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

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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 *Champscephalus 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

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

82 The Antarctic ichthyofauna consists of highly endemic species, dominated by a single
83 taxonomic group, the perciform suborder *Notothenioidei*. They account for about 35% of all
84 known Antarctic fish species ¹⁶. In high Antarctic shelf areas the notothenioids form up to
85 98% of the total fish abundance. They occupy a multitude of niches and inhabit benthic to
86 epibenthic, semipelagic, cryopelagic and pelagic habitats ¹. Their habitat temperatures
87 typically range from -1.8 to 2°C ¹⁷.

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

therefore is to what extent this evolutionary specialization may become a drawback in the capability of Antarctic fish to face the rapid changes in the Southern Ocean.

While eurytherm fish possess the capacities to adjust enzymatic capacities during thermal acclimation^{27, 28}, the enzyme machinery of Antarctic fish is generally very limited in their thermal response, particularly when it comes to exposure to multiple ambient stressors^{29, 30}. Considering the evolutionary adaptation and high stenothermy of physiological functions in these fish, knowledge about their xenobiotics metabolism capacities is a crucial point to determine their vulnerability to anthropogenic influences. Yet, no data exist on the thermal flexibility of the phase I and II enzymes or biotransformation rates of xenobiotics in Antarctic fish.

The aim of the present study was to assess if highly stenothermal Antarctic fish can metabolize organic pollutants and at which rates, and if their biotransformation system has the plasticity to respond to rising temperatures. For our study, we chose four notothenioid fish species, two possessing red blood cells and two icefish species, which lack hemoglobin. The red-blooded, semipelagic marbled rockcod *Notothenia rossii* feeds on benthos and plankton²¹, the humped rockcod *Gobionotothen gibberifrons* is a benthic opportunistic feeder¹⁷. The planktivorous mackerel icefish, *Champsocephalus gunnari* shows a rather active, benthopelagic lifestyle, and the blackfin icefish, *Chaenocephalus aceratus*, is a rather sedentary form which is usually found resting on the sea floor and mainly feeds on fish when adult³¹. These Antarctic species were compared to the well-studied rainbow trout of temperate latitudes (*Oncorhynchus mykiss*). Using a biochemical approach, we measured activities of representative enzymes for the metabolism of xenobiotics in phase I, i.e. CYP1A (measured as ethoxyresorufin-*O*-deethylase (EROD)) – and in phase II, i.e. glutathione-*S*-transferase (GST) in liver, the central organ for xenobiotic metabolism. The enzyme activities were

determined at the habitat temperatures of the experimental animals (Antarctic fish: 0°C, rainbow trout: 12°C) and during rising assay temperatures as a measure for the thermal flexibility of the enzymes in the different species. As enzyme activities are no direct measure of the actual metabolic capacities, we additionally examined *in vitro* rates of xenobiotic metabolic turnover. To this end, we conducted a substrate-depletion approach with liver S9 fractions and benzo(*a*)pyrene (BaP) as prototypic substrate, as it is established for metabolism measurements with temperate fish species at habitat temperatures³², and measured BaP metabolite production as well. The metabolic rate determinations were performed at the habitat temperatures of the experimental animals.

MATERIALS AND METHODS

Fish capture and handling

Antarctic fish were caught with a 140 feet commercially sized bottom trawl down to 500 m during a four weeks cruise in March 2012 (ANT XXVIII/4) with the research vessel *RV Polarstern*. Sampling sites were between the Elephant Island - South Shetland Island region and the northern tip of the Antarctic Peninsula. Only fish netted alive and without any macroscopically visible damage were used for the sampling. Directly after capture, the fish were anesthetized and dissected immediately to avoid necrotic tissue alterations. The whole liver of each fish was sampled, transferred immediately to -80°C and stored there for further analyses in the home laboratory. The following, sexually mature, species were used in this study: *C. aceratus* (standard length 53-50 cm, weight 1206-1866 g, *n* = 4 females, *n* = 2 males), *C. gunnari* (standard length 34-47 cm, weight 312-748 g, *n* = 6 females), *G. 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 Na₂-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 12.096g, 1°C, 20 minutes followed by two centrifugations at 100.000g, 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 Lowry³³ using bovine serum albumin as standard.

