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The effect of chronic heat stress on cortisol levels in the Antarctic fish *Pagothenia borchgrevinki*

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Abstract. Radioimmunoassay was used to determine levels of the stress-inducible glucocorticoid, cortisol, circulating in the plasma of the extremely stenothermal Antarctic fish *Pagothenia borchgrevinki* at rest and after heat stress. Fish sampled immediately after capture (−1.9 °C) had low cortisol levels (10.4 ± 1.4 ng ml^{-1}, mean ± SEM) as did fish which were laboratory rested for 3 days. Sudden exposure to 5 °C (48 h) resulted in a peak cortisol value after 3 h (69.9 ± 6.8 ng ml^{-1}) whereas exposure to 8 °C (6 h) resulted in a peak value after 1 h (73.5 ± 8.0 ng ml^{-1}). At both temperatures levels remained significantly elevated ($p < 0.05$) for the entire period of exposure. Increased temperature also resulted in a significant change in haemoglobin, haematocrit and mean cell haemoglobin concentration (MCHC) ($p < 0.05$). Plasma lactate was significantly elevated only after exposure to 8 °C ($p < 0.05$). Plasma cortisol levels from *P. borchgrevinki* are reported here for the first time and show this cryopelagic Antarctic species to have an unusual hormonal stress profile.
Key words. Cortisol; stress; heat; Antarctic; fish.

Introduction

The stress response of *Pagothenia borchgrevinki* has been investigated with respect to factors such as anaesthesia[1], handling[2] and periods of elevated temperature[3,4]. Changes in haematological parameters have previously been used to describe the stress resulting from interference during experimental procedures. However, the primary response to such manipulation in teleost fish involves a hormonal flush of catecholamines and corticosteroids. Adrenaline release is rapid, but transient. Cardiac output and oxygen carrying capacity increase[5,6], and there is evidence for erythrocyte swelling and increased oxygen affinity of haemoglobin[7,8]. In contrast, elevation of corticosteroids is more sustained and generated by activity of the hypothalamo-pituitary-interrenal (HPI) axis[9]. Cortisol appears to be the dominant corticosteroid in most teleosts[10] and the plasma level of cortisol is now well established as a reliable indicator of stress in fish[11]. In addition, cortisol has been recently shown to have an interactive effect with catecholamines[12]. In trout red blood cells, cortisol stimulated the production of additional β-adrenoreceptors, thereby increasing receptor availability and enhancing erythrocyte sensitivity to circulating catecholamines[12].

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P. borchgrevinki is a common cryopelagic Antarctic notothenioid living at a year round temperature of −1.9 °C. Previous studies which have used this fish indicate it to be somewhat resilient to capture and transport, and able to recover quickly under laboratory holding conditions[4,13]. Freezing is avoided by the presence of glycoprotein antifreeze molecules and elevated levels of ions in the body fluids which both act to depress the freezing point of the fish. Reduced metabolism at such an extremely low temperature is a result of the kinetics of the metabolic processes, although rates are usually greater than that expected by extrapolation from temperate species; a phenomenon called metabolic cold adaptation[14]. Various biochemical indices in Antarctic fish exhibit different magnitudes of temperature compensation compared with temperate species. For example, protein synthetic rates are 2- to 3-fold higher than predicted, while the levels of enzymes responsible for fatty acid catabolism in red muscle are up to 5-fold greater in nototheniids than in temperate teleosts[15]. This range highlights the uncertainty of predicting the time profile and magnitude of the corticosteroid stress response in Antarctic fish from data in the current literature.

Therefore the aim of this study was to determine baseline plasma cortisol levels in *P. borchgrevinki* and monitor changes during confinement and exposure to increased temperature. Secondary stress parameters (plasma lactate levels, haemoglobin concentration, haematocrit and MCHC) were measured for comparison with plasma cortisol levels and to afford a better understanding of the primary stress response in *P. borchgrevinki*.

