fish.

INTRODUCTION

Antarctica is one of the world's most isolated and environmentally stable habitats due to its separation from other world oceans by deep-water current patterns and the Antarctic Circumpolar Current [1]. Nonetheless, the Antarctic Ocean is impacted by man-made stressors as well, such as rising seawater and surface temperatures up to four degrees by the end of this century [2-4] and the increase of chemical contamination of the Antarctic environment, particularly by persistent organic pollutants (POPs) [5-7]. Additionally, expanding scientific activities, fisheries and tourism in the Antarctic can cause local contamination by organic chemical pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), particularly around Antarctic research stations, at levels which are comparable to highly polluted marine sites in other areas of the world [5, 6, 7].

These anthropogenic, lipophilic organic chemicals can be taken up via a physicochemically driven, passive partitioning of the chemicals from the water phase into the lipid phase of the organism and thereby bioaccumulate in the marine biota. The chemical body burdens, however, are mainly determined by endogenous biotransformation capacities of the exposed organisms. The main site of xenobiotic biotransformation in fish as well as in other vertebrates is the liver, where chemicals are processed and metabolized by enzymes [8]. Particularly the biotransformation enzymes of the P450 families, such as CYP1A, are responsible for the conversion of lipophilic organic chemicals into more water-soluble metabolites (phase I metabolism) which, after conjugation to endogenous substrates such as glutathione or glucuronic acid (phase II metabolism) can be excreted via bile [8-10]. In principal, biotransformation represents an adaptive detoxification process as it leads to reduced accumulation of toxic compounds in the organism. However, it can also result in a

toxification through the generation of highly reactive intermediates and metabolites [11]. Yet, the elimination or metabolism rates for contaminants greatly vary between different fish species, which makes it difficult to define biotransformation capacities of individual sub-orders or families of fish [12-15]. Given the role of biotransformation in both toxicokinetics and toxicodynamics, knowledge about species differences is crucial when it comes to determine the toxicological sensitivity or tolerance towards xenobiotics of different fish species.

The Antarctic ichthyofauna consists of highly endemic species, dominated by a single taxonomic group, the perciform suborder *Notothenioidei*. They account for about 35% of all known Antarctic fish species [16]. In high Antarctic shelf areas the notothenioids form up to 98% of the total fish abundance. They occupy a multitude of niches and inhabit benthic to epibenthic, semipelagic, cryopelagic and pelagic habitats [1]. Their habitat temperatures typically range from -1.8 to 2°C [17].

Notothenioid fishes are characterized by a variety of evolutionary adaptations to live in the permanently cold waters of Antarctica. Adaptations include, for example, an extreme stenothermy, the evolution of antifreeze glycoproteins (AFGPs) [18], and relatively high intracellular concentrations of lipids which may be used as energy stores [19], aid gas diffusion [20] and play a role in buoyancy [21]. These adaptations, however, also involve functional losses, such as the complete absence of red blood cells, or extremely low metabolic rates and narrow thermal tolerance ranges [22]. The few stenotherm Antarctic fish analysed so far appear not to possess any abilities to compensate their aerobic metabolism in response to chronic warmth-exposure [23, 24]. Furthermore, the Antarctic clade of notothenioids lacks the heat shock response, which is considered to be one of the most conserved biological processes across evolution [25]. So far, it is unclear to what extent the evolutionary adaptation of Antarctic fish has led to losses of genes and regulatory elements, which are essential for the adaptation to environmental changes [26]. Until the beginning of the 20th century, the isolated Antarctic environment was largely unaffected by anthropogenic influences. The critical question

102 therefore is to what extent this evolutionary specialization may become a drawback in the
103 capability of Antarctic fish to face the rapid changes in the Southern Ocean.
104
105 While eurytherm fish possess the capacities to adjust enzymatic capacities during thermal
106 acclimation [27, 28], the enzyme machinery of Antarctic fish is generally very limited in their
107 thermal response, particularly when it comes to exposure to multiple ambient stressors [29, 30].
108 Considering the evolutionary adaptation and high stenothermy of physiological functions in
109 these fish, knowledge about their xenobiotics metabolism capacities is a crucial point to
110 determine their vulnerability to anthropogenic influences. Yet, no data exist on the thermal
111 flexibility of the phase I and II enzymes or biotransformation rates of xenobiotics in Antarctic
112 fish.
113
114 The aim of the present study was to assess if highly stenothermal Antarctic fish can
115 metabolize organic pollutants and at which rates, and if their biotransformation system has the
116 plasticity to respond to rising temperatures. For our study, we chose four notothenioid fish
117 species, two possessing red blood cells and two icefish species, which lack hemoglobin. The
118 red-blooded, semipelagic marbled rockcod *Notothenia rossii* feeds on benthos and plankton
119 [21], the humped rockcod *Gobionotothen gibberifrons* is a benthic opportunistic feeder [17]. The
120 planktivorous mackerel icefish, *Champsocephalus gunnari* shows a rather active, bentho-
121 pelagic lifestyle, and the blackfin icefish, *Chaenocephalus aceratus,* is a rather sedentary
122 form which is usually found resting on the sea floor and mainly feeds on fish when adult [31].
123 These Antarctic species were compared to the well-studied rainbow trout of temperate
124 latitudes (*Oncorhynchus mykiss*). Using a biochemical approach, we measured activities of
125 representative enzymes for the metabolism of xenobiotics in phase I, i.e. CYP1A (measured
126 as ethoxyresorufin-*O*-deethylase (EROD)) – and in phase II, i.e. glutathione-*S*-transferase
127 (GST) in liver, the central organ for xenobiotic metabolism. The enzyme activities were

128 determined at the habitat temperatures of the experimental animals (Antarctic fish: 0°C,
129 rainbow trout: 12°C) and during rising assay temperatures as a measure for the thermal
130 flexibility of the enzymes in the different species. As enzyme activities are no direct measure
131 of the actual metabolic capacities, we additionally examined *in vitro* rates of xenobiotic
132 metabolic turnover. To this end, we conducted a substrate-depletion approach with liver S9
133 fractions and benzo(*a*)pyrene (BaP) as prototypic substrate, as it is established for metabolism
134 measurements with temperate fish species at habitat temperatures [32], and measured BaP
135 metabolite production as well. The metabolic rate determinations were performed at the
136 habitat temperatures of the experimental animals.
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138
139 MATERIALS AND METHODS
140
141 *Fish capture and handling*
142
143 Antarctic fish were caught with a 140 feet commercially sized bottom trawl down to 500 m
144 during a four weeks cruise in March 2012 (ANT XXVIII/4) with the research vessel *RV*
145 *Polarstern.* Sampling sites were between the Elephant Island - South Shetland Island region
146 and the northern tip of the Antarctic Peninsula. Only fish netted alive and without any
147 macroscopically visible damage were used for the sampling. Directly after capture, the fish
148 were anesthetized and dissected immediately to avoid necrotic tissue alterations. The whole
149 liver of each fish was sampled, transferred immediately to -80°C and stored there for further
150 analyses in the home laboratory. The following, sexually mature, species were used in this
151 study: *C. aceratus* (standard length 53-50 cm, weight 1206-1866 g, $n = 4$ females, $n = 2$
152 males), *C. gunnari* (standard length 34-47 cm, weight 312-748 g, $n = 6$ females), *G.*
153 *gibberifrons* (standard length 44-48 cm, weight 1214-1400 g, $n = 6$ females), *N. rossii*

(standard length 39-50 cm, weight 878-2972 g, *n* = 3 females, *n* = 3 males). No gender-related differences were observed in our measurements.

As a reference organism, we used *O. mykiss* which were raised in outdoor, flow-through water systems of the Centre for Fish and Wildlife Health, University of Bern. Fish were held at ambient temperatures ranging from 10°C during winter to 19°C during summer on a natural day-night cycle. Fish were fed daily with commercial dry pellets, accounting for 1.5 % body weight. The experimental animals were all female, had an age of 18 to 25 month and a weight of 222.7-467.4 g (*n* = 6). Sampling took place in March 2014 at a water temperature of 12 – 13 °C. Fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS222, 0.25 g/l), and killed by severing their spinal cord. Livers were excised, immediately shock-frozen in liquid nitrogen and stored at -80°C for further analysis.

Tissue preparation

Frozen liver samples were ground under liquid nitrogen and then slowly homogenized on ice with a Potter-Elvehjem type homogenizer (Sartorius AG, Germany) in Tris buffer pH 7.4 at 0°C containing 50 mM Tris, 250 mM Sucrose, 1 mM Na_2-EDTA, 150 mM KCl, 1 mM DTT (dithiothreitol), 0.25 mM PMSF (phenylmethanesulfonyl fluoride) and 20% glycerol. The S9, cytosolic and microsomal fractions were obtained by three successive centrifugations at 12.096*g*, 1°C, 20 minutes followed by two centrifugations at 100.000*g,* 1°C for 60 minutes each. The microsomal pellets were dissolved in Tris buffer (pH 7.4 at 0°C) containing 20% glycerol. Protein concentration in S9 (1.51 – 26.41 mg/ml), cytosol (0.93 – 18.08 mg/ml) and microsomal (1.43 – 14.71 mg/ml) fractions was determined after Lowry [33] using bovine serum albumin as standard.