Biochemical assays

180

181 Cytochrome P4501A activity was assessed using the spectrofluorometric EROD

182 (ethoxyresorufin-*O*-deethylase) assay following a modified protocol by Burke and Mayer ³⁴.

183 The assays were run at different temperatures from 0 to 21°C (Antarctic fish) and from 12 to

184 33°C (rainbow trout) in steps of 3°C. Measurements were carried out in water-cooled cuvette

185 holders of the spectrophotometer or fluorescence spectrometer, respectively, controlled by a

186 thermostat (Lauda, Königshofen, Germany). Each reaction mix consisted of 15 µl microsomal

187 sample in 100 mM Tris-phosphate buffer (pH 7.4 at 0°C), 10 µM 7-ethoxyresorufin as

188 substrate in a final volume of 1 ml. Reaction was started by adding 100 µM NADPH to the

189 cuvette. The resorufin production was measured in duplicates during 20 minutes in a Perkin

190 Elmer LS 55 (PerkinElmer Life and Analytical Sciences, Switzerland) at 544/590 nm

191 excitation/emission wavelengths, respectively. Quantification was performed using a

192 resorufin calibration curve from 0.078 to 10 pmol/ml and the activity was calculated as pmol

193 resorufin generated per minute of reaction time per mg microsomal protein.

194 Glutathione-S-transferase (GST) activity was assayed in the cytosolic fraction as described in

195 Habig ³⁵ and Harvey ³⁶. The assay mixture contained 0.1 mg/ml final protein concentration,

196 100 mM phosphate buffer (pH 7.4 at 0°C), 2 mM glutathione (GSH, reduced form) and 1 mM

197 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance was measured

198 for 10 min at 340 nm in a Helios alpha spectrophotometer (Thermo Fisher Scientific Inc.,

199 Switzerland) at different assay temperatures from 0 to 21°C in steps of 3°C. The

200 concentration of CDBN-glutathione conjugate in the samples was calculated according to

201 Beer's law using a path length of 1 cm and molar absorption coefficient of 9.6 mM⁻¹ cm⁻¹.

202 GST activities are given as nmol per minute reaction time per mg cytosolic protein.

203 The total hepatic S9 cytochrome P450 (CYP) content was determined using the dithionite-

204 difference spectrophotometry method modified after Matsubara et al. ³⁷ and Guengerich et al.

205 ³⁸. Absorbance readings of CO-treated and sodium dithionite-treated S9 fractions were taken

at 420, 450 and 490 nm in a microplate reader (Enspire, Perkin Elmer Life and Analytical Sciences, Switzerland). Total CYP content was calculated according to Beer's law using a path length (b) of 0.63 cm and a molar extinction coefficient (ϵ) of $91 \text{ mM}^{-1} \text{ cm}^{-1}$.

In vitro benzo(a)pyrene (BaP) metabolism

In vitro substrate-depletion experiments were conducted with S9 fractions following a modified protocol after Harris³⁹ and Johanning et al.³². The BaP-incubation assay was performed in a 100 mM phosphate buffer containing 1 mM Na_2EDTA , 0.72 mM NADPH, S9 protein concentrations of 0.5 mg/ml and substrate concentrations of 0.5 – 2 μM BaP (incubation conditions: 0.5 μM BaP: *C. aceratus*; 1 μM BaP: *C. gunnari*, *N. rossii*; 2 μM : *G. gibberifrons*, *O. mykiss*). In preliminary experiments, we tested different protein concentrations, substrate concentrations and incubation times to assess the kinetics of substrate depletion for each fish species. We thus could establish assay conditions for a log-linear elimination of BaP in Antarctic fish and rainbow trout.

