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Seasonality of the red blood cell stress response in rainbow trout, *Oncorhynchus mykiss*

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Running head: Na^+/H^+ exchange in trout red blood cells

Summary

The β-adrenergic stress response in red blood cells (RBCs) of rainbow trout shows seasonal changes in expression. We have explored the mechanisms underpinning this response by following over a period of 27 months changes in β-adrenergic receptor (β-AR) binding characteristics, β-adrenergically stimulated RBC Na^+/H^+ exchanger (βNHE) activity, together with β-AR and βNHE mRNA levels and plasma steroid hormone and lactate levels. These parameters were measured at approximately monthly intervals in a single population of fish held under semi-natural conditions. Membrane-bound, high-affinity β-ARs were present in RBCs at all sampling times, varying from 668 ± 112 to 2654 ± 882 receptors $cell^{-1}$ (mean ± SEM; n=8). βNHE activity, however, was reduced by 57 and 34% in December 1999 and February 2001, respectively, compared to an otherwise sustained influx that averaged 110.4 ± 2.3 mmol l^{-1} RBCs h^{-1} (n = 119). Only one reduction coincided with a spawning period but both were preceded by transient increases in circulating testosterone. βNHE activity measured under standard conditions was not correlated with the number or affinity of β-ARs nor with water temperature, but both β-AR numbers and βNHE activity were positively related to their respective mRNA levels (P = 0.005 and 0.038, respectively). Pharmaceutical intervention in the transduction cascade linking the β-AR and βNHE failed to indicate any failure of the transduction elements in RBCs displaying low βNHE activity. Similarly, we failed to demonstrate any link between seasonal cortisol fluctuations and seasonally reduced βNHE activity. However, the βNHE activity of age-separated RBC fractions showed that younger RBCs had a significantly higher βNHE response than older RBCs, consistent with the seasonal reductions in βNHE being linked to turnover of red cells and

erythropoiesis. Testosterone is known to induce erythropoiesis and we conclude that seasonal reductions in βNHE are not caused by changes in β−AR numbers, but may be linked to testosterone-induced erythropoiesis.

Key words: adrenergic receptor, Na^+/H^+ exchanger, estradiol, testosterone, cortisol, seasonal changes

Introduction

The red blood cell β-adrenergic response is of crucial importance for oxygen delivery to the tissues in numerous fish species during stressful events such as burst swimming or environmental hypoxia (Primmett et al. 1986; Nikinmaa, 1992). In salmonid fish, the magnitude of the β-adrenergic response appears to vary seasonally. Thus, Cossins and Kilbey (1989) showed that *in vitro* the adrenergically stimulated H^+ efflux in rainbow trout (*Oncorhynchus mykiss*) red blood cells (RBCs) was gradually reduced during winter and increased abruptly early in the following spring. Nikinmaa and Jensen (1986) found that neither exercise nor adrenaline injection of rainbow trout in winter affected RBC volume or the pH gradient across the RBC membrane, in contrast to summer fish. These authors suggested that the number or affinity of the β-adrenergic receptors (β-ARs) might be seasonally reduced, or that the activity of some other step in the cascade leading to Na^+/H^+ exchanger (βNHE) activation might be altered. Seasonality in magnitude and temperature sensitivity of the β-adrenergic response has also been shown *in vitro* in RBCs of Arctic charr (*Salvelinus alpinus*; Lecklin and Nikinmaa, 1999). By contrast,

Tetens et al. (1988) found no difference in the magnitude and affinity of the β-adrenergic response of rainbow trout RBCs *in vitro* in winter and summer fish.

The seasonal loss of βNHE activity is likely to impair the normal respiratory response to stress and this might negatively affect stress tolerance. Understanding the underlying mechanisms of this seasonally reduced β-adrenergic responsiveness and its physiological significance requires an intimate understanding of how red cell biology varies over the seasonal timescale and how this relates to circulating hormones. Thus, a reduction in rainbow trout RBC adrenergic responsiveness and flounder Na^+/H^+ exchanger activity has been circumstantially linked to the reproductive cycle (Lecklin and Nikinmaa, 1999; Weaver et al., 1999, respectively). Furthermore, plasma cortisol levels also show seasonal variation in salmonid fish (Pickering and Christie, 1981) and Reid and Perry (1991) have shown an up-regulation of RBC β-AR numbers in response to highly elevated plasma cortisol level. Also, Perry et al. (1996) observed an increased RBC βNHE responsiveness when cortisol was elevated through repeated physical stress.