Biochemical assays

Cytochrome P4501A activity was assessed using the spectrofluorometric EROD (ethoxyresorufin-*O*-deethylase) assay following a modified protocol by Burke and Mayer [34]. The assays were run at different temperatures from 0 to 21°C (Antarctic fish) and from 12 to 33°C (rainbow trout) in steps of 3°C. Measurements were carried out in water-cooled cuvette holders of the spectrophotometer or fluorescence spectrometer, respectively, controlled by a thermostat (Lauda, Königshofen, Germany). Each reaction mix consisted of 15 µl microsomal sample in 100 mM Tris-phosphate buffer (pH 7.4 at 0°C), 10 µM 7-ethoxyresorufin as substrate in a final volume of 1 ml. Reaction was started by adding 100 µM NADPH to the cuvette. The resorufin production was measured in duplicates during 20 minutes in a Perkin Elmer LS 55 (PerkinElmer Life and Analytical Sciences, Switzerland) at 544/590 nm excitation/emission wavelengths, respectively. Quantification was performed using a resorufin calibration curve from 0.078 to 10 pmol/ml and the activity was calculated as pmol resorufin generated per minute of reaction time per mg microsomal protein.

Glutathione-S-transferase (GST) activity was assayed in the cytosolic fraction as described in Habig [35] and Harvey [36]. The assay mixture contained 0.1 mg/ml final protein concentration, 100 mM phosphate buffer (pH 7.4 at 0°C), 2 mM glutathione (GSH, reduced form) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance was measured for 10 min at 340 nm in a Helios alpha spectrophotometer (Thermo Fisher Scientific Inc., Switzerland) at different assay temperatures from 0 to 21°C in steps of 3°C. The concentration of CDNB-glutathione conjugate in the samples was calculated according to Beer's law using a path length of 1 cm and molar absorption coefficient of 9.6 mM^{-1} cm^{-1}. GST activities are given as nmol per minute reaction time per mg cytosolic protein.

The total hepatic S9 cytochrome P450 (CYP) content was determined using the dithionite-difference spectrophotometry method modified after Matsubara et al. [37] and Guengerich et al. [38]. Absorbance readings of CO-treated and sodium dithionite-treated S9 fractions were taken

206 at 420, 450 and 490 nm in a microplate reader (Enspire, Perkin Elmer Life and Analytical
207 Sciences, Switzerland). Total CYP content was calculated according to Beer's law using a
208 path length (*b*) of 0.63 cm and a molar extinction coefficient (ε) of 91 mM^{-1} cm^{-1}.
209
210 *In vitro benzo(a)pyrene (BaP) metabolism*
211
212 In vitro substrate-depletion experiments were conducted with S9 fractions following a
213 modified protocol after Harris [39] and Johanning et al. [32]. The BaP-incubation assay was
214 performed in a 100 mM phosphate buffer containing 1 mM Na_2EDTA, 0.72 mM NADPH, S9
215 protein concentrations of 0.5 mg/ml and substrate concentrations of 0.5 – 2 µM BaP
216 (incubation conditions: 0.5 µM BaP: *C. aceratus*; 1 µM BaP: *C. gunnari*, *N. rossii*; 2 µM: *G.*
217 *gibberifrons, O. mykiss*). In preliminary experiments, we tested different protein
218 concentrations, substrate concentrations and incubation times to assess the kinetics of
219 substrate depletion for each fish species. We thus could establish assay conditions for a log-
220 linear elimination of BaP in Antarctic fish and rainbow trout.
221 All S9 incubation assays were conducted at the physiological temperatures and pH of
222 Antarctic fish (0°C, pH 7.4) and rainbow trout (12°C, pH 7.8). In the control assays, the
223 reaction was immediately stopped after BaP exposure. Furthermore, heat-inactivated S9
224 controls were run for quality control.
225 In the first set of BaP incubations, we aimed to determine the BaP metabolism rate. For this
226 purpose, the BaP depletion reaction was initiated by addition of BaP and stopped after 0, 10,
227 20, 30 and 60 minutes by addition of five ml ice-cold acetonitrile. Each time point was
228 sampled in duplicate. Afterwards, they were extracted and centrifuged at 10.000g, 4°C, 10
229 minutes [39].
230 In the second set of BaP incubations, we aimed to measure the BaP metabolites formed during
231 BaP metabolism. S9 fractions (2 mg/ml protein) of *O. mykiss, G. gibberifrons* (red-blooded)

and *C. gunnari* (white-blooded) were incubated with 2 µM BaP and stopped after one hour, two hours, four hours and eight hours by addition of five ml ice-cold acetonitrile. The extended incubation period was chosen to obtain a sufficient amount of metabolites in the Antarctic fish, which were found to deplete BaP slower compared to *O. mykiss*. Each time point was sampled in duplicate. Afterwards, the samples were extracted [41]: the organic phase was dried down under nitrogen, dissolved in acetonitrile and analyzed in the HPLC. Quantification of BaP in the samples (limit of quantification: 0.0695 ng/µl) was performed based on a BaP calibration curve with external eight standards, ranging from the analyte's method detection level of 0.0625 to 8 ng/µl BaP/ 0.25 to 3171 µM BaP, respectively. For the BaP metabolites, a calibration curve with eight external standards between 0.0695g and 8 ng/µl (0.25 to 3171 µM) was prepared for the following metabolites (obtained by MRI Global, Kansas City, Missouri, US and Aptochem, Montreal, Canada): Benzo(*a*)pyrene-*trans*-7,8-dihydrodiol (7,8-diol); 3-Hydroxybenzo(*a*)pyrene (3-OH); 9- Hydroxybenzo(*a*)pyrene (9-OH); Benzo(*a*)pyrene-1,6-dione (1,6-dione), Benzo(*a*)pyrene-6,12-dione (6,12-dione). 1,6-dione and 6,12 dione both could not be detected during the measurements. The standard curves were linear for all substances. The detection limit for each substance was defined as the concentration that resulted in a peak three-times above the baseline (0.0626 ng/µl). Analysis was performed using a Dionex HPLC system (Dionex P680 HPLC pump, ASI-100 automated sample injector, RF-2000 sample detector; Thermo Fisher Scientific, Switzerland). 100µl were injected onto a C18 reversed-phase column (Supelcosil LC-PAH C18, 150 × 4.6 mm, 5µm, Sigma-Aldrich, USA). Measurement conditions were as follows: flow rate 1 ml/min, 30/70 acetonitrile/water for 5 minutes, linear gradient to 85/15 acetonitrile/water in 35 minutes, 85/15 acetonitrile/water for 10 minutes, return to 30/70 acetonitrile/water within 10 minutes. Fluorescence was monitored at excitation/emission wavelengths 320/430 nm.

Data analysis

The temperature coefficient Q_{10} was calculated for the temperature ranges 0-21°C (Antarctic fish) and 12-33°C (rainbow trout) according to the formula:

$$Q_{10} = (\text{enzyme activity}_{(2)}/\text{enzyme activity}_{(1)})^{10/(T_2 - T_1)}$$

Arrhenius break temperatures (ABTs, the temperature above which the enzymatic activity fails to increase and/or drops off) of EROD and GST activities were determined following a method by [41, 42]. The enzyme activities were log transformed and plotted as a function of temperature. The two linear regression lines that best fitted the data and with the least residuals were selected using Microsoft Excel (Version 2010, Microsoft Co.). The ABT was determined graphically via the intersection of these two lines.

For the *in vitro* BaP metabolism analysis, the measured BaP concentrations of the S9 incubations were log-transformed at each time point and then plotted against reaction time to develop a linear regression. Slopes from each species were compared for significant differences using a Student's t test. The slopes were also used to calculate the first order depletion rate constant (k_e) according to the following formula:

$$K_e = -2.3 \times \text{Slope}$$

K_e was used to calculate the *in vitro* intrinsic clearance rate ($CL_{IN\ VITRO,\ INT}$; $ml*h^{-1}*mg\ protein^{-1}$) per mg S9 protein [32]. Liver weights were not available for all fish, thus the intrinsic hepatic clearance per gram of liver could not be calculated. All data were tested for normality (Kolmogorov-Smirnov) and homogeneity of variance. Significant differences ($P < 0.05$) between species were determined by analysis of variance (ANOVA, with Tukey Post-Test). Statistical analyses were performed with Prism 5, GraphPad Software (San Diego, USA). An ANOVA was conducted to exclude an influence on enzyme activities by gender differences

283 using the R-software (http://www.R-project.org/). Values are reported as mean ± standard
284 error of the mean (SEM).
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286
287 RESULTS
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289 *EROD and GST activities*
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291 In the present study, we measured EROD activity at 0°C to obtain an insight into the CYP1A
292 activities of Antarctic fish at their natural habitat temperatures around 0°C. In the Antarctic
293 fish species analyzed in our study, hepatic EROD activity (in pmol*min^{-1}*mg^{-1} protein) was
294 highest in the two white-blooded species, *C. gunnari* (4.4 ± 1.5) and *C. aceratus* (3.4 ± 1.8),
295 and slightly (non-significantly) lower in the two red-blooded species, *G. gibberifrons* (2.9 ±
296 1.5) and *N. rossii* with 2.3 ± 0.4. The hepatic EROD activity of rainbow trout (15.4 ± 1.7,
297 measured at 12°C) was significantly higher (ANOVA, Tukey Post-Test, $P = 0.0034$) than
298 EROD activities of all Antarctic fish species at 0°C (Figure 1). The Q_{10} values between the
299 EROD activities measured at 12°C in *O. mykiss* and at 0°C in the Antarctic fish were 6.5 for
300 *G. gibberifrons,* 5.1 for the icefish *C. aceratus,* 4.6 for *N. rossii*, and 3.1 for the white-blooded
301 *C. gunnari*.
302 The activities of the phase II enzyme GST (all values given in nmol*min^{-1}*mg protein^{-1}),
303 were highest for *O. mykiss* (178.0 ± 5.2, measured at 12°C). All Antarctic fish species
304 displayed significantly lower GST activities (*N. rossii* 63.6 ± 2.6; *G. gibberifrons* 98.6 ± 20.2;
305 *C. aceratus:* 22.5 ± 3.9; *C. gunnari* 34. 8 ± 10.3; ANOVA with Tukey Post-Test, $P < 0.0001$)
306 than *O. mykiss.* The two white-blooded species and the red-blooded *N. rossii* had a
307 significantly lower activity compared to the red-blooded *G. gibberifrons* (Figure 2). The Q_{10}
308 between the GST activities measured at 12°C in *O. mykiss* and at 0°C in the Antarctic fish