All S9 incubation assays were conducted at the physiological temperatures and pH of Antarctic fish (0°C, pH 7.4) and rainbow trout (12°C, pH 7.8). In the control assays, the reaction was immediately stopped after BaP exposure. Furthermore, heat-inactivated S9 controls were run for quality control.

In the first set of BaP incubations, we aimed to determine the BaP metabolism rate. For this purpose, the BaP depletion reaction was initiated by addition of BaP and stopped after 0, 10, 20, 30 and 60 minutes by addition of five ml ice-cold acetonitrile. Each time point was sampled in duplicate. Afterwards, they were extracted and centrifuged at 10.000g, 4°C, 10 minutes³⁹.

In the second set of BaP incubations, we aimed to measure the BaP metabolites formed during BaP metabolism. S9 fractions (2 mg/ml protein) of *O. mykiss*, *G. gibberifrons* (red-blooded)

and *C. gunnari* (white-blooded) were incubated with 2 μ M 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 extracted⁴¹: 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/ μ l) 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/ μ l BaP/ 0.25 to 3171 μ M BaP, respectively. For the BaP metabolites, a calibration curve with eight external standards between 0.0695g and 8 ng/ μ l (0.25 to 3171 μ M) 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/ μ l). 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). 100 μ l were injected onto a C18 reversed-phase column (Supelcosil LC-PAH C18, 150 $\times$ 4.6 mm, 5 μ m, 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

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

261 $Q_{10} = (\text{enzyme activity}_{(2)} / \text{enzyme activity}_{(1)})^{10/(T_2 - T_1)}$

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

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

273

274 $K_e = -2.3 \times \text{Slope}$

275

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

using the R-software (<http://www.R-project.org/>). Values are reported as mean $\pm$ standard error of the mean (SEM).

RESULTS

EROD and GST activities

In the present study, we measured EROD activity at 0°C to obtain an insight into the CYP1A activities of Antarctic fish at their natural habitat temperatures around 0°C. In the Antarctic fish species analyzed in our study, hepatic EROD activity (in pmol*min⁻¹*mg⁻¹ protein) was highest in the two white-blooded species, *C. gunnari* (4.4 $\pm$ 1.5) and *C. aceratus* (3.4 $\pm$ 1.8), and slightly (non-significantly) lower in the two red-blooded species, *G. gibberifrons* (2.9 $\pm$ 1.5) and *N. rossii* with 2.3 $\pm$ 0.4. The hepatic EROD activity of rainbow trout (15.4 $\pm$ 1.7, measured at 12°C) was significantly higher (ANOVA, Tukey Post-Test, $P = 0.0034$) than EROD activities of all Antarctic fish species at 0°C (Figure 1). The Q₁₀ values between the EROD activities measured at 12°C in *O. mykiss* and at 0°C in the Antarctic fish were 6.5 for *G. gibberifrons*, 5.1 for the icefish *C. aceratus*, 4.6 for *N. rossii*, and 3.1 for the white-blooded *C. gunnari*.

The activities of the phase II enzyme GST (all values given in nmol*min⁻¹*mg protein⁻¹), were highest for *O. mykiss* (178.0 $\pm$ 5.2, measured at 12°C). All Antarctic fish species displayed significantly lower GST activities (*N. rossii* 63.6 $\pm$ 2.6; *G. gibberifrons* 98.6 $\pm$ 20.2; *C. aceratus*: 22.5 $\pm$ 3.9; *C. gunnari* 34.8 $\pm$ 10.3; ANOVA with Tukey Post-Test, $P < 0.0001$) than *O. mykiss*. The two white-blooded species and the red-blooded *N. rossii* had a significantly lower activity compared to the red-blooded *G. gibberifrons* (Figure 2). The Q₁₀ between the GST activities measured at 12°C in *O. mykiss* and at 0°C in the Antarctic fish

was highest in the two icefish species: 5.2 for *C. aceratus*, 3.6 for *C. gunnari*, *N. rossii* 1.7; *G. gibberifrons* 1.5.