Thus, in order to throw light on the occurrence, duration and magnitude of seasonal down-regulation of the β-adrenergic response and to identify the underlying mechanisms, we have undertaken an extensive monthly monitoring programme over 27 months. We have sought to relate changes in RBC adrenergic responsiveness to the number and affinity of RBC β-ARs and also to link changes in both to a range of other physiological parameters including reproductive condition and circulating levels of cortisol and reproductive hormones. In particular, we have sought measures of erythropoietic activity, since Lecklin et al. (2000) have shown that immature RBCs have a higher adrenergic responsiveness than mature RBCs. Thus, we have determined the

cellular amounts of transcripts for both the β-AR and βNHE through the seasons. Finally, we employed known pharmacological activators of the βNHE response to probe the effectiveness during winter and summer of the transduction pathway linking β-AR with adrenergically activated NHE activity.

Materials and Methods

Animals and sampling procedures

Rainbow trout (*Oncorhynchus mykiss*, Stirling strain, hatched March 1997, mean mass 728 g at the start of the study) were transferred to eight 1000 l outdoor holding tanks in October 1999, 50 fish per tank, with a constant flow (35 l min^{-1}) of Windermere lake water. The fish were fed commercial feed three times per week according to the manufacturer's recommendations, and their mean mass increased to 1642 and 1882 g in December 2000 and 2001, respectively. Water temperature was electronically logged at 6-h intervals. At approximately monthly intervals, for 27 months, 8 female and 8 male fish (until April 2000), two fish from each holding tank, were netted into a bucket containing anaesthetic (2-phenoxyethanol; 1:2000; v/v). Blood (approx. 8-10 ml) was sampled from the caudal vessels into heparinised syringes, transferred to polypropylene tubes, and kept on ice. Between May 2000 and May 2001 a second blood sample (~1 ml), for determination of β-adrenoreceptor and βNHE mRNA levels, was collected into sterile syringes and transferred to sterile micro-centrifuge tubes before being stored, unwashed, at -80ºC. The fish then were killed by a sharp blow to the head, length and weight measured, after which the fish were pithed and sexed. The gonads were weighed in male and unovulated female fish.

Red blood cell β-adrenergic receptor determination

Freshly drawn blood was washed three times in isotonic saline (145 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM D-glucose and 10 mM HEPES, pH 7.9 and an osmolality of 319 ± 1 mosm kg^{-1}; mean ± SEM; n=4) to remove the buffy coat. The red cells were re-suspended in fresh saline at a haematocrit (Hct) of approx. 30%. Plasma removed after the first spin was frozen (-20^{o}C) in aliquots (200 µl) for subsequent hormone and metabolite assays. High-affinity β-adrenergic receptors were measured using CGP 12177 as a β-adrenergic agonist according to Marttila and Nikinmaa (1988). Briefly, duplicate samples (50 µl) of the RBC suspension were added to a series of tubes containing 450 µl saline with final concentrations of 0.3-4.3 nM of [^{3}H]-CGP 12177 (((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]Benzimidazol-2-one)); specific activity 1.85 TBq $mmol^{-1}$; Amersham) either alone (total binding; B_t) or in the presence of excess unlabelled CGP 12177 (gift from Novartis Pharma AG, Switzerland) (non-specific binding; B_{ns}). The samples were mixed and incubated for 6 h at 4ºC, during which time they were regularly agitated. The RBCs were counted using a haemocytometer (Weber Scientific International Ltd.). At the end of the incubation period, 400 µl of each individual sample was filtered through glass microfibre filters (2.5 cm Ø, retention ≥1.0 µm; Whatman) held on a vacuum filtration manifold (Millipore, Model 1225) attached to a vacuum pump. The filters were washed 3 times with 2 ml ice-cold saline to remove unbound ligand. The filters were placed in vials containing 5 ml of scintillation cocktail (Eco-Safe, Meridian), shaken vigorously and stored in the dark at 4ºC for 72 h before liquid scintillation counting (Tri-Carb 2100 TR, Packard). The activity present in the

incubation media was determined by counting of the stock standard solution activities. Counts for B_t and B_{ns} were multiplied by 1.25 to account for the fact that only 80% of the total volume of the samples was counted. Values for specifically bound ligand (B_s) were determined by subtraction of B_{ns} from B_t and subsequently converted to fmol tube^{-1} by division by the specific activity. Receptor density (B_{max}) and affinity (K_d) were determined by use of non-linear regression analysis (Sigmaplot 4, Jandel Scientific) of B_s as a function of free [^{3}H]-CGP 12177 (total [[^{3}H]-CGP] – B_t). B_{max} values were then converted from fmol tube^{-1} to receptors cell^{-1} by multiplication by (Avogadro's number fmol^{-1} cells tube^{-1}).