309 was highest in the two icefish species: 5.2 for *C. aceratus,* 3.6 for *C. gunnari, N. rossii* 1.7; *G.*
310 *gibberifrons* 1.5.
311 No significant difference were observed between the liver CYP450 content of rainbow trout
312 and the Antarctic fish, or between the red- and white-blooded Antarctic species (ANOVA,
313 Tukey Post-Test, $P = 0.6189$; Table 1).
314
315 *Thermal capacities of biotransformation enzymes*
316 In *O. mykiss*, EROD activities rose non-significantly (Linear regression analysis, $P = 0.2036$)
317 with assay temperature from 12 to 33°C, while GST activities showed a significant increase
318 with assay temperatures up to 33°C (Linear regression analysis, $P < 0.0001$) (Figures 3 & 4).
319 In contrast, the Antarctic fish *C. gunnari* and the two red-blooded species, *N. rossii* and *G.*
320 *gibberifrons*, showed stable EROD activities with rising assay temperatures until a moderate
321 drop in enzyme activity between 3 and 6°C in *N. rossii*, 9 and 12°C in *C. gunnari* and
322 between 12 and 15°C in *G. gibberifrons* (Figure 3). The EROD activity of the icefish *C.*
323 *aceratus* displayed a sharp drop in enzyme activity between the 3 and 6°C assay.
324 The Arrhenius plots of the GST activity revealed increasing enzyme capacities with rising
325 assay temperatures in all four Antarctic fish species. The ABTs were lowest in *C. aceratus*
326 and *N. rossii* between 12 and 15°C, and beyond 18°C in both *G. gibberifrons* and *C. gunnari*
327 (Figure 4).
328
329 *Biotransformation of benzo(a)pyrene (BaP)*
330
331 *BaP depletion rate:* The S9 substrate-depletion approach of BaP revealed a measurable, but
332 low substrate (BaP) depletion by the S9 fraction of the Antarctic fish compared to trout
333 (Figure 5). The slopes of the substrate depletion rate were similar between all four Antarctic
334 fish species (Linear regression analysis, $P = 0.6424$), and significantly lower compared to

rainbow trout (Linear regression analysis, $P < 0.0001$). The depletion rate constant (k_e; per hour) was highest in *O. mykiss* (measured at 12°C), about four times higher than in the red-blooded Antarctic fish at 0°C (Table 2). Among the Antarctic fish, k_e was two times higher in red-blooded Antarctic fish than in the two white-blooded species. The intrinsic clearance rate ($Cl_{IN\ VITRO,\ INT,}$ $ml*h^{-1}mg\ protein^{-1}$) for trout (6.4) liver S9-fraction was four times higher than the highest $Cl_{IN\ VITRO,\ INT}$ of the Antarctic fish (*N. rossii,* 1.8). It was lowest in the red-blooded *G. gibberifrons* and the white-blooded *C. gunnari* (Table 2).

BaP metabolite formation: In *O. mykiss*, BaP metabolites firstly occurred after four hours BaP incubation. After eight hours BaP incubation, 7,8 diol levels were 0.52 ± 0.09 $ng*mg\ protein^{-1}$, 3-OH levels were 7.8 ± 1.5 $ng*mg\ protein^{-1}$ and 9-OH 1.8 ± 0.5 $ng*mg\ protein^{-1}$. From the two Antarctic fish species investigated, only *C. gunnari* showed 2.9 ± 0.07 $ng*mg\ protein^{-1}$ 3-OH after eight hours incubation to 2 µM BaP. At time point zero of the incubation, BaP levels started with 108.2 ± 21.3 $ng*mg\ protein^{-1}$ in *O. mykiss*, 78.7 ± 20.4 *G. gibberifrons* and 76.3 ± 4.1 $ng*mg\ protein^{-1}$ in *C. gunnari,* and were depleted to 21.7 ± 3.6 $ng*mg\ protein^{-1}$ in *O. mykiss*, 83.14 ± 40.4 $ng*mg\ protein^{-1}$ in *G. gibberifrons* and 70.2 ± 30.3 $ng*mg\ protein^{-1}$ in *C. gunnari* (Figure 6).

DISCUSSION

Biotransformation enzyme activities

In the first part of our study, we aimed to determine the activities of CYP1A (measured as EROD activity) and phase II (exemplified as GST activity) biotransformation enzymes of Antarctic fish, and compared them to enzyme activities of the model species *O. mykiss.* Importantly, we performed the measurements at the environmental temperatures of the

investigated species, i.e. -1.8 to +1°C for the four Antarctic species [42], and 12°C for rainbow trout. This is in contrast to the few previous studies which measured biotransformation enzyme activities of the Antarctic fish *Chionodraco hamatus* and *Trematomus bernacchii* at supraphysiological temperatures around 20°C [43-47]. Also for temperate fish species it is known that water and assay temperature can drastically affect EROD activity and thus should be adjusted to the ecological temperature range of the experimental animal [48].Yet, the biotransformation enzyme activities measured in our study for the Antarctic fish are generally within the same order or magnitude compared to the few studies which dealt with biotransformation enzyme activities in red-blooded Antarctic fish so far [14, 44, 45, 47, 50]. However, the low EROD thermal flexibility and ABTs of our Antarctic fish species reflect functional or structural limitations of enzyme at warmer assay temperatures [51]. Thus, enzyme the EROD activities of Antarctic fish measured at room temperature may not mirror the actual physiological capacity of their xenobiotics metabolism system. When compared to rainbow trout, the EROD activities of Antarctic fish at their environmental temperature of 0°C are all significantly lower than the EROD activities of *O. mykiss* at 12°C.

As most physiological reactions, such as enzymatic activities, follow the temperature coefficient (Q_{10}). The simple Q_{10} relationship describes an uncompensated change of a physiological rate with a temperature change of 10°C,which yields Q_{10} values of two to three [52]. According to this relationship, enzymes of Antarctic animals seem to work at lower speeds, simply due to their cold environmental temperatures. Thus, the usage of the Q_{10} relationship can be a useful method to compare enzyme activities at their optimal temperature, to which they have been physiologically adapted. Yet, is has to be taken into account that a simple extrapolation of physiological rates between different temperatures according to Q_{10}, cannot ultimately reflect enzymatic activities at temperatures, which lay outside their optimal thermal range.

A comparison of the Q_{10} values between the EROD activities of *O. mykiss* (12°C) and of the

Antarctic fish (0°C) revealed high Q_{10} values above three. Such values indicate that CYP1A activities of Antarctic fish show no temperature compensation and are actually much lower than if trout EROD activities at 12°C were extrapolated down to 0°C. Thus, although Antarctic fish possess mechanistically fully functional enzymes at their habitat temperature, they seem to be incapable to overcome the decelerating thermodynamic effects of temperature in the Southern Ocean, similarly as it has been suggested for other aerobic enzymes in polar fish recently [53]. Our EROD data, and particularly Q_{10} values between the Antarctic and temperate fish species therefore emphasize that Antarctic fish in fact possess extremely low CYP1A capacities at their habitat temperatures.

In case of the GST activity, we measured similar activities in our rainbow trout compared to values reported earlier for *O. mykiss* at room temperatures (507-559 nmol nmol*min^{-1}*mg protein^{-1}, [54, 55]). In case of Antarctic fish, we only found literature values on GST activities measured at 20°C in *T. bernacchii,* which are about two times higher (180-213 nmol min^{-1} mg protein^{-1}, [14, 56]) than the GST activities of the red-blooded Antarctic species of our study, measured at 0°C. Despite the species difference between our and previous studies, the different GST activities are likely related to the assay temperature of 20°C used in these experiments. However, the difference in GST activity between our values at 0°C and the values of *T. bernacchii* around 20°C did not follow the typical temperature dependency of a Q_{10} between two and three, but yields a Q_{10} around one. Thus, our GST data measured at 0°C indicate that the GST activity of Antarctic fish is much lower at ecologically relevant temperatures than expected based on room temperature measurements.

Although the GST activities of the two red-blooded Antarctic species (at 0°C) were not significantly lower than the ones of rainbow trout at 12°C, the enzyme activities did not exactly follow the regular temperature dependency for biological rates. In fact, the Q_{10} between *N. rossii, G. gibberifrons* and *O. mykiss* was below two and thus reflects that the GST activities of the two Antarctic species would be still lower than the ones of *O. mykiss*

413 also if their GST activities are extrapolated up to the values of *O. mykiss* at 12°C, and taking
414 into account the generally low metabolic rates of Antarctic fish. The same holds true for the
415 icefish, where the low GST activities at 0°C and trout GST activity at 12°C yields a Q_{10} far
416 above two. This underlines the low absolute GST activities of icefish measured at their habitat
417 temperature of 0°C and thereby their evolutionarily highly cold-adapted enzyme activities.
418 However, it remains difficult to predict the actual detoxification capacities of the different
419 Antarctic fish solely from their enzymatic activities under a single assay condition. In the
420 second part of our study, we thus assessed the capacities of both EROD and GST as
421 representatives for phase I and II metabolism in terms of their thermal flexibility. The
422 activities of most metabolic enzymes usually follow rising assay temperatures until they reach
423 their capacity or structural limit, which is represented by the ABT. Yet, subcellular systems,
424 such as enzymes, but also organelles usually cover a wider range of thermal tolerance than
425 those of the whole organism [57, 58]. Therefore, enzymatic thermal limits frequently are beyond
426 the critical, lethal temperature limits for the whole organisms and do not reflect actual
427 metabolic capacities of an animal. Furthermore, thermal tolerance is highly correlated to the
428 rate of warming. Acute temperature limits are usually higher than the chronic tolerance
429 towards the warmth at both the cellular and organismic level [59, 60]. Nevertheless, acute thermal
430 profiles of (metabolic) enzymes and their ABTs can provide information on the general
431 thermal sensitivities of differently thermally adapted fishes and potentially their general
432 thermal acclimation capacities [61-63].
433 In *O. mykiss*, EROD activities remained stable within the thermal range assayed in our study,
434 which is typical for eurytherm species [64]. In contrast, none of the Antarctic fish showed an
435 increase of EROD activities with rising assay temperature, and only *N. rossii, G. gibberifrons*
436 and *C. gunnari* could maintain EROD activities stable with rising assay temperatures until a
437 steady drop in enzyme activity beyond the ABTs. Particularly *C. aceratus* displayed a very
438 low thermal flexibility, which was visible in drastically decreasing enzyme activities with