No significant difference were observed between the liver CYP450 content of rainbow trout and the Antarctic fish, or between the red- and white-blooded Antarctic species (ANOVA, Tukey Post-Test, $P = 0.6189$; Table 1).

Thermal capacities of biotransformation enzymes

In *O. mykiss*, EROD activities rose non-significantly (Linear regression analysis, $P = 0.2036$) with assay temperature from 12 to 33°C, while GST activities showed a significant increase with assay temperatures up to 33°C (Linear regression analysis, $P < 0.0001$) (Figures 3 & 4).

In contrast, the Antarctic fish *C. gunnari* and the two red-blooded species, *N. rossii* and *G. gibberifrons*, showed stable EROD activities with rising assay temperatures until a moderate drop in enzyme activity between 3 and 6°C in *N. rossii*, 9 and 12°C in *C. gunnari* and between 12 and 15°C in *G. gibberifrons* (Figure 3). The EROD activity of the icefish *C. aceratus* displayed a sharp drop in enzyme activity between the 3 and 6°C assay.

The Arrhenius plots of the GST activity revealed increasing enzyme capacities with rising assay temperatures in all four Antarctic fish species. The ABTs were lowest in *C. aceratus* and *N. rossii* between 12 and 15°C, and beyond 18°C in both *G. gibberifrons* and *C. gunnari* (Figure 4).

Biotransformation of benzo(a)pyrene (BaP)

BaP depletion rate: The S9 substrate-depletion approach of BaP revealed a measurable, but low substrate (BaP) depletion by the S9 fraction of the Antarctic fish compared to trout (Figure 5). The slopes of the substrate depletion rate were similar between all four Antarctic 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 ($Cl_{IN\ VITRO,\ INT}$, $ml \cdot h^{-1} \cdot mg\ protein^{-1}$) 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 $0.52 \pm 0.09\ ng \cdot mg\ protein^{-1}$, 3-OH levels were $7.8 \pm 1.5\ ng \cdot mg\ protein^{-1}$ and 9-OH $1.8 \pm 0.5\ ng \cdot mg\ protein^{-1}$. From the two Antarctic fish species investigated, only *C. gunnari* showed $2.9 \pm 0.07\ ng \cdot mg\ protein^{-1}$ 3-OH after eight hours incubation to 2 μM BaP. At time point zero of the incubation, BaP levels started with $108.2 \pm 21.3\ ng \cdot mg\ protein^{-1}$ in *O. mykiss*, 78.7 ± 20.4 in *G. gibberifrons* and $76.3 \pm 4.1\ ng \cdot mg\ protein^{-1}$ in *C. gunnari*, and were depleted to $21.7 \pm 3.6\ ng \cdot mg\ protein^{-1}$ in *O. mykiss*, $83.14 \pm 40.4\ ng \cdot mg\ protein^{-1}$ in *G. gibberifrons* and $70.2 \pm 30.3\ ng \cdot mg\ protein^{-1}$ 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