Cortisol may influence the number of RBC membrane-bound β-adrenergic receptors upon exposure to a stressor (Reid and Perry, 1991). To establish whether seasonal fluctuations in plasma cortisol concentrations influence the number of β-adrenergic receptors, we incubated sub-samples of RBC suspensions under anoxic conditions at 5ºC for 1 h prior to incubation with labelled and unlabelled ligand (July 2000-March 2001). The subsequent procedures were as described above.

Red blood cell βNHE activity measurements

Samples of 1.2 ml washed RBC suspension were placed in rotating Eschweiler tonometers thermostatted at 15ºC and equilibrated with humidified N_2 (anoxic condition) for 60 min. After 50 min of equilibration, ouabain ($5*10^{-4}$ M final concentration) was added and duplicate samples of the suspension were taken for Hct determination. The RBC suspension was transferred from the tonometer flask into polypropylene test tubes containing saline with ouabain ($5*10^{-4}$ M final concentration; 0.5% DMSO v/v vehicle)

and ^{22}Na (approx. 0.05 MBq ml^{-1}) equilibrated to the same conditions as the blood cells, resulting in a 10-fold RBC dilution (this was defined as time zero). The ^{22}Na influx was measured in unstimulated cells (basal flux) and in cells stimulated by 10^{-5} M (final concentration) isoproterenol added at 5 min.

Triplicate samples (300 µl) from control and treated RBC suspensions were taken at 5 and 10 min. The samples were centrifuged (Eppendorf, Model 5410) and the supernatant removed, whereupon the RBCs were washed 3 times in ice-cold isotonic HEPES-containing $MgCl_2$ solution (adjusted to pH 7.9). The remaining RBC pellet was lysed in 0.5 ml 0.05% Triton-X solution and deproteinised by subsequent addition of 0.5 ml 5% trichloroacetic acid. The samples were centrifuged for 2 min and 0.5 ml of the supernatant was counted (Tri-Carb 2100 TR, Packard) in 5 ml scintillation cocktail (Eco-Safe, Meridian). In addition, triplicate samples of 200 µl extracellular medium were counted for each experiment. The Na^+ influx (mmol Na l^{-1} RBC h^{-1}) was calculated as:

$$((((X*A_2)-(X*A_1))Y^{-1}*10^3)(t_2-t_1)^{-1})60$$

where X denotes µmol Na^+ equivalent to 1 cpm; A_1 and A_2 denote ^{22}Na (cpm) in triplicate samples at time 1 and 2, respectively; Y denotes µl packed RBCs (determined from the Hct value) and t_1 and t_2 denote time in minutes.

Plasma cortisol, sex steroids and lactate

Plasma cortisol, testosterone, and estradiol-17β concentrations were measured by previously validated radioimmunoassays (cortisol: Pickering et al., 1987; testosterone: Pottinger and Pickering, 1985; estradiol-17β: Pottinger and Pickering, 1990). Plasma

lactate concentration was determined enzymatically using lactate oxidase and peroxidase followed by spectrophometric analysis (Roche, Switzerland).

Effects of repeated disturbance stress on adrenergic responsiveness

Eighty rainbow trout from a stock population were divided evenly between four 1000 l outdoor holding tanks (conditions as above) and acclimated for two weeks. At the onset of the experiment the water level in two of the tanks was dropped to approx. 10 cm for approx. 5 min once or twice daily for a two-week period. The control tanks were left undisturbed. Upon sampling three fish from each tank (mean mass ± SEM: 1773 ± 92 g; n=12) were anaesthetised and blood was collected and prepared for Na^+ influx measurements exactly as described above. The abundance of RBC β-adrenergic receptors was determined for RBC suspensions incubated under normoxic and anoxic conditions as described above.

The effects of isoproterenol, forskolin, dibutyryl cAMP and calyculin A on RBC Na^+ influx

Six rainbow trout from the stock population (mean mass ± SEM: 1847 ± 83 g) were netted into anaesthetic and blood was sampled, washed and stored overnight for Na^+ influx measurements as described above. The Na^+ influx was measured as described above 5 min after addition of (i) isoproterenol (10^{-5} M; non-selective β-AR agonist), (ii) forskolin ($1.5*10^{-4}$ M; adenylate cyclase activator), (iii) the membrane permeable cAMP analogue dibutyryl cAMP (10^{-3} M), or (iv) the phosphatase inhibitor calyculin A (10^{-7} M). In addition, the Na^+ influx was measured in unstimulated RBCs.