Materials and methods

Specimens of the Antarctic fish *P. borchgrevinki* were collected during November and December 1992 by jigging lures on hand lines just under the annual sea-ice in the vicinity of New Zealand's permanent Antarctic base on Ross Island in McMurdo Sound, Antarctica (77 °S, 165 °E). One set of control samples was taken immediately upon capture with blood samples drawn within 60 s of capture. Blood was taken by caudal puncture, without the aid of anaesthesia[1], using 21 gauge needles and 3 ml syringes with the dead space filled with sodium heparin solution (5000 I.U. ml^{-1}). Another set of samples was taken from fish which had been transported to the temporary laboratory located on the sea-ice and allowed to recover for 3 days. Prior to sampling these fish were kept undisturbed in a covered 200 l white plastic fish bin with fresh sea-water continually pumped in at a rate of 2000 l h^{-1}. Ambient sea-water temperature was −1.9 °C and a size range of *P. borchgrevinki* of 80–120 g was used.

A system of reticulated aquaria (65 l) was supplied with fresh running sea-water for experiments at −1.9 and 5 °C. Filtration ensured no toxic buildup of nitrogenous waste and airstones maintained seawater oxygen content close to 100% saturation[15]. Groups of five fish were quickly netted and transferred to each of the tanks and sampled after the allotted time period (30 min, 1, 3, 6, 12, 24 and 48 h). Each fish was sampled once and then sacrificed. The procedure was also carried out at the extreme water temperature of 8 °C. However, two fatalities in the group exposed for 6 h suggested that fish exposed for 12 h or longer would not survive at all and therefore the experiment was concluded at this point.

Haematocrit and haemoglobin concentrations [Hb] were determined by the method of Dacie and Lewis[16] immediately after sampling. Mean cell haemoglobin concentration, used as an index of changes in erythrocyte volume, was estimated as; MCHC = [Hb]/Hct. Remaining whole blood was spun at 8000 × *g* for 5 min and the plasma removed and placed into 0.5 ml Eppendorf tubes. Samples were then frozen in liquid nitrogen for storage and transport back to New Zealand for analysis. Blood lactate was analysed using Sigma enzymatic test chemicals and metabolic standards (Bulletin no. 826-UV). Plasma cortisol was measured by radioimmunoassay after extraction with ethyl acetate as described by Pankhurst and Conroy[17]. Extraction efficiency was determined by recovery of [^{3}H] cortisol added to the sample, and was in excess of 90%. Assays were conducted with [1,2,6,7-^{3}H]cortisol and an antibody purchased from Bioanalysis Ltd, Cardiff, Wales. The assay detection limit was 0.3 ng ml^{-1}.

All data were analysed in a two way factorial design using the general linear models (GLM) procedure of the SAS statistical package. Inter group differences were determined by a posteriori multiple comparisons (Tukey's HSD) with significance set at the 5% level.

Table. Resting values in *P. borchgrevinki* immediately after capture and values obtained after 72 h recovery in laboratory aquaria (n).

	Immediately after capture (5)	Recovery in aquaria (72 h) (9)
Plasma cortisol (ng ml^{-1})	13.4 ± 3.2	17.7 ± 6.4
Plasma lactate (mmol l^{-1})	0.318 ± 0.052	0.362 ± 0.065
Haematocrit (% RBC)	21.0 ± 3.4	16.4 ± 1.1
Haemoglobin (g l^{-1})	28.0 ± 4.0	24.1 ± 2.0
MCHC (g l^{-1})	136.9 ± 3.7	145.5 ± 4.6

Values are means ± SEM.
There was no significant difference between the values obtained using each method ($p > 0.05$).

Results

There was no significant difference in haematocrit, haemoglobin concentration, MCHC, plasma lactate and plasma cortisol levels in fish sampled immediately after line capture and after 3 days recovery from capture in the 200 l holding aquaria (table).