439 increasing temperatures beyond the 3°C assay. Usually, most aerobic and anaerobic
440 enzymatic activities increase with rising assay temperature in Antarctic fish [63, 65], but this
441 relation appears not to hold true for EROD activities. Such high temperature sensitivities of
442 EROD when compared to rainbow trout could be related to structural and functional
443 peculiarities, e.g. in the protein tertiary structure [66], which then involve general functional
444 failures of the enzyme towards warmer temperatures. The functional limitations in enzyme
445 activities, which seem to occur just a few degrees above the physiological temperature limit
446 of those Antarctic fish [67], clearly mirror the evolutionary adaptation to the cold and extreme
447 stenothermy of these species, which most likely also transfers to the xenobiotic metabolism in
448 Antarctic fish.
449
450 The Arrhenius plots of the GST activity showed increasing enzyme activities with rising
451 assay temperatures in all four Antarctic fish species and were generally less temperature
452 sensitive than the EROD activities. Also the ABTs of GST in Antarctic fish were much higher
453 compared to the ABTs of EROD, a clear sign for a higher thermal flexibility of the phase II
454 than of the phase I enzymes.
455 Earlier studies emphasize that the phase I cytochrome P450-dependent monooxygenase
456 system is responsible for the oxidation of organic pollutants such as PAHs and PCBs [68, 69].
457 Thus, it may be mainly the thermal inflexibility of cytochrome P4501A (reflected by EROD
458 activity) that could limit the xenobiotics metabolism capability of Antarctic fish during future
459 seawater warming and pollution.
460
461 Yet, GST showed similar species differences in its thermal flexibility as the EROD activities:
462 ABTs were lowest in *C. aceratus* and *N. rossii,* and higher, i.e. beyond 18°C, in both *G.*
463 *gibberifrons* and *C. gunnari.* Such species differences, as we observed them in the thermal
464 flexibility of the detoxification capacities, can be also found in the tolerance of the whole

organism towards environmental changes. Previous studies document a comparably low acute and chronic heat tolerance in *N. rossii* [70, 71], which could be related to low thermal capacities of their oxidative metabolism [63]. Also for the white-blooded *C. aceratus* an extremely low thermal tolerance is hypothesized, which is putatively related to the low hematocrit in this species [72]. Thus, these two species appear particularly sensitive to ambient thermal influence, which is also reflected at the enzymatic level. In contrast, species like *G. gibberifrons* appear to possess a slightly higher thermal tolerance and a putatively higher physiological flexibility to environmental changes at the organismic level [72]. The low thermal plasticity and high stenothermy of enzymatic function in species like *N. rossii* and *C. aceratus* could indicate that some species might be more at risk when it comes to multiple stressor exposure, such as warming and pollutants, in the future. Yet, the physiological mechanisms underlying such differences in thermal sensitivity between Antarctic fish are difficult to predict at present, and so far we have no indications for a relation to the absence or presence of hemoglobin or their trophic position.

Metabolism capacities for xenobiotics

The actual biotransformation rates in Antarctic fish are completely unknown so far. Here, we used a substrate-depletion approach in hepatic S9 fractions as a proxy for the metabolism capacity of the intact fish, as demonstrated by Johanning et al. [32] and Laue et al. [73], with BaP as a model substrate.

The slope of BaP depletion in rainbow trout, measured in S9 fractions of frozen liver tissue [32], is in a comparable range to the values measured in the present study. Our results demonstrate that also Antarctic fish possess a capacity for xenobiotic metabolism at their physiological optimum temperature, although the rates are very low. This is in agreement with the finding of the EROD measurements on the absence of cold compensation. The presence of xenobiotic

491 metabolism capacity in Antarctic fish species is also suggested from the findings of Yu et al.
492 [74] on the presence of PAH metabolites in the bile of *N. gibberifrons* exposed to Diesel Fuel.
493
494 In a comparison of the depletion rate constants (k_e; per hour) and the intrinsic clearance rate
495 ($Cl_{IN\ VITRO,\ INT}$) of the Antarctic species to those of rainbow trout, the rates of the former are
496 significantly lower than in *O. mykiss*. Even when the higher metabolic rates of *O. mykiss* are
497 considered, a down-extrapolation of the clearance rates of rainbow trout using a Q_{10} of two
498 would reveal three to seven times lower clearance rates in Antarctic fish compared to trout.
499 Such low BaP biotransformation rates are furthermore mirrored by the low EROD activities
500 in the Antarctic fish. A calculation of the BaP to EROD rates revealed a BaP/EROD rate of
501 2.4 for *O. mykiss,* 1.6 for *N. rossii,* 0.7 for *G. gibberifrons,* 1.2 for *C. aceratus* and 0.4 for *C.*
502 *gunnari.* In other words, in comparison to rainbow trout, the BaP metabolism per unit EROD
503 activity is only half as efficient in *N. rossii* and *C. aceratus* than in *O. mykiss*, three times
504 lower in *G. gibberifrons* and six times lower in *C. gunnari*. Furthermore, our data on the high
505 thermal sensitivity and putatively low structural flexibility of the EROD and GST activities
506 corroborates our findings of generally low detoxification capacities in Antarctic fish.
507
508 Among the Antarctic fish, k_e was two times higher in red-blooded Antarctic fish than in the
509 two white-blooded species. This is a first indicator on species differences in xenobiotic
510 metabolism within the Antarctic fish species that could be related to their physiological
511 differences. Thus, the intrinsic clearance rates, which express BaP metabolism per mg S9
512 protein and allows for comparing the metabolic capacities per S9 protein, was two orders of
513 magnitude higher in *C. aceratus* when compared to *C. gunnari,* and three-times higher in *N.*
514 *rossii* compared to *G. gibberifrons*.
515

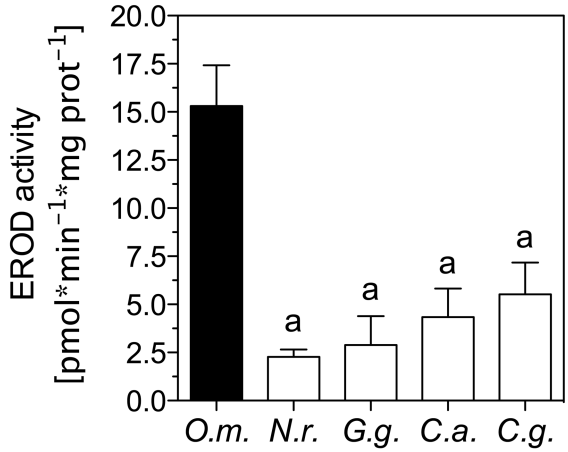
516 In temperate fish species, biotransformation of BaP are frequently related with their tissue
517 CYP content and biotransformation enzyme activities [10]. However, the CYP content of our
518 rainbow trout were not higher than in the Antarctic fish, and the review by Livingstone [10]
519 reports a total CYP content in liver of temperate fish of 322 + 35 pmol mg^{-1}, which lies in the
520 range of the Antarctic fish we measured in our study. Our data therefore show the general
521 presence of the detoxification enzymes that, however, do not necessarily parallel the actual
522 biotransformation capacities of the detoxification enzymes, as we demonstrate with the BaP
523 metabolism rates of the Antarctic fish.
524
525 The toxicity of BaP can be attributed to the reaction of BaP metabolites formed. Our studies
526 on the metabolite formation of *O. mykiss*, the red-blooded *G. gibberifrons* and the white-
527 blooded *C. gunnari* showed that rainbow trout produced all three analyzed metabolites at
528 substantial levels (3-OH: 32.9 % of total BaP metabolites, followed by 9-OH (7.9 %) and 7,8-
529 diol (1.9 %)). In contrast, the metabolite formation rates of the Antarctic fish species were
530 mostly below detection level. The only exception was *C. gunnari,* where 6.8 % of 3-OH was
531 produced. Similarly, other animal species, including rainbow trout, form 3-OH as the major
532 metabolite during BaP metabolism [75, 76].
533 CYP1-mediated EROD activity is usually highly correlated to BaP metabolism, such as the
534 formation of 7,8-diol that is mainly catalyzed by CYP1A1. The BaP/EROD rate, which was
535 lowest in *G. gibberifrons*, thus parallels our findings of absent BaP metabolites in this species.
536 Interestingly, the BaP/EROD rate was even lower in the icefish *C. gunnari* than in *G.*
537 *gibberifrons,* Yet, it showed the formation of 3-OH. The lack of correlation between EROD
538 activity and BaP metabolite formation in this species suggests that also other CYP isoforms
539 may be involved in BaP metabolism in this icefish species [77]. In sum, the low or absent
540 metabolite formation corroborate our findings from the BaP depletion measurements, i.e. that

metabolic capacities of the Antarctic species are very low, when compared to temperate fish species.