361 investigated species, i.e. -1.8 to +1°C for the four Antarctic species⁴², and 12°C for rainbow
362 trout. This is in contrast to the few previous studies which measured biotransformation
363 enzyme activities of the Antarctic fish *Chionodraco hamatus* and *Trematomus bernacchii* at
364 supraphysiological temperatures around 20°C⁴³⁻⁴⁷. Also for temperate fish species it is known
365 that water and assay temperature can drastically affect EROD activity and thus should be
366 adjusted to the ecological temperature range of the experimental animal⁴⁸. Yet, the
367 biotransformation enzyme activities measured in our study for the Antarctic fish are generally
368 within the same order or magnitude compared to the few studies which dealt with
369 biotransformation enzyme activities in red-blooded Antarctic fish so far^{14, 44, 45, 47, 50}.
370 However, the low EROD thermal flexibility and ABTs of our Antarctic fish species reflect
371 functional or structural limitations of enzyme at warmer assay temperatures⁵¹. Thus, enzyme
372 the EROD activities of Antarctic fish measured at room temperature may not mirror the actual
373 physiological capacity of their xenobiotics metabolism system. When compared to rainbow
374 trout, the EROD activities of Antarctic fish at their environmental temperature of 0°C are all
375 significantly lower than the EROD activities of *O. mykiss* at 12°C.
376 As most physiological reactions, such as enzymatic activities, follow the temperature
377 coefficient (Q_{10}). The simple Q_{10} relationship describes an uncompensated change of a
378 physiological rate with a temperature change of 10°C, which yields Q_{10} values of two to three
379⁵². According to this relationship, enzymes of Antarctic animals seem to work at lower
380 speeds, simply due to their cold environmental temperatures. Thus, the usage of the Q_{10}
381 relationship can be a useful method to compare enzyme activities at their optimal temperature,
382 to which they have been physiologically adapted. Yet, it has to be taken into account that a
383 simple extrapolation of physiological rates between different temperatures according to Q_{10} ,
384 cannot ultimately reflect enzymatic activities at temperatures, which lay outside their optimal
385 thermal range.
386 A comparison of the Q_{10} values between the EROD activities of *O. mykiss* (12°C) and of the

387 Antarctic fish (0°C) revealed high Q_{10} values above three. Such values indicate that CYP1A
388 activities of Antarctic fish show no temperature compensation and are actually much lower
389 than if trout EROD activities at 12°C were extrapolated down to 0°C. Thus, although
390 Antarctic fish possess mechanistically fully functional enzymes at their habitat temperature,
391 they seem to be incapable to overcome the decelerating thermodynamic effects of temperature
392 in the Southern Ocean, similarly as it has been suggested for other aerobic enzymes in polar
393 fish recently⁵³. Our EROD data, and particularly Q_{10} values between the Antarctic and
394 temperate fish species therefore emphasize that Antarctic fish in fact possess extremely low
395 CYP1A capacities at their habitat temperatures.

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

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

also if their GST activities are extrapolated up to the values of *O. mykiss* at 12°C, and taking into account the generally low metabolic rates of Antarctic fish. The same holds true for the icefish, where the low GST activities at 0°C and trout GST activity at 12°C yields a Q_{10} far above two. This underlines the low absolute GST activities of icefish measured at their habitat temperature of 0°C and thereby their evolutionarily highly cold-adapted enzyme activities. However, it remains difficult to predict the actual detoxification capacities of the different Antarctic fish solely from their enzymatic activities under a single assay condition. In the second part of our study, we thus assessed the capacities of both EROD and GST as representatives for phase I and II metabolism in terms of their thermal flexibility. The activities of most metabolic enzymes usually follow rising assay temperatures until they reach their capacity or structural limit, which is represented by the ABT. Yet, subcellular systems, such as enzymes, but also organelles usually cover a wider range of thermal tolerance than those of the whole organism^{57, 58}. Therefore, enzymatic thermal limits frequently are beyond the critical, lethal temperature limits for the whole organisms and do not reflect actual metabolic capacities of an animal. Furthermore, thermal tolerance is highly correlated to the rate of warming. Acute temperature limits are usually higher than the chronic tolerance towards the warmth at both the cellular and organismic level^{59, 60}. Nevertheless, acute thermal profiles of (metabolic) enzymes and their ABTs can provide information on the general thermal sensitivities of differently thermally adapted fishes and potentially their general thermal acclimation capacities⁶¹⁻⁶³.