βNHE activity in RBCs separated according to density (age)

Blood was sampled, washed and stored as described above from rainbow trout (Shasta strain; mean mass ± SEM: 937 ± 68 g; n=6) during October-November 2001. This treatment assured that the RBC membrane transporters and cell volume were in an un-stimulated steady-state condition (Bourne and Cossins, 1982). After overnight storage the RBCs were washed once and re-suspended in saline containing 1% bovine serum albumin (BSA) at an Hct of approximately 80%. The RBCs were separated into age fractions by fixed-angle (30º) centrifugation (10,000 g; 4ºC; 15 min) in narrow tubes (diameter 4 mm, length 45 mm, volume 0.5 ml) (Speckner et al., 1989; optimized for trout by Phillips et al., 2000). The youngest, least dense, cells are located in the top layer, whereas the older more dense cells are located in the middle and bottom of the tube. The tubes were cut into a top (24 ± 2%), middle (60 ± 3%) and bottom (15 ± 1%) fraction, containing RBCs of increasing age, which were washed in isotonic saline 3 times to remove BSA. The mean cellular haemoglobin concentration (MCHC), determined as [Hb]/Hct, was used to verify that the cells were separated according to age. MCHC is lower in younger cells than in older cells (Speckner et al., 1989; Lund et al., 2000). Adrenergically stimulated Na^+ influx was measured in each of the different cell fractions and also in the original unseparated population of RBCs.

RBC βNHE and β3b receptor mRNA determinations

Total RNA was isolated from frozen tissue by homogenisation in guanidinium thiocyanate (Chomczynski and Sacchi, 1987) using Trizol Reagent (Invitrogen). After

treatment with DNase I (5 units per µg RNA; Invitrogen) to remove any remaining genomic DNA, the quality of the RNA was assessed by gel electrophoresis. cDNA was synthesised from 1-2 µg RNA using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). Previous studies (J. Nickerson, 2003) have demonstrated that the trout RBC β-AR most closely resembles the β_3-AR of mammals or the β_{4c}-AR of turkey RBCs (Chen et al., 1994). Because the trout RBC β-AR is found exclusively in the blood, it has been termed B_{3b}-AR (J. Nickerson, 2003). β_{3b}-AR or βNHE mRNA levels were assessed by Q-PCR on duplicate samples of cDNA (1 µl) using a Hot StarTaq Master Mix kit (Qiagen) and a Stratagene MX-4000 multiplex quantitative PCR system. CYBR Green (Molecular Probes Inc.) and ROX (Stratagene) were used as DNA and reference dyes, respectively. The PCR conditions (final reaction volume = 20 µl) were as follows: cDNA template = 1.0 µl; forward and reverse [primer] = 150 pmol l^{-1}; [Mg^{2+}] = 2.0 mmol l^{-1}; CYBR green = 1:50,000 final dilution; ROX = 1:30,000 final dilution; dNTP = 200 µmol l^{-1}. The annealing and extension temperatures over 40 cycles were 58ºC (45 sec) and 72ºC (60 sec), respectively. The following primer pairs were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi):

actin forward	5'-CAC CAT GAA GAT CAA GAT CAT YGC-3'
actin reverse	5'-ATT TRC GGT GGA CGA TGG AG-3'
β_{3b} forward	5'-CTT GGG CTA TGG TGG CAG TA-3'
β_{3b} reverse	5'-CCA TGA TAA TGC CCA AGG TC-3'
βNHE forward	5'-GGG TAA TGC GTC AGA CAA CC-3'

βNHE reverse 5”-CCA TGA TAA TGC CCA AGG TC-3’

The specificity of the primers was verified by cloning (TOPO TA cloning kit; Invitrogen) and sequencing of the amplified products. To ensure that CYBR green was not being incorporated into primer dimers or non-specific amplicons during the Q-PCR runs, PCR products were analysed by gel electrophoresis in initial experiments; single bands of expected size were obtained in all instances. Furthermore, the construction of CYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. Relative expression of mRNA levels was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Data presentation and statistics

Throughout, the data are presented as mean ± SEM. The data sets were analysed using analysis of variance (ANOVA, Genstat 5, Lawes Agricultural Trust) with, for the seasonal study, individual fish, tank and time as factors; for the implant studies, fish, tank and treatment as factors; and for the separated cell study, fish and cell fraction as factors. Significant differences between times, treatment groups or fractions were determined using the estimated standard error of the differences between means. Where mean and variance were found to be interdependent, the data were log-transformed prior to analysis. Sigma Plot 4.0 was used to assess statistical significance of fitted linear and non-linear regressions.