Finally, our data emphasize that the measurement of the actual metabolism rates for xenobiotics, rather than single measurements of phase I or II enzyme activities, are an important and valuable tool to determine the physiological susceptibility of Antarctic fish to organic pollutants.

In summary, both red- and white-blooded Antarctic fish species investigated herein possess lower biotransformation enzyme activities as it would be expected considering the typical Q_{10} relationship in comparison to temperate fish. Importantly, future climate warming is expected to increase the levels of contaminants in the Southern Ocean. This will concomitantly result in an increasing diffusion of lipophilic xenobiotics into the tissue of Antarctic fish. Our data, however, revealed no capacity of Antarctic fish to increase their biotransformation enzyme activities with warmer temperatures. Such low detoxification enzyme activities and the limited thermal plasticity of those enzymes will consequently result in a relatively higher bioaccumulation of xenobiotics in the tissues of Antarctic fish compared to temperate species. Complex environmental stressor interactions such as climate warming and pollutants may therefore make Antarctic fish much more susceptible to anthropogenic contaminants than it can be expected for fishes from temperate zones. In conclusion, our study highlights the importance of considering the distinct biotransformation rates and metabolism capacities of Antarctic fish for future assessments of the actual risk of these fish towards anthropogenic pollution and warming.

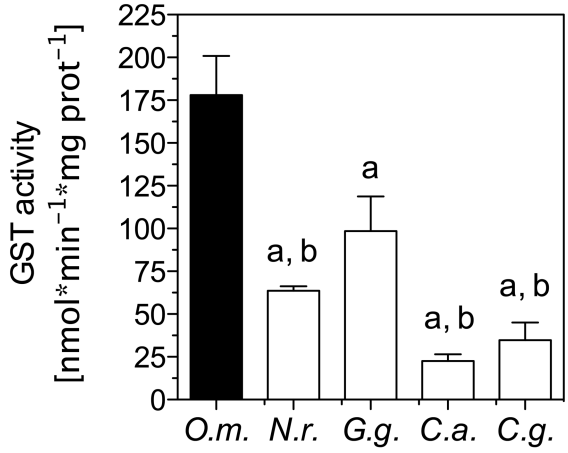
FIGURES



567

568 **Figure 1**. Hepatic microsomal EROD activities of Antarctic fish (white bars) *vs.* trout (black
569 bar) measured at the fish's habitat temperature of 0 or 12°C, respectively. Red-blooded: *N.r.*,
570 *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*, *Champsocephalus*
571 *gunnari*, *C.a.*, *Chaenocephalus aceratus*; *O.m.*, *Onchorhynchus mykiss*. $N = 6$ per species,
572 data are means ± SEM. [a] Significantly different from *O.m.*, $p < 0.05$ (ANOVA, Tukey Post-
573 Test).

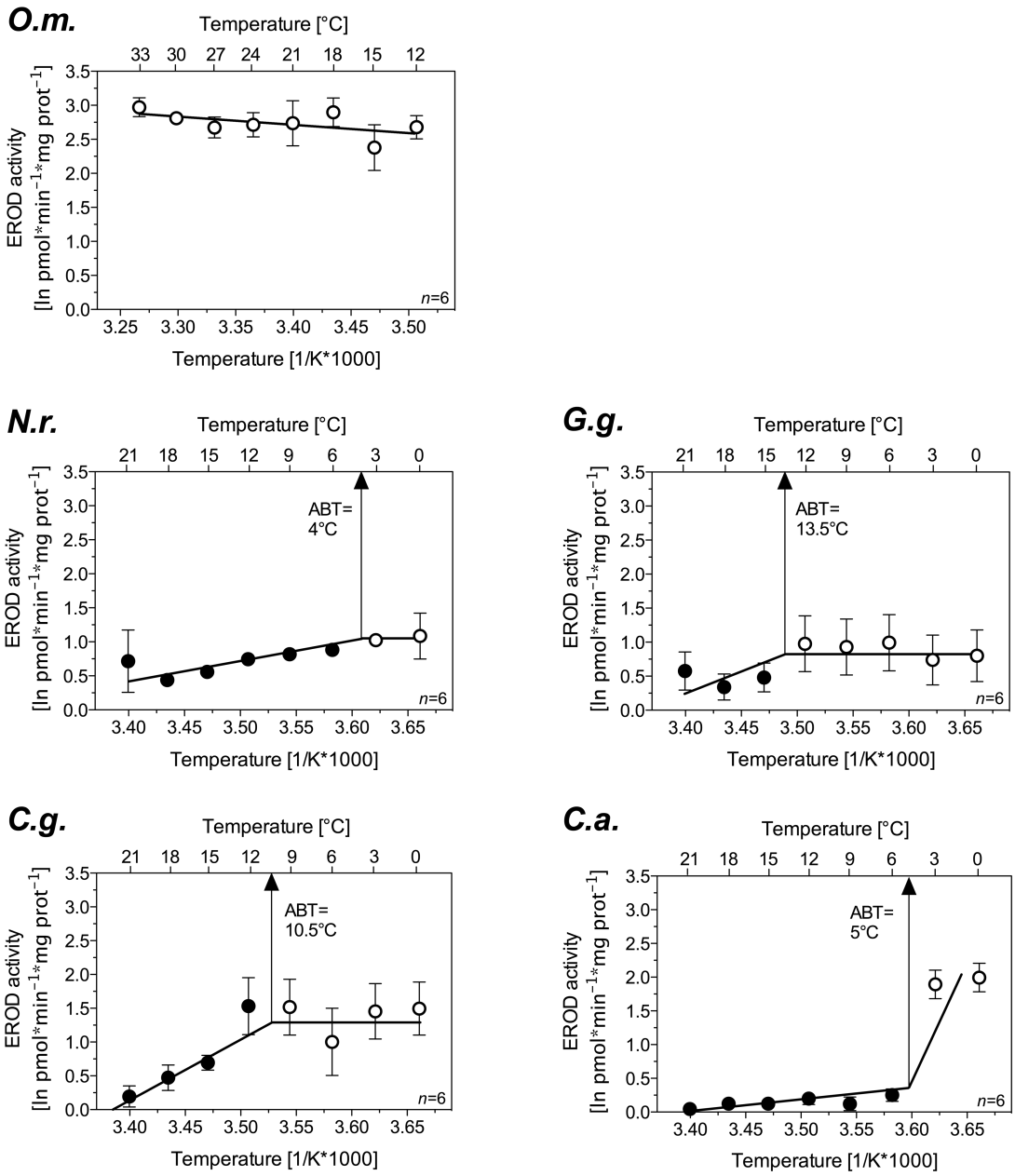
574



575

576 **Figure 2**. GST activities determined in liver S9 fractions of Antarctic fish (white bars) and
577 trout (black bar) measured at the fish's habitat temperature of 0 or 12°C, respectively. Red-

578 blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*,
579 *Champsocephalus gunnari*, *C.a.*, *Chaenocephalus aceratus*; *O.m.*, *Onchorhynchus mykiss*. *N*
580 = 6 per species, data are means ± SEM. [a] Significantly different from *O.m.*, [b] significantly
581 different from *G.g.*, $p < 0.05$ (ANOVA, Tukey Post-Test).
582



583

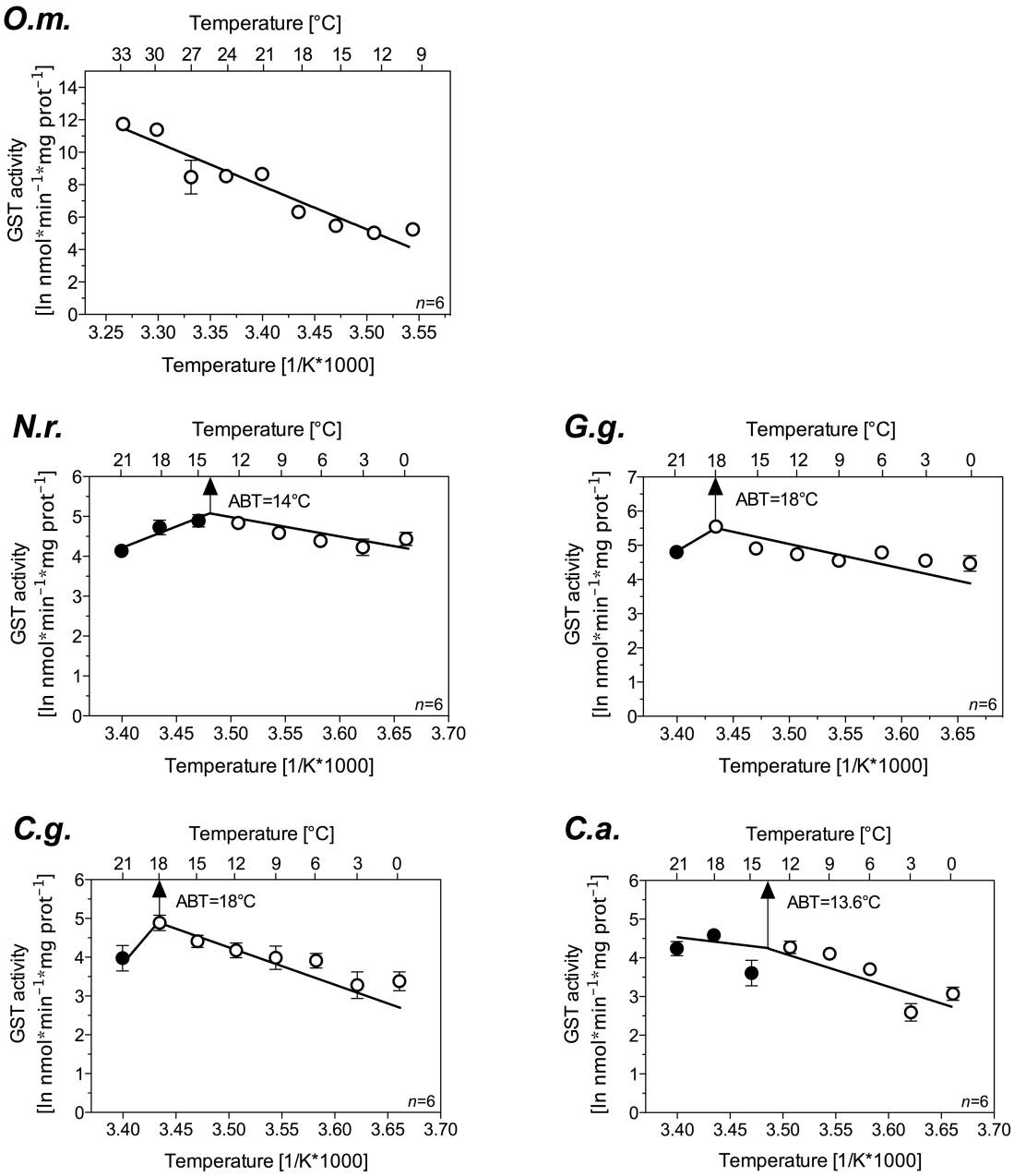
584 **Figure 3**. Arrhenius plots for EROD activities of Antarctic fish and rainbow trout. Red-
585 blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*,