In *O. mykiss*, EROD activities remained stable within the thermal range assayed in our study, which is typical for eurytherm species⁶⁴. In contrast, none of the Antarctic fish showed an increase of EROD activities with rising assay temperature, and only *N. rossii*, *G. gibberifrons* and *C. gunnari* could maintain EROD activities stable with rising assay temperatures until a steady drop in enzyme activity beyond the ABTs. Particularly *C. aceratus* displayed a very low thermal flexibility, which was visible in drastically decreasing enzyme activities with

increasing temperatures beyond the 3°C assay. Usually, most aerobic and anaerobic enzymatic activities increase with rising assay temperature in Antarctic fish^{63, 65}, but this relation appears not to hold true for EROD activities. Such high temperature sensitivities of EROD when compared to rainbow trout could be related to structural and functional peculiarities, e.g. in the protein tertiary structure⁶⁶, which then involve general functional failures of the enzyme towards warmer temperatures. The functional limitations in enzyme activities, which seem to occur just a few degrees above the physiological temperature limit of those Antarctic fish⁶⁷, clearly mirror the evolutionary adaptation to the cold and extreme stenothermy of these species, which most likely also transfers to the xenobiotic metabolism in Antarctic fish.

The Arrhenius plots of the GST activity showed increasing enzyme activities with rising assay temperatures in all four Antarctic fish species and were generally less temperature sensitive than the EROD activities. Also the ABTs of GST in Antarctic fish were much higher compared to the ABTs of EROD, a clear sign for a higher thermal flexibility of the phase II than of the phase I enzymes.

Earlier studies emphasize that the phase I cytochrome P450-dependent monooxygenase system is responsible for the oxidation of organic pollutants such as PAHs and PCBs^{68, 69}. Thus, it may be mainly the thermal inflexibility of cytochrome P4501A (reflected by EROD activity) that could limit the xenobiotics metabolism capability of Antarctic fish during future seawater warming and pollution.

Yet, GST showed similar species differences in its thermal flexibility as the EROD activities: ABTs were lowest in *C. aceratus* and *N. rossii*, and higher, i.e. beyond 18°C, in both *G. gibberifrons* and *C. gunnari*. Such species differences, as we observed them in the thermal 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 metabolism⁶³. Also for the white-blooded *C. aceratus* an extremely low thermal tolerance is hypothesized, which is putatively related to the low hematocrit in this species⁷². 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 level⁷². 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 tissue³², 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

metabolism capacity in Antarctic fish species is also suggested from the findings of Yu et al.
⁷⁴ on the presence of PAH metabolites in the bile of *N. gibberifrons* exposed to Diesel Fuel.

In a comparison of the depletion rate constants (k_e ; per hour) and the intrinsic clearance rate ($Cl_{IN\ VITRO, INT}$) of the Antarctic species to those of rainbow trout, the rates of the former are significantly lower than in *O. mykiss*. Even when the higher metabolic rates of *O. mykiss* are considered, a down-extrapolation of the clearance rates of rainbow trout using a Q_{10} of two would reveal three to seven times lower clearance rates in Antarctic fish compared to trout. Such low BaP biotransformation rates are furthermore mirrored by the low EROD activities in the Antarctic fish. A calculation of the BaP to EROD rates revealed a BaP/EROD rate of 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. gunnari*. In other words, in comparison to rainbow trout, the BaP metabolism per unit EROD activity is only half as efficient in *N. rossii* and *C. aceratus* than in *O. mykiss*, three times lower in *G. gibberifrons* and six times lower in *C. gunnari*. Furthermore, our data on the high thermal sensitivity and putatively low structural flexibility of the EROD and GST activities corroborates our findings of generally low detoxification capacities in Antarctic fish.

Among the Antarctic fish, k_e was two times higher in red-blooded Antarctic fish than in the two white-blooded species. This is a first indicator on species differences in xenobiotic metabolism within the Antarctic fish species that could be related to their physiological differences. Thus, the intrinsic clearance rates, which express BaP metabolism per mg S9 protein and allows for comparing the metabolic capacities per S9 protein, was two orders of magnitude higher in *C. aceratus* when compared to *C. gunnari*, and three-times higher in *N. rossii* compared to *G. gibberifrons*.