Results

Only data for female fish are presented. Because of differential mortality rates during the previous spawning season (male rainbow trout tend to mature one year earlier than females, and are more vulnerable to fungal infections when sexually mature) sufficient numbers of male fish were available only for the initial five samples. Over the five-month period, which included one sample with a reduced β-adrenergic responsiveness (December 1999; Fig. 2), we observed no statistically significant sex-related differences in β-AR characteristics or βNHE activity. Maximum androgen and estrogen levels occurred at the same time in males as in female fish (data not shown). These limited data suggest that our observations of female fish are also valid for male fish.

Seasonal variations in RBC β-AR characteristics and βNHE activity

Figure 1 presents the time course over 27 months of β-adrenergic receptor numbers together with the measured water temperatures. The number of β-adrenergic receptors (β-ARs) varied seasonally from a minimum of 668 ± 112 to a maximum of 2654 ± 882 receptors cell^{-1} (n=8; Fig. 1). During late summer 2000 the β-AR number increased, remained high until late autumn and then decreased into the winter. A similar though less pronounced rhythm was evident in 2001 (Fig. 1). Throughout this period the β-AR number was positively related in a linear fashion to the average ambient water temperature (P=0.002, r^2=0.417; Fig. 1 inset). The equilibrium dissociation constant (K_d) for CGP binding to the RBC β-ARs varied between 0.31 ± 0.08 and 1.35 ± 0.26 nM (n=8;

not illustrated), but did not correlate with the ambient water temperature (linear regression: P=0.77).

Reid and Perry (1991) showed that cortisol applied through an osmotic pump can pre-adapt the RBCs to cope with a stressor by elevating the number of membrane-bound β-adrenergic receptors, and this potentially increases adrenergic responsiveness. During our routine procedures for measuring β-adrenergic responsiveness we also stressed the RBCs by incubating cells throughout under anoxic conditions. We therefore examined whether seasonal fluctuations in cortisol, with elevated levels during the spawning periods (Fig. 2), influenced the number or affinity of functional β-ARs when incubated under oxygenated or anoxic conditions. There was, however, no statistical difference in β-AR characteristics (number and affinity) between oxygenated and anoxically treated RBC suspensions during the entire period between July 2000 and March 2001 (data not shown).

The average isoproterenol-stimulated Na^+ influx was 110.4 ± 2.3 mmol l^{-1} RBCs h^{-1} (n=128) during the months where the β-adrenergic response was not reduced (Fig. 2). A down-regulated response was arbitrarily defined as any flux lower than 85 mmol l^{-1} RBCs h^{-1}. In December 1999 and February 2001 the βNHE activity was reduced by 57 and 34%, respectively, compared to the average value, whereas during the third winter of the study there was no statistically significant reduction in the βNHE activity (Fig. 2). Surprisingly, there was no obvious dependence of the magnitude of the Na^+ influx on the number (linear regression: P=0.83; n=163) or affinity (linear regression: P=0.68; n=163) of the β-ARs. These data suggest that the seasonal changes in βNHE activity do not reflect changes in the binding characteristics of the hormone to the receptors. Also, the

Na^+ influx did not correlate with ambient water temperature (linear regression: P=0.73; non-linear regression: P=0.72), i.e. the temperature at which the fish were acclimated, which agrees with the results obtained by Cossins and Kilbey (1989).

Effects of repeated disturbance stress on β-adrenergic responsiveness

RBC β-adrenergic receptor density has been shown to decrease in response to chronically elevated plasma catecholamine levels (Gilmour et al., 1994). We exposed fish to repeated daily stress over a 2-week period in January 2001, when the βNHE responsiveness was normal, to elevate plasma catecholamine levels and test if unidentified stressors in the holding facilities would affect the β-adrenergic responsiveness. The βNHE activity was not affected by repeated physical stress with Na influx values of 129.7 ± 4.7 and 128.8 ± 7.4 mmol l^{-1} RBCs h^{-1} in control and disturbed fish, respectively.