Transfer to the reduced volume aquaria (65 l) in the experimental apparatus ('confinement') led to no apparent change in behaviour at ambient temperature. However, exposure to 5 °C and 8 °C resulted in an initial increase in activity and a sustained rise in the rate of opercular movements. The skin of the fish changed from the normal silver grey to a dark grey colour. After approximately 15 min the fish settled down, opercular rates decreased somewhat and they displayed little spontaneous activity throughout the period of exposure. Skin colour remained dark for the duration of exposure. Initial levels of cortisol from fish caught by hand line were low (13.4 ± 3.2 ng ml^{-1}). Fish in aquaria at −1.9 °C showed no significant rise in cortisol levels over the 48 h of confinement. The small rise observed in the 1, 3 and 6 h samples (see fig. 1) was not significant ($p > 0.05$) due to the large variation in the values recorded, for example, after 1 h the plasma cortisol level was 42.1 ± 16.6 (mean ± SEM). Mean cortisol levels returned to control values after 12 h. Conversely, fish placed into water at 5 °C showed a significant elevation in plasma cortisol levels ($p < 0.05$), peaking after 3 h and remaining elevated for the duration of the exposure, indicating a state of chronic stress (fig. 1). At 8 °C peak cortisol values were recorded in fish after 1 h of exposure and remained significantly greater than control levels throughout the experiment ($p < 0.05$).

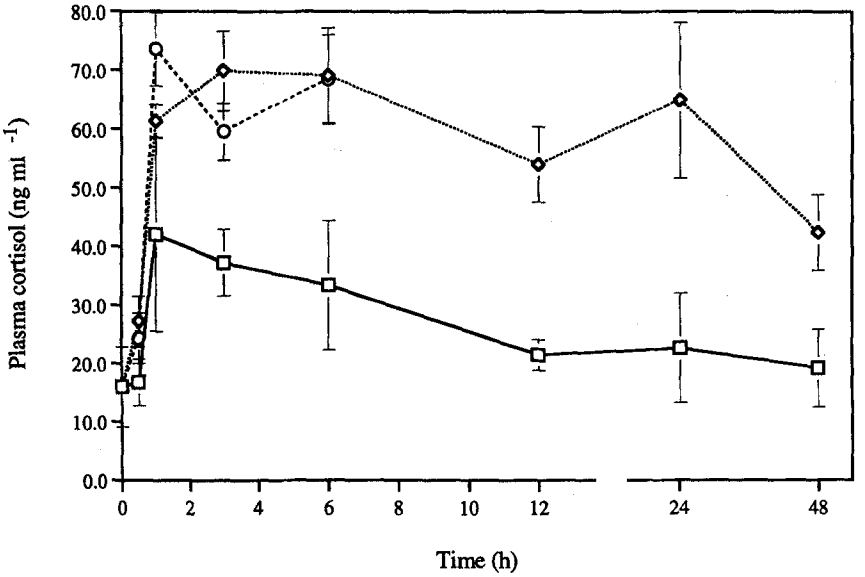

Figure 1. Plasma cortisol levels in *P. borchgrevinki* subject to confinement and chronic heat stress. Groups of 5 fish were transferred to 'confinement' aquaria at −1.9 °C (□) for time periods up to 48 h. The experiment was repeated at 5 °C (◇) for 48 h, and 8 °C (○) for 6 h. See 'Materials and methods' for detailed protocol.