586 *Champsocephalus gunnari*, *C.a.*, *Chaenocephalus aceratus; O.m.*, *Onchorhynchus mykiss.*

587 *Open* and *closed* circles represent data below and above the Arrhenius break temperature

588 (ABT), respectively. Values are means ± SEM (n = 6).

589



590

591 **Figure 4**. Arrhenius plots for GST activities of Antarctic fish and rainbow trout. Red-

592 blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*,

593 *Champsocephalus gunnari*, *C.a.*, *Chaenocephalus aceratus; O.m.*, *Onchorhynchus mykiss.*

Open and *closed* circles represent data below and above the Arrhenius break temperature (ABT), respectively. Values are means ± SEM (n = 6).

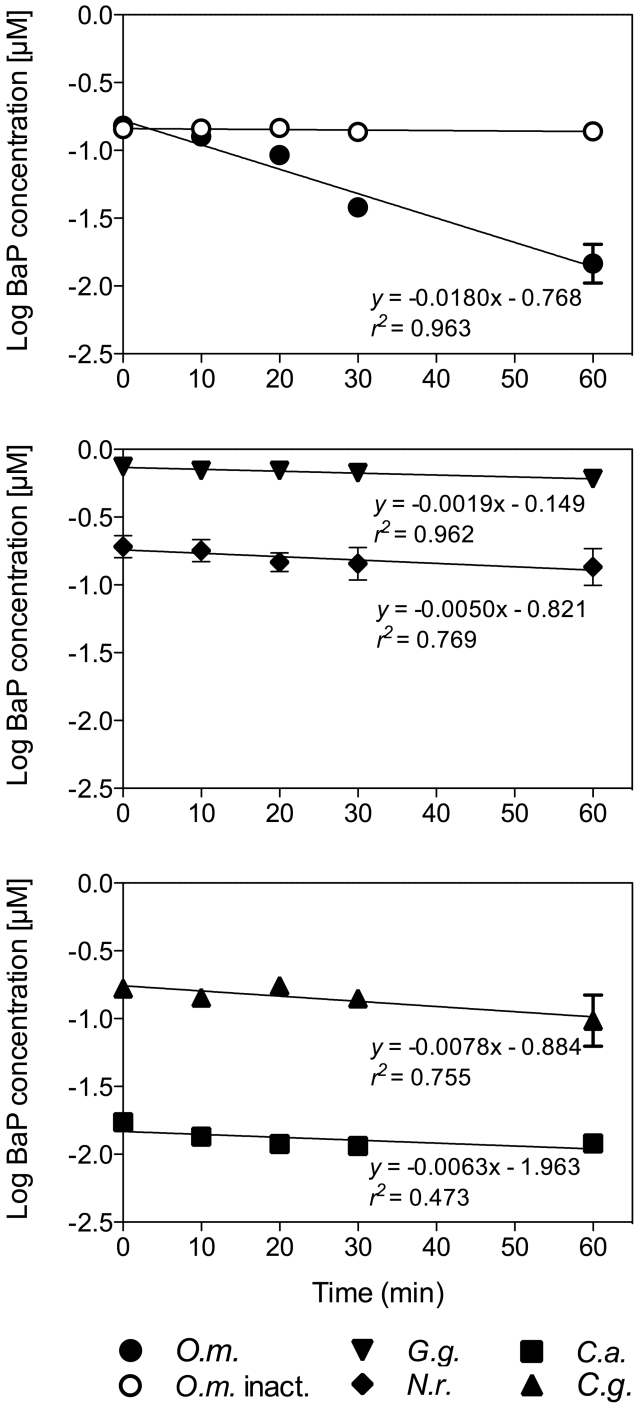


Figure 5. Biotransformation of benzo(*a*)pyrene (BaP) by Antarctic fish and trout liver S9 fractions. Red-blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*, *Champsocephalus gunnari*, *C.a.*, *Chaenocephalus aceratus*; *O.m.*, *Onchorhynchus mykiss*. Measured concentrations of BaP are plotted as log-transformed values and used for linear regression analysis. Incubation concentrations: 0.5 µM BaP: *C. aceratus*; 1 µM BaP: *C. gunnari*, *N. rossii*; 2 µM: *G. gibberifrons, O. mykiss*. Grey circles denote heat-inactive S9 of trout (*O.m.* inact.), heat-inactive values of Antarctic fish are not displayed for simplification (n = 3 per species and time point (mean ± SEM), each individual was measured in duplicates).

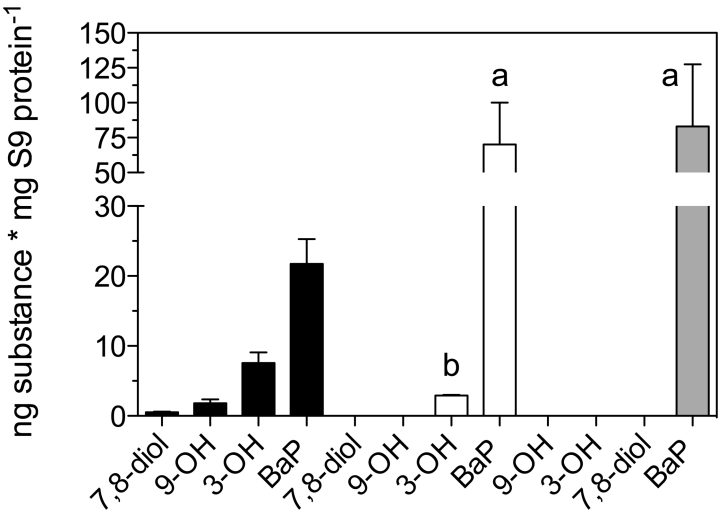


Figure 6: Benzo(*a*)pyrene (BaP) metabolites in liver S9 fractions of *O. mykiss* (black bars), *C. gunnari* (white bars) and *G. gibberifrons* (grey bars). Metabolites were determined after 8 hours incubation with 2 µM BaP. 7,8-diol: Benzo(*a*)pyrene-*trans*-7,8-dihydrodiol; 3-OH: 3-Hydroxybenzo(*a*)pyrene; 9-OH: 9- Hydroxybenzo(*a*)pyrene. N = 3 per species (mean ± SEM). [a] Significantly different from *O.mykiss*, [b] significantly different from *C. gunnari.*, $p <$ 0.05 (Paired t-test/ ANOVA, Tukey Post-Test)

617 TABLES

618

619 **Table 1**. Cytochrome P450 (CYP) content of S9 fractions of Antarctic fish

Species	CYP content (pmol CYP*mg S9 protein^{-1})[a]
O. mykiss	119.3 ± 22.5
N. rossii	295 ± 126.5
G. gibberifrons	186 ± 8.0
C. aceratus	305.9 ± 123.8
C. gunnari	157.2 ± 41.9

620 CYP = Cytochrome P450; values are presented as mean ± SEM, n=4-6. [a] Significantly
621 different to *O. mykiss* (ANOVA, Tukey Post-Test, $P < 0.05$)

622

623 **Table 2**. *In vitro* depletion and intrinsic clearance rate of benzo(*a*)pyrene (BaP) by rainbow
624 trout and Antarctic fish liver S9 fractions

Species	k_e (1/h)	Intrinsic hepatic clearance rate $Cl_{IN\ VITRO,\ INT,}$ (ml*h^{-1}*mg protein^{-1})*
O. mykiss	0.0412 ± 0.002	6.431 ± 0.56
N. rossii	0.0110 ± 0.003	1.747 ± 0.43*
G. gibberifrons	0.0113 ± 0.009	0.522 ± 0.16*
C. aceratus	0.0049 ± 0.002	1.203 ± 0.31*
C. gunnari	0.0045 ± 0.001	0.616 ± 0.18*

K_e = depletion rate constant; data are presented as mean ± SEM. * Based on four replicate determinations. [a] Significantly different to *O. mykiss* (ANOVA, Tukey Post-Test, $P < 0.05$)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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651 REFERENCES