A comparison of the effects of isoproterenol, forskolin, dibutyryl cAMP and calyculin A

Having established that the seasonal reduction in βNHE activity was not linked to seasonal changes in β-AR number or affinity, we looked for other causal factors. Binding of catecholamine to the membrane-bound β-AR triggers an adenylate cyclase-catalysed synthesis of cAMP (Mahe et al., 1985), which in turn stimulates protein kinase A to activate the βNHE (Guizouarn et al., 1993). Accordingly, we tested whether the cause of the seasonally reduced adrenergic responsiveness lay in the transduction pathway linking the receptor with the transporting effector using chemical compounds known to stimulate the βNHE by intervening at different points on a possibly long transduction pathway. None of the compounds stimulated the Na^+ influx to a greater extent than isoproterenol

(data not shown). More significantly in the present context, the extent to which these compounds stimulated the βNHE relative to stimulation by isoproterenol was similar in RBCs with a seasonally reduced response compared to RBCs with a normal response (Table 1). Thus, the reduced isoproterenol-induced βNHE activity could not be rescued by any of the transduction activators, indicating that the seasonal diminution of βNHE activity was not linked to any of the steps lying between the β-AR and the βNHE. This together with the absence of any changes in the binding affinity and number of β-ARs, suggests that the winter loss of βNHE activity was linked to changes in the βNHE itself.

Red blood cell βNHE and β3b mRNA levels

The seasonal variation in β3b mRNA levels is shown in Fig. 3. The relative quantity of β3b mRNA was more-or-less constant between May and October, decreased markedly by approx. 90% between October and December, and remained at low levels between December and May. The data showed that the level of β3b mRNA, like the receptor number per cell, was correlated in a linear fashion with the ambient water temperature (P=0.01; r^2=0.632) and, also, that the number of receptors depended in a linear fashion (P=0.005; r^2=0.755) on the level of β3b mRNA present in the RBCs (Fig. 3 inset).

Fig. 4 illustrates the corresponding changes in βNHE mRNA. The relative quantity increased between May and November, decreased markedly between November and December and increased again progressively between December and May (Fig. 4 inset). Examination of the levels of βNHE mRNA at different times of the year showed that the βNHE activity had a one-sampling time delayed hyperbolic dependency on the mRNA level (P=0.038; r^2=0.538) present in the cell (Fig. 4).

βNHE activity of age-separated RBCs

After separation, the top layer contained 23.9 ± 2.9%, the middle layer 61.5 ± 3.0% and the bottom layer 14.7 ± 1.1% of the cells (n=6). The values for MCHC are presented in Table 2. MCHC increased from the top to the bottom fractions of RBCs, showing that the cells had been separated according to age. Based on measurements for the individual fractions, MCHC of the un-separated RBCs suspension was calculated to be 4.19 mmol l^{-1}, which is within the 0.05 significance limits of the measured value. The activity of the βNHE of the top fraction RBCs was 23.6 and 35.5 mmol l^{-1} RBCs h^{-1} higher than that of the lower and bottom fractions, respectively (P<0.05 and P<0.01, respectively; Table 2).

Seasonal variations in plasma estradiol, testosterone and lactate

The seasonal fluctuations in plasma sex steroids in female fish within the experimental population are illustrated together with the βNHE activity in Fig. 5. Plasma testosterone levels in the female fish increased steeply just prior to or concomitantly with the observed major changes in the RBC βNHE activity (Fig. 5). It is noticeable that the maximal levels of plasma testosterone observed in successive years decreased gradually and in a statistically significant manner; i.e. in the third year of the study, the plasma testosterone level was reduced compared to the level in the first (P<0.01) and second (P<0.001) year of the study. Plasma estradiol levels varied in a very predictable manner, i.e. the maximal levels and the timing of the increases and decreases were similar each year (Fig. 5).

Plasma lactate varied throughout the study seasonally with minimal values of 1.13 ± 0.11 mM (n=16) in February and maximal values of 2.54 ± 0.24 mM (n=16) in August.

The response to β-adrenergic stimulation, which lowers the risk of anoxic metabolism and lactic acid production, was also lowest in the winter (see above). The lactate data, therefore, indicates that despite the reduction in β-adrenergic responsiveness, the oxygen uptake from the water was entirely sufficient to prevent activation of anaerobic metabolism under conditions of routine activity in the well-aerated holding facilities.