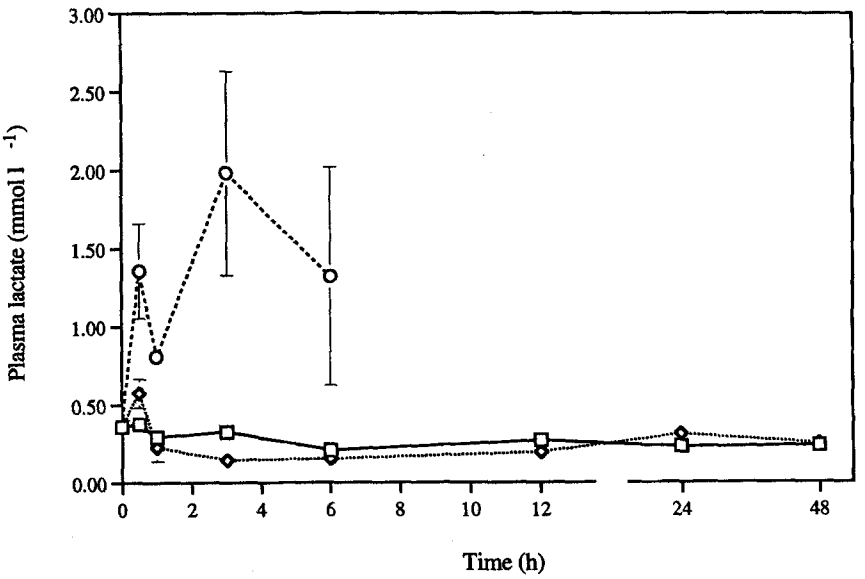

Figure 2. Plasma lactate levels in *P. borchgrevinki* subject to confinement and thermal stress. Fish were exposed to three temperatures: −1.9 °C (□), 5 °C (◇), and 8 °C (○).

Plasma lactate was elevated only after exposure to 8 °C seawater (fig. 2). Statistically significant values were recorded after 30 min ($p < 0.05$) and plasma levels remained high for the duration of the experiment (6 h). Resting lactate levels of 0.360 ± 0.059 mmol l^{-1} were similar to those recorded previously for *P. borchgrevinki*[13]. Peak values attained in this study resulting from thermal stress (8 °C) were 40% higher than those reported by Davison et al.[13] to develop in *P. borchgrevinki* after being exercised to exhaustion.

Haematocrit was elevated in response to confinement stress and increased further in a stepwise fashion with the addition of heat stress (fig. 3a). A bimodal trend was observed with all three treatments, that is, two rises followed each time by a fall. The onset of the second rises in haematocrit values was accelerated by increasing temperature, but after 48 h haematocrit had returned to values seen in control fish.

Haemoglobin control values from this study (26.0 ± 2.0 g l^{-1}) compare favourably with those recorded previously for *P. borchgrevinki* laboratory acclimated to −1.5 °C (25.4 ± 5.2 g l^{-1})[3]. Fish in 65 l aquaria at −1.9 °C had the greatest [Hb], rising to 42.9 ± 4.6 mmol l^{-1} after 30 min, followed by a significant fall ($p < 0.05$) to 16.3 ± 3.3 mmol l^{-1} after 6 h (fig. 3b). After 48 h, [Hb] had returned to control levels. Expo-