652 1. Mintenbeck, K.; Barrera-Oro, E. R.; Brey, T.; Jacob, U.; Knust, R.; Mark, F. C.;
653 Moreira, E.; Strobel, A.; Arntz, W. E., 5 - Impact of Climate Change on Fishes in Complex
654 Antarctic Ecosystems. In *Adv. Ecol. Res.,* Ute, J.; Guy, W., Eds. Academic Press: 2012; Vol.
655 Volume 46, Burlington, pp 351-426.
656 2. Meredith; P., M.; King; C., J., *Rapid climate change in the ocean west of the Antarctic*
657 *Peninsula during the second half of the 20th century*. American Geophysical Union:
658 Washington, DC, ETATS-UNIS, 2005; Vol. 32.
659 3. Turner, J.; Lachlan-Cope, T. A.; Colwell, S.; Marshall, G. J.; Connolley, W. M.,
660 Significant Warming of the Antarctic Winter Troposphere. *Science* **2006,** *311*, (5769), 1914-
661 1917.
662 4. IPCC *Fifth Assessment Report of the Intergovernmental Panel on Climate Change*;
663 2014.
664 5 . SCAR, S. C. o. A. R. *Persistent Organic Pollutants (POPs) in the Antarctic*
665 *environment - A Review of Findings*; Scott Polar Research Institute, Cambridge, UK: 2009.
666 6. Nash, S. B., Persistent organic pollutants in Antarctica: current and future research
667 priorities. *J. Environ. Monit.* **2011,** *13*, (3), 497-504.
668 7. Curtosi, A.; Pelletier, E.; Vodopivez, C. L.; Mac Cormack, W. P., Distribution of
669 PAHs in the water column, sediments and biota of Potter Cove, South Shetland Islands,
670 Antarctica. *Ant. Sci.* **2009,** *21*, (04), 329-339.
671 8. Hinton, D. E.; Segner, H.; Au, D. W.; Kullman, S. W.; Hardman, R. C., Liver toxicity.
672 *The toxicology of fishes* **2008**, 327-400.
673 9. Parkinson, A., An overview of current cytochrome P450 technology for assessing the
674 safety and efficacy of new materials. *Toxicol. Pathol.* **1996,** *24*, (1), 45-57.
675 10. Livingstone, D., The fate of organic xenobiotics in aquatic ecosystems: quantitative
676 and qualitative differences in biotransformation by invertebrates and fish. *Comp. Biochem.*
677 *Physiol. A* **1998,** *120*, (1), 43-49.
678 11. Regoli, F.; Giuliani, M. E., Oxidative pathways of chemical toxicity and oxidative
679 stress biomarkers in marine organisms. *Mar. Envirn. Res.* **2014,** *93*, 106-117.
680 12. Roberts, S. C.; Noyes, P. D.; Gallagher, E. P.; Stapleton, H. M., Species-specific
681 differences and structure− activity relationships in the debromination of PBDE congeners in
682 three fish species. *Environ. Sci. Technol.* **2011,** *45*, (5), 1999-2005.
683 13. Bearr, J. S.; Mitchelmore, C. L.; Roberts, S. C.; Stapleton, H. M., Species specific
684 differences in the *in vitro* metabolism of the flame retardant mixture, Firemaster sup® BZ-54.
685 *Aquat. Toxicol.* **2012,** *124*, 41-47.
686 14. Ghosh, R.; Lokman, P. M.; Lamare, M. D.; Metcalf, V. J.; Burritt, D. J.; Davison, W.;
687 Hageman, K. J., Changes in physiological responses of an Antarctic fish, the emerald rock
688 cod (*Trematomus bernacchii*), following exposure to polybrominated diphenyl ethers
689 (PBDEs). *Aquat. Toxicol.* **2013,** *128*, 91-100.
690 15. Lyssimachou, A.; Thibaut, R.; Gisbert, E.; Porte, C., Gemfibrozil modulates
691 cytochrome P450 and peroxisome proliferation-inducible enzymes in the liver of the yellow
692 European eel (*Anguilla anguilla*). *Environ. Sci. Pollut. Res.* **2014,** *21*, (2), 862-871.
693 16. Arntz, W. E., The Magellan-Antarctic connection: links and frontiers at southern high
694 latitudes. Summary review. *Scientia Marina* **2005,** *69*, 359.
695 17. Eastman, J. T., *Antarctic Fish Biology: Evolution in a unique environment*. Academic
696 Press: New York, 1993.
697 18. Matschiner, M.; Hanel, R.; Salzburger, W., On the origin and trigger of the
698 notothenioid adaptive radiation. *PLoS ONE* **2011,** *6*, (4), e18911.
699 19. Crockett, E.; Sidell, B., Some pathways of energy metabolism are cold adapted in
700 Antarctic fishes. *Physiol. Zool.* **1990,** *63*, (3), 472-488.

20. Kamler, E.; Krasicka, B.; Rakusa-Suszczewski, S., Comparison of lipid content and fatty acid composition in muscle and liver of two notothenioid fishes from Admiralty Bay (Antarctica): an eco-physiological perspective. *Polar Biol.* **2001,** *24*, (10), 735-743.
21. Barrera-Oro, E., The role of fish in the Antarctic marine food web: differences between inshore and offshore waters in the southern Scotia Arc and west Antarctic Peninsula. *Ant. Sci.* **2002,** *14*, (04), 293-309.
22. Pörtner, H. O., Climate-dependent evolution of Antarctic ectotherms: An integrative analysis. *Deep Sea Research Part II: Topical Studies in Oceanography* **2006,** *53*, (8-10), 1071-1104.
23. Weinstein, R. B.; Somero, G. N., Effects of temperature on mitochondrial function in the Antarctic fish *Trematomus bernacchii*. *J. Comp. Physiol., B* **1998,** *168*, (3), 190-196.
24. Strobel, A.; Leo, E.; Pörtner, H. O.; Mark, F. C., Elevated temperature and PCO2 shift metabolic pathways in differentially oxidative tissues of Notothenia rossii. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2013,** *166*, (1), 48-57.
25. Sleadd, I. M.; Lee, M.; Hassumani, D. O.; Stecyk, T. M.; Zeitz, O. K.; Buckley, B. A., Sub-lethal heat stress causes apoptosis in an Antarctic fish that lacks an inducible heat shock response. *J Therm. Biol.* **2014,** *44*, 119-125.
26. Patarnello, T.; Verde, C.; Di Prisco, G.; Bargelloni, L.; Zane, L., How will fish that evolved at constant sub- zero temperatures cope with global warming? Notothenioids as a case study. *Bioessays* **2011,** *33*, (4), 260-268.
27. Cai, Y.; Adelman, I., Temperature acclimation in respiratory and cytochrome *c* oxidase activity in common carp (*Cyprinus carpio*). *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **1990,** *95*, (1), 139-144.
28. Hardewig, I.; van Dijk, P. L.; Moyes, C. D.; Portner, H. O., Temperature-dependent expression of cytochrome-c oxidase in Antarctic and temperate fish. *Am J Physiol* **1999,** *277*, (2 Pt 2), R508-16.
29. Seebacher, F.; Davison, W.; Lowe, C. J.; Franklin, C. E., A falsification of the thermal specialization paradigm: compensation for elevated temperatures in Antarctic fishes. *Biol. Lett.* **2005,** *1*, (2), 151-4.
30. Strobel, A.; Leo, E.; Pörtner, H. O.; Mark, F. C., Elevated temperature and PCO2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii. Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2013,** *166*, (1), 48-57.
31. Flores, H.; Kock, K.-H.; Wilhelms, S.; Jones, C. D., Diet of two icefish species from the South Shetland Islands and Elephant Island, *Champsocephalus gunnari* and *Chaenocephalus aceratus*. *Polar Biol.* **2004,** *27*, (2), 119-129.
32. Johanning, K.; Hancock, G.; Escher, B.; Adekola, A.; Bernhard, M. J.; Cowan-Ellsberry, C.; Domoradzki, J.; Dyer, S.; Eickhoff, C.; Embry, M., Assessment of metabolic stability using the rainbow trout (*Oncorhynchus mykiss*) liver S9 fraction. *Curr. Protoc. Toxicol.* **2012**, 14.10. 1-14.10. 28.
33. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J., Protein measurements with the folin phenol reagent. *J. Biochem.* **1951,** *193*, 265-275.
34. Burke, M. D.; Mayer, R. T., Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **1974,** *2*, (6), 583-588.
35. Habig, W. H.; Pabst, M. J.; Jakoby, W. B., Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **1974,** *249*, (22), 7130-7139.
36. Harvey, H. R.; Taylor, K. A.; Pie, H. V.; Mitchelmore, C. L., Polycyclic aromatic and aliphatic hydrocarbons in Chukchi Sea biota and sediments and their toxicological response in the Arctic cod, *Boreogadus saida*. *Deep Sea Res. Part II: Top. Stud. Oceanogr.* **2014,** *102*, 32-55.