Discussion

Seasonal variations in the βNHE activity

In agreement with previous studies (Cossins and Kilbey 1989), the magnitude of the RBC β-adrenergic response, as indicated by βNHE activity measured under standard conditions, varied seasonally. Responses declined significantly below normal levels during some winter months and recovered during the early spring. As before, the spring recovery occurred prior to any increase in ambient temperature (Cossins and Kilbey 1989). However, the timing of the down-regulation was not consistent over the life of the experimental trout population; during the first winter the response was minimal in December, 2-3 months prior to spawning, whereas during the following winter the maximal reduction coincided exactly with the spawning period in February. The inconsistency in timing of the reduced βNHE activity was somewhat unexpected, but is in accordance with the timing of peaks in plasma testosterone levels (see below). One of the factors that varied during our study was the age of the fish. To our knowledge no studies have previously looked into possible age-related variations in timing of parameters involved in the reproductive cycle or the stress response. Also, the magnitude of reduction varied during the study; thus it was larger (57% of the average βNHE

activity) during the first winter than during the following winter (34% of the average βNHE activity) whilst there was no significant reduction in the third winter. In part this may be explained by our sampling point not coinciding exactly with time of the maximal reduction but might represent a trend that is associated with increasing age or size.

In considering the magnitude of the winter reduction in β-adrenergic responsiveness, it is worth pointing out that the trout RBC βNHE activity is markedly temperature dependent with a Q_{10} of 7.9 over the temperature range 0 to 19ºC (Cossins and Kilbey, 1990). Thus, assay of βNHE activity *in vitro* at a standard temperature 15ºC irrespective of seasonally varying environmental temperature must, therefore, profoundly underestimate the seasonal fluctuation in the *in vivo* RBC β-adrenergic response. Thus, the flux of 72 mmol l^{-1} RBCs h^{-1} measured *in vitro* in February 2001 at 15ºC would be reduced *in vivo* to approx. 9 mmol l^{-1} RBCs h^{-1} at the prevailing water temperature at that time.

Repeated physical stress and confinement both elevate plasma catecholamine and cortisol levels, with opposite effects on the β-adrenergic response (Perry et al., 1996). Thus, exposure of rainbow trout to daily physical stress reduced the number of membrane-bound β-ARs determined both under normoxic and hypoxic conditions, but elevated adrenergic responsiveness due to increased β-AR affinity (Perry et al., 1996). We therefore considered the possibility that (unidentified) seasonal differences in the degree of stress to which the experimental fish were exposed could have influenced RBC adrenergic responsiveness and contributed to the seasonal variation in RBC function observed during this study. However, at a time of the year when the β-adrenergic response was normal, exposure of the fish to a regime of repeated daily disturbance stress

did not change the βNHE responsiveness. The seasonal reductions in adrenergic responsiveness, therefore, seem to occur independently of increased stress levels.

An obvious factor that might underlie the seasonal variations in RBC β-adrenergic response is the effectiveness of the cellular β-AR system. Previous work has implicated altered β-AR numbers in the enhanced response to hypoxia (Reid and Perry, 1991; Marttila and Nikinmaa, 1988), however, the fluctuations in β−AR numbers or affinity in the present study were not in any way related to the seasonal changes in βNHE activity. An alternative explanation is that there was some impairment during winter in the effectiveness of the transduction pathway linking the β−AR with the βNHE. We stimulated the βNHE in a number of receptor-independent ways, using forskolin, calyculin A, which activate the βNHE by effects on adenylate cyclase and protein phosphatase, respectively (Seamon et al., 1981; Guizouarn et al., 1995), and the membrane permeable cAMP analogue dibutyryl cAMP. We compared the effect of these compounds on the βNHE activity of winter fish with reduced β-adrenergic responsiveness with those in summer fish with the normal high β-adrenergic responsiveness. If winter-suppression were due to impairment at a specific step in this pathway we would expect a larger effect of pharmacological activation at a downstream step, relative to the effects of the β-adrenergic agonist, isoproterenol, acting alone. We found that all three compounds stimulated the βNHE to the same extent in RBCs with a reduction in βNHE activity compared to RBCs with a normal response. This indicates no rescue of β-adrenergic response in RBCs of winter fish by downstream activation; the red cells from animals collected at different times of the year behaved identically with respect to transduction manipulation when tested under common experimental conditions.

Whilst we cannot exclude the possibility that there is a critical step lying downstream of the calyculin A-sensitive protein phosphatase, we suggest that the limiting step during winter months in the β-adrenergic response was not upstream to the βNHE. Given that it was not due to changes in β-AR numbers, we therefore conclude that it was due to variations in the number or affinity of the exchanger itself or properties of its microenvironment.