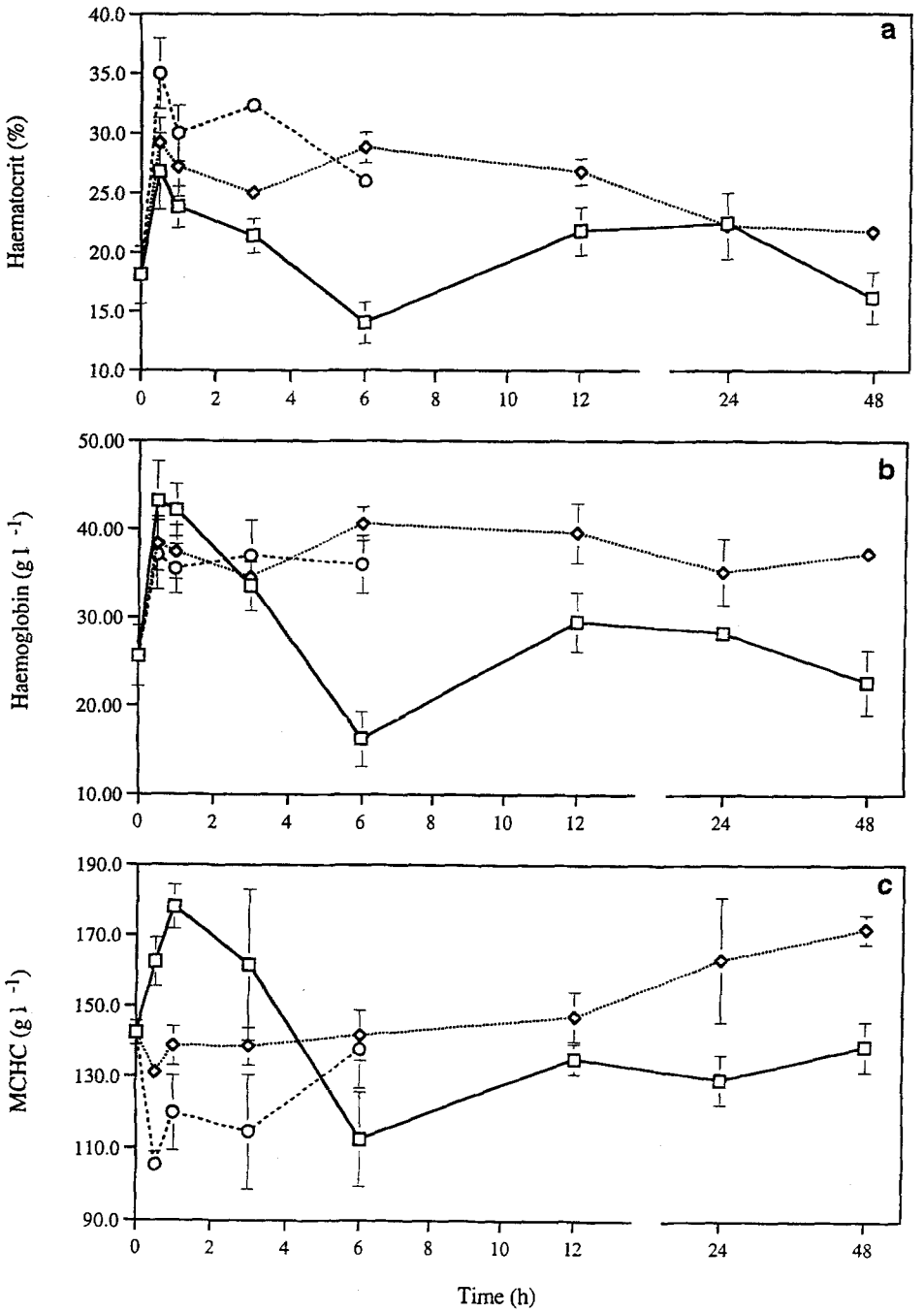

Figure 3. Changes in haematological parameters in *P. borchgrevinki* exposed to water at three different temperatures: −1.9 °C (□), 5 °C (◇), and 8 °C (○). From top to bottom: *a* haematocrit, *b* haemoglobin concentration, *c* mean cell haemoglobin concentration.

sure to increased temperature (5 °C and 8 °C) also caused an initial increase but of a smaller magnitude ($38.4 \pm 3.3\ g\ l^{-1}$ and $37.1 \pm 4.0\ g\ l^{-1}$ respectively) and [Hb] remained significantly greater than control during both periods of exposure ($p < 0.05$).

Significant changes in *P. borchgrevinki* red cell volume ($p < 0.05$) were apparent in response to confinement stress (cell shrinking), and at 8 °C (cell swelling) was indicated by MCHC (fig. 3c). Red cell volume was relatively stable throughout the period of exposure to 5 °C.

Discussion

Basal levels of plasma cortisol measured in *P. borchgrevinki* were slightly greater than those recorded in other marine teleosts[18]. Lower values for blue mao mao (*Scorpis violaceus*), snapper (*Pagrus auratus*) and blue cod (*Parapercis colias*) have been recorded but in fish sampled underwater using scuba apparatus[18]. However, this method was not attempted given the harsh diving conditions of the Antarctic. Wells and co-workers[19] found that haematological parameters in *P. borchgre-*

vinki with chronically implanted cannulae were not significantly different to values obtained by the 'grab-and-stab' method and therefore sampling of these fish immediately after capture will afford acceptable results. This is particularly the case for *P. borchgrevinki* which is found within the first 2–5 m of the water column, and therefore capture and sampling may be completed in less than 60 s.