37. Matsubara, T.; Koike, M.; Touchi, A.; Tochino, Y.; Sugeno, K., Quantitative determination of cytochrome P450 in rat liver homogenate. *Anal. Biochem.* **1976,** *75*, (2), 596-603.
38. Guengerich, F. P.; Martin, M. V.; Sohl, C. D.; Cheng, Q., Measurement of cytochrome P450 and NADPH–cytochrome P450 reductase. *Nature protocols* **2009,** *4*, (9), 1245-1251.
39. Harris, D. L.; Huderson, A. C.; Niaz, M. S.; Ford, J. J.; Archibong, A. E.; Ramesh, A., Comparative metabolism of benzo (a) pyrene by ovarian microsomes of various species. *Environ. Toxicol.* **2009,** *24*, (6), 603-609.
40. Nickerson, D. M.; Facey, D. E.; Grossman, G. D., Estimating physiological thresholds with continuous two-phase regression. *Physiol. Zool.* **1989,** *62*, (4), 866-887.
41. Dahlhoff, E. A.; Somero, G. N., Effects of temperature on mitochondria from abalone (genus Haliotis): adaptive plasticity and its limits. *J. Exp. Biol.* **1993**; *185*, 151-168.
42. Arntz, W. E., The Magellan-Antarctic connection: links and frontiers at southern high latitudes. Summary review. *Scientia Marina* **2005,** *69*, 359.
43. Miller, H. C.; Mills, G. N.; Bembo, D.; Macdonald, J. A.; Evans, C. W., Induction of cytochrome P4501A (CYP1A) in *Trematomus bernacchii* as an indicator of environmental pollution in Antarctica: assessment by quantitative RT-PCR. *Aquat. Toxicol.* **1998,** *44*, (3), 183-193.
44. Di Bello, D.; Vaccaro, E.; Longo, V.; Regoli, F.; Nigro, M.; Benedetti, M.; Gervasi, P. G.; Pretti, C., Presence and inducibility by β-naphthoflavone of CYP1A1, CYP1B1 and phase II enzymes in *Trematomus bernacchii*, an Antarctic fish. *Aquat. Toxicol.* **2007,** *84*, (1), 19-26.
45. Lurman, G. J.; Macdonald, J. A.; Evans, C. W., Evaluating the impact of environmental pollution on fish in McMurdo Sound, Antarctica: A biomarker approach. *Polar Sci.* **2010,** *3*, (4), 246-253.
46. Regoli, F.; Nigro, M.; Benedetti, M.; Gorbi, S.; Pretti, C.; Gervasi, P. G.; Fattorini, D., Interactions between metabolism of trace metals and xenobiotic agonists of the aryl hydrocarbon receptor in the antarctic fish *Trematomus bernacchii*: environmental perspectives. *Environ. Toxicol. Chem.* **2005,** *24*, (6), 1475-1482.
47. Focardi, S.; Fossi, M.; Lari, L.; Casini, S.; Leonzio, C.; Meidel, S.; Nigro, M., Induction of MFO activity in the antarctic fish *Pagothenia bernacchii*: Preliminary results. *Mar. Environ. Res.* **1995,** *39*, (1), 97-100.
50. Rodrigues, E.; Suda, C. N.; Rodrigues Junior, E.; de Oliveira, M. F.; Carvalho, C. d. S.; Vani, G. S., Antarctic fish metabolic responses as potential biomarkers of environmental impact. *Oecologia Australis* **2011,** *15*, (1), 124-149.
51. Marshall, C. J., Cold-adapted enzymes. *Trends Biotechnol.* **1997,** *15*, (9), 359-64.
52. Precht, H.; Christophersen, J.; Hensel, H.; Larcher, W., *Temperature and life*. Springer-Verlag: Berlin, Heidelberg, 1973.
53. White, C. R.; Alton, L. A.; Frappell, P. B., Metabolic cold adaptation in fishes occurs at the level of whole animal, mitochondria and enzyme. *Proc. R. Soc. B* **2011**, rspb20112060.
54. Connors, K. A.; Du, B.; Fitzsimmons, P. N.; Hoffman, A. D.; Chambliss, C. K.; Nichols, J. W.; Brooks, B. W., Comparative pharmaceutical metabolism by rainbow trout (Oncorhynchus mykiss) liver S9 fractions. *Environ. Toxicol. Chem.* **2013,** *32*, (8), 1810-1818.
55. Stephensen, E.; Adolfsson-Erici, M.; Hulander, M.; Parkkonen, J.; Förlin, L., Rubber additives induce oxidative stress in rainbow trout. *Aquat. Toxicol.* **2005,** *75*, (2), 136-143.
56. Benedetti, M.; Martuccio, G.; Fattorini, D.; Canapa, A.; Barucca, M.; Nigro, M.; Regoli, F., Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. *Aquat. Toxicol.* **2007,** *85*, (3), 167-175.
57. Pörtner, H. O.; Mark, F. C.; Bock, C., Oxygen limited thermal tolerance in fish? Answers obtained by nuclear magnetic resonance techniques. *Respir. Physiol. Neurobiol.* **2004,** *141*, (3), 243-60.

802 58. Mark, F. C.; Hirse, T.; Pörtner, H. O., Thermal sensitivity of cellular energy budgets in
803 some Antarctic fish hepatocytes. *Polar Biol.* **2005,** *28*, (11), 805-814.
804 59. Peck, L. S.; Clark, M. S.; Morley, S. A.; Massey, A.; Rossetti, H., Animal temperature
805 limits and ecological relevance: effects of size, activity and rates of change. *Functional*
806 *Ecology* **2009,** *23*, (2), 248-256.
807 60. Bilyk, K. T.; DeVries, A. L., Heat tolerance and its plasticity in Antarctic fishes.
808 *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2011,** *158*, (4), 382-390.
809 61. Weinstein, R. B.; Somero, G. N., Effects of temperature on mitochondrial function in
810 the Antarctic fish *Trematomus bernacchii*. *J. Comp. Physiol. B* **998,** *168*, (3), 190-196.
811 62. Mark, F. C.; Hirse, T.; Pörtner, H. O., Thermal sensitivity of cellular energy budgets in
812 some Antarctic fish hepatocytes. *Polar Biol.* **2005,** *28*, (11), 805-814.
813 63. Mark, F. C.; Lucassen, M.; Strobel, A.; Barrera-Oro, E.; Koschnick, N.; Zane, L.;
814 Patarnello, T.; Portner, H. O.; Papetti, C., Mitochondrial function in Antarctic nototheniids
815 with ND6 translocation. *PLoS ONE* **2012,** *7*, (2), e31860.
816 64. Pörtner, H.; Lucassen, M.; Storch, D., Metabolic biochemistry: its role in thermal
817 tolerance and in the capacities of physiological and ecological function. *Fish Physiol.* **2005,**
818 *22*, 79.
819 65. Somero, G., Linking biogeography to physiology: Evolutionary and acclimatory
820 adjustments of thermal limits. *Front. Zool.* **2005,** *2*, (1), 1.
821 67. Pörtner, H. O., Oxygen- and capacity-limitation of thermal tolerance: a matrix for
822 integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **2010,** *213*, (6),
823 881-93.
824 68. Förlin, L.; Lemaire, P.; Livingstone, D. R., Comparative studies of hepatic xenobiotic
825 metabolizing and antioxidant enzymes in different fish species. *Mar. Environ. Res.* **1995,** *39*,
826 (1), 201-204.
827 69. Oliva, M.; Gravato, C.; Guilhermino, L.; Galindo-Riaño, M. D.; Perales, J. A., EROD
828 activity and cytochrome P4501A induction in liver and gills of Senegal sole *Solea*
829 *senegalensis* from a polluted Huelva Estuary (SW Spain). *Comp. Biochem. Physiol. Part C*
830 **2014,** *166*, 134-144.
831 70. Bilyk, K. T.; DeVries, A. L., Heat tolerance and its plasticity in Antarctic fishes.
832 *Comp. Biochem. Physiol. Part A* **2011,** *158*, (4), 382-390.
833 71. Strobel, A.; Bennecke, S.; Leo, E.; Mintenbeck, K.; Pörtner, H. O.; Mark, F.,
834 Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and
835 PCO2. *Front. Zool.* **2012,** *9*, (1), 28.
836 72. Beers, J. M.; Sidell, B. D., Thermal tolerance of Antarctic notothenioid fishes
837 correlates with level of circulating hemoglobin. *Physio. Biochem. Zool.: PBZ* **2011,** *84*, (4),
838 353-62.
839 73. Laue, H.; Gfeller, H.; Jenner, K. J.; Nichols, J. W.; Kern, S.; Natsch, A., Predicting the
840 Bioconcentration of Fragrance Ingredients by Rainbow Trout Using Measured Rates of in
841 Vitro Intrinsic Clearance. *Environ. Sci. Technol.* **2014,** *48*, (16), 9486-9495.
842 74. Yu, Y.; Wade, T.; Fang, J.; McDonald, S.; Brooks, J., Gas chromatographic-mass
843 spectrometric analysis of polycyclic aromatic hydrocarbon metabolites in Antarctic fish
844 (*Notothenia gibberifrons*) injected with Diesel Fuel Arctic. *Arch. Environ. Contam. Toxicol.*
845 **1995,** *29*, (2), 241-246.
846 75. Miranda, C.; Chung, W.; Wang-Buhler, J.-L.; Musafia-Jeknic, T.; Baird, W.; Buhler,
847 D., Comparative in vitro metabolism of benzo [a] pyrene by recombinant zebrafish CYP1A
848 and liver microsomes from β-naphthoflavone-treated rainbow trout. *Aquat. Toxicol.* **2006,** *80*,
849 (2), 101-108.
850 76. Möller, A.-M.; Hermsen, C.; Floehr, T.; Lamoree, M. H.; Segner, H., Tissue-Specific
851 Metabolism of Benzo [a] pyrene in Rainbow Trout (*Oncorhynchus mykiss*): A Comparison
852 between the Liver and Immune Organs. *Drug Metab. Dispos.* **2014,** *42*, (1), 111-118.

853 77. Willett, K. L.; Gardinali, P. R.; Lienesch, L. A.; Di Giulio, R. T., Comparative
854 metabolism and excretion of benzo (a) pyrene in 2 species of ictalurid catfish. *Toxicol. Sci.*
855 **2000,** *58*, (1), 68-76.
856

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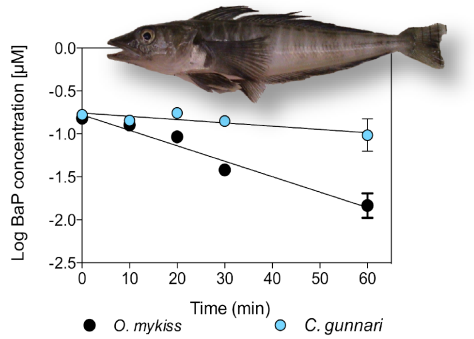

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