In temperate fish species, biotransformation of BaP are frequently related with their tissue CYP content and biotransformation enzyme activities¹⁰. However, the CYP content of our rainbow trout were not higher than in the Antarctic fish, and the review by Livingstone¹⁰ reports a total CYP content in liver of temperate fish of $322 \pm 35 \text{ pmol mg}^{-1}$, which lies in the range of the Antarctic fish we measured in our study. Our data therefore show the general presence of the detoxification enzymes that, however, do not necessarily parallel the actual biotransformation capacities of the detoxification enzymes, as we demonstrate with the BaP metabolism rates of the Antarctic fish.

The toxicity of BaP can be attributed to the reaction of BaP metabolites formed. Our studies on the metabolite formation of *O. mykiss*, the red-blooded *G. gibberifrons* and the white-blooded *C. gunnari* showed that rainbow trout produced all three analyzed metabolites at substantial levels (3-OH: 32.9 % of total BaP metabolites, followed by 9-OH (7.9 %) and 7,8-diol (1.9 %)). In contrast, the metabolite formation rates of the Antarctic fish species were mostly below detection level. The only exception was *C. gunnari*, where 6.8 % of 3-OH was produced. Similarly, other animal species, including rainbow trout, form 3-OH as the major metabolite during BaP metabolism^{75, 76}.

CYP1-mediated EROD activity is usually highly correlated to BaP metabolism, such as the formation of 7,8-diol that is mainly catalyzed by CYP1A1. The BaP/EROD rate, which was lowest in *G. gibberifrons*, thus parallels our findings of absent BaP metabolites in this species. Interestingly, the BaP/EROD rate was even lower in the icefish *C. gunnari* than in *G. gibberifrons*. Yet, it showed the formation of 3-OH. The lack of correlation between EROD activity and BaP metabolite formation in this species suggests that also other CYP isoforms may be involved in BaP metabolism in this icefish species⁷⁷. In sum, the low or absent 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

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

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

blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*,
Champscephalus gunnari, *C.a.*, *Chaenocephalus aceratus*; *O.m.*, *Onchorhynchus mykiss*. *N*
= 6 per species, data are means $\pm$ SEM. ^a Significantly different from *O.m.*, ^b significantly
different from *G.g.*, $p < 0.05$ (ANOVA, Tukey Post-Test).

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

Champscephalus 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 $\pm$ SEM ($n = 6$).

Figure 4. Arrhenius plots for GST activities of Antarctic fish and rainbow trout. Red-blooded: *N.r.*, *Notothenia rossii*, *G.g.*, *Gobionotothen gibberifrons*; white-blooded: *C.g.*, *Champscephalus gunnari*, *C.a.*, *Chaenocephalus aceratus*; *O.m.*, *Onchorhynchus mykiss*.

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

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Figure 5. Biotransformation of benzo(a)pyrene (BaP) by Antarctic fish and trout liver S9 fractions. Red-blooded: *N.r.*, *Notothernia 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 μ M BaP: *C. aceratus*; 1 μ M BaP: *C. gunnari*, *N. rossii*; 2 μ M: *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 $\pm$ 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 μ M 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 $\pm$ 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
<i>O. mykiss</i>	119.3 ± 22.5
<i>N. rossii</i>	295 ± 126.5
<i>G. gibberifrons</i>	186 ± 8.0
<i>C. aceratus</i>	305.9 ± 123.8
<i>C. gunnari</i>	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) [*]
<i>O. mykiss</i>	0.0412 ± 0.002	6.431 ± 0.56
<i>N. rossii</i>	0.0110 ± 0.003	1.747 ± 0.43*
<i>G. gibberifrons</i>	0.0113 ± 0.009	0.522 ± 0.16*
<i>C. aceratus</i>	0.0049 ± 0.002	1.203 ± 0.31*
<i>C. gunnari</i>	0.0045 ± 0.001	0.616 ± 0.18*

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

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