Plasma cortisol levels were not significantly elevated by confinement at −1.9 °C and an apparent increase after 1 h is most likely due to the stress of handling and manipulation (fig. 1). In contrast, blue mao mao (*Scorpis violaceus*) placed in larger (200 l) aquaria exhibit a sustained elevation of plasma cortisol levels indicating that confinement in this species represents a significant stressor[20]. The response latency of 1 h seen in *P. borchgrevinki* (this study) is much longer than that seen in other species. Increased plasma cortisol levels in response to confinement is usually apparent after 5–10 min in both marine fish, e.g. blue mao mao *S. violaceus*[20], Napper *Pagrus auratus*[18], and freshwater trout[22]. This delayed response may be due to the ambient sea-water temperature in Antarctic waters being −1.9 °C. However, in marine red drum (*Scianops ocellatus*) at 26 °C[23], blue mao mao (*Scorpis violaceus*) at 15 °C[20], and trout at 4 °C[22], latency for each was in the region of 10 min, and Pankhurst et al.[20] suggested that most species will exhibit a detectable response within 15 min. Blue mao mao sampled during summer in warmer waters (19–21 °C) responded more quickly to confinement stress (5 min) than fish treated identically during winter (13–15 °C; 10 min) which suggests the difference in ambient sea-water temperature to be responsible[20]. However, given the data above, it seems unlikely that the delayed response in *P. borchgrevinki* is simply a temperature effect but more likely a result of a less sensitive HPI axis.

Temperature was chosen as a stressor for *P. borchgrevinki* due to its very small range of tolerance, from −1.9 to 5 °C[24]. Without knowing what response would be evoked, a stressor anticipated to produce a maximal response was desired. Franklin et al.[4] exposed Antarctic fish to brief periods of severe temperature stress (10 °C) and reported large physiological disturbances, followed by a full recovery after 12 h at ambient seawater temperature (−1.9 °C). For *P. borchgrevinki*, 8 °C still represents a temperature in excess of its critical thermal maximum.

Plasma cortisol levels increased significantly ($p < 0.05$) during heat stress (5 °C) with a detectable response after 30 min and a peak response (69.9 ± 6.8 ng ml^{-1}) after 3 h (see fig. 1). Exposure to the higher temperature (8 °C) did not generate higher peak cortisol levels. From this we can conclude that both treatments eventually induced the maximal response from the HPI axis. Responses of a similar magnitude have been reported for other marine species. For example, snapper (*P. auratus*) after capture using longline and transfer to a laboratory holding tank had plasma cortisol levels of 76 ng ml^{-1} (ref. 18). However, the value of 73.5 ng ml^{-1} obtained from *P. borchgrevinki* in this study is amongst the lowest reported from any fish. The peak response in *P. borchgrevinki* at 5 and 8 °C occurred after 3 h and 1 h respectively, the more severe stress reducing the time to onset of peak cortisol release by 2 h. Such a response supports the work of Pickering and Pottinger[25] who suggested that the severity of the stressor is responsible for the rate of plasma elevation of cortisol levels in salmonids.

For this cryopelagic Antarctic notothenioid fish, increased ambient water temperature represents an unusual and severe form of stress as shown by the rapid changes in the parameters measured in fish exposed to 5 and 8 °C. However, in fish confined at the normal seawater temperature of −1.9 °C (fig. 1), there was evidence of recovery within the 48 h period of confinement. This is in direct contrast to temperate species in which confinement results in chronically elevated plasma cortisol levels[20,21]. The observed recovery may reflect the habit of *P. borchgrevinki* to lodge itself into small fissures in the platelet ice to rest. Without a swim bladder, Antarctic notothenioids are negatively buoyant and therefore must swim constantly to maintain their position in the water column. Occupying small spaces is therefore not an unusual situation for *P. borchgrevinki* to find itself in and this probably accounts for the cortisol levels approaching baseline values in confined fish after 48 h.