The linkage between seasonal changes in β–adrenergic responsiveness and reduced expression of the βNHE is supported by measurements of βNHE transcript expression. We found that transcript amounts declined in the autumn and increased in the spring, the changes in the mRNA levels preceding by one sampling period the spring increase in βNHE activity. The changes in βNHE mRNA levels could be caused either by seasonal effects on the transcriptional activity in the circulating population of RBCs or by seasonality in erythropoietic activity coupled with age-dependent changes in RBC transcriptional activity. Age-dependent reductions in mRNA levels have been reported for carbonic anhydrase and the Band 3 anion exchanger in rainbow trout RBCs (Lund et al., 2000). At the physiological level Lecklin et al. (2000) showed that the volume increase following β–adrenergic stimulation was considerably lower in mature than in immature RBCs, suggesting lower βNHE activity in the older cells. Consistent with this observation, age-dependent reductions in enzyme activity levels have been reported for citrate synthase, cytochrome oxidase, lactate dehydrogenase and pyruvate kinase in rainbow trout RBCs (Phillips et al., 2000). Age-dependent decreases in activity levels therefore seem to be widespread at both the transcriptional and functional levels.

Erythropoietic activity and the release of newly synthesized RBCs into the

circulation is reduced in rainbow trout during the winter (Lane, 1979), and in Baltic salmon, the proportion of immature cells in circulation decreases during the winter (Härdig and Höglund, 1984). Seasonal changes in the age profile of the circulating RBCs make the age-dependency of the RBC β-adrenergic volume response and enzyme activities of great interest in the interpretation of seasonal changes in RBC function. We show with density-separated RBCs that the βNHE activity of the top (youngest RBCs) of the separated fractions was significantly higher than that of the middle and bottom fractions, containing older RBCs, which is consistent with the changing age profile of circulating RBCs underpinning the seasonally reduced β–adrenergic responsiveness. In the present study, we do not have any measure of the erythropoietic activity. However, previous work in the same fish holding facilities at CEH Windermere showed that the circulating RBC number decreased during autumn and winter in mature females of the closely related brown trout, *Salmo trutta* (Pottinger and Pickering, 1987). The critical factor might be influence of low water temperatures during late autumn and winter upon erythropoietic activity (Hevesy et al., 1964), progressively reducing the proportion of young RBCs with a high β-adrenergic responsiveness in circulation.

Linking circulating hormones and erythropoietic activity with the βNHE activity

Nevertheless, elevated levels of plasma estradiol were observed prior to and concomitant with the seasonally reduced βNHE activity in December 1999 and January-February 2001. It remains, to be investigated whether seasonal elevations of plasma estradiol play any role in the β-adrenergic response of the circulating RBCs.

Testosterone is known to stimulate erythropoiesis in humans (Barcelo et al., 1999; Snyder et al., 2000) and birds (Jones and Johansen, 1972; Robinzon and Rogers, 1979; Thapliyal et al., 1982) and is suspected to have the same effect in fish (Pottinger and Pickering, 1987). Thus, we suggest that the increased testosterone levels observed in the present work during the spawning period may, by stimulating erythropoiesis, have promoted the rapid increase in adrenergic responsiveness during winter or early spring, the increasing proportion of immature RBCs with increased βNHE activity explaining the enhanced β–adrenergic responsiveness. The increases in β–adrenergic responsiveness were observed 1-2 sampling periods after the increases in plasma testosterone and decreases in βNHE activity. This delay is consistent with the lengthy lag time known to precede the entry of immature RBCs into the circulation (Lecklin et al., 2000).

Seasonality in the β-adrenergic receptor characteristics

We provide the first evidence linking, in a linear fashion, the abundance of a receptor, i.e. the β-adrenoceptor, and of its cognate transcript with the ambient water temperature. The spring increase in water temperature was closely associated with an increase in β3b-receptor mRNA and subsequently to an increase in the number of functional receptors. This seems appropriate, as oxygen consumption increases with a rise in water temperature and stimulation of the β-AR optimizes oxygen transport (Nikinmaa, 1992). It is paradoxical, therefore, that the increase in receptor β-AR numbers did not elevate βNHE activity, and consequently, in functional terms, does not seem to improve oxygen transport. No other parameter monitored during the present study co-varied with the β3b mRNA levels and β-AR numbers. However, we cannot exclude the idea that the

influence of temperature on β3b mRNA levels and β-AR numbers was indirect. How would the lower ambient temperatures routinely experienced by fish in Northern USA, Canada and Scandinavia (0-2ºC) affect seasonal fluctuations in β-ARs? Extrapolation of the linear relationship between β-AR numbers and ambient temperature to 1ºC predicts 549 receptors $cell^{-1}$. This might be entirely sufficient