Blood lactate concentrations have been used previously as an indicator of stress[26], but this may be inappropriate if lactate is a normal product of routine anaerobic muscle metabolism. In *P. borchgrevinki* these levels are very low[13] and even after exercising fish to exhaustion plasma lactate concentrations only reach levels comparable to those in resting (non-exercised) temperate teleosts[27]. Exposure to 8 °C resulted in a significant increase in plasma lactate versus baseline, probably as a consequence of the physical reaction to this extreme temperature shock as well as the increased opercular rate which continued for the duration of the exposure (fig. 2). There was no apparent influence of plasma cortisol on lactate production, which is consistent with studies on rainbow trout, *Oncorhynchus mykiss*[28].

Confinement of *P. borchgrevinki* resulted in increased haematocrit and [Hb] (fig. 3a and b), despite a significant reduction in red blood cell size as indicated by an increased mean cell haemoglobin concentration (MCHC) (fig. 3c). The increase in haematocrit and [Hb] is probably due to contraction of the spleen, which serves as a store for erythrocytes[6]. Wells et al.[29] observed a 39% reduction in splenic mass of *P. borchgrevinki* in response to hypoxia, and Ling and Wells[30]

reported at 25% reduction in spleen [Hb] in the marine teleost, *Girella tricuspidata* in response to capture stress. Work by Nilsson and Grove[31] on the cod *Gadus morhua* suggests splenic contraction in teleosts may be a result of increased levels of circulating catecholamines. In this study, simple calculations show that the increases in haematocrit and [Hb] in response to 1 h of confinement may be totally accounted for by an increased number of circulating erythrocytes, presumably expelled from the spleen. Subsequent changes in the direction of baseline values may represent partial recovery and acclimation to the imposed thermal environment.

After 1 h exposure at 5 and 8 °C erythrocyte volume was greater than that seen as a result of confinement at −1.9 °C (fig. 3c). Red blood cell swelling in rainbow trout *Oncorhynchus mykiss*, has been shown to be a response to increased levels of circulating catecholamines[32,33] which activate the Na^+/H^+ exchanger located on the red blood cell membrane[12]. This mechanism causes red cell swelling due to the net influx of Na^+ and Cl^- into the cells and is enhanced with higher catecholamine concentrations[34]. This would explain the observed cell swelling in *P. borchgrevinki* at 5 °C and 8 °C. Increased circulating catecholamines also raises blood oxygen affinity, and Soivio and Nikinmaa[35] suggest that in rainbow trout (*O. mykiss*) this is a direct result of stress. Although catecholamine levels were not measured in this study. Antarctic fish have been found to exhibit a catecholamine profile in response to stress in which a short period of forced exertion resulted in transient increases in both adrenaline (4 nmol l^{-1} to 20 nmol l^{-1}) and noradrenaline (2 nmol l^{-1} to 40 nmol l^{-1}) (T. Lowe, pers. commun.). While the profile resulting from exposure to chronic stress remains uncertain, the rapid increase in haematocrit and decrease in MCHC can be largely explained by increased red cell swelling (due to an acute catecholamine response[12]) with exposure to increased temperature.

Cortisol also has a direct influence on the responsiveness of red blood cells to catecholamines, particularly under conditions of chronic stress. Perry and Reid[12] have described a mechanism whereby cortisol stimulates increased production of (uncoupled) β-adrenoreceptors via interaction with red blood cell corticosteroid receptors. The hormone-receptor complex binds to chromatin stimulating an increase in β-adrenoreceptor mRNA production and subsequently protein biosynthesis. Additional stress (hypoxia) mobilised β-adrenoreceptors to the surface of trout red blood cells thereby enhancing their responsiveness to catecholamines. Hence, the interactive effects of cortisol and catecholamines may ensure the homeostatic control of red blood cell intracellular pH regulation and therefore oxygen transport.

Under conditions of chronic stress Perry and Reid[12] suggest that cortisol pre-adapts the teleost erythrocyte to receive additional physiological inputs that ultimately enhance respiratory performance beyond that which would be possible in the absence of chronically elevated cortisol. Therefore, not only is cortisol a accurate indicator of the physiological response to stress but is essential to any explanation of the observed response. In this study levels of plasma cortisol from an Antarctic teleost are presented for the first time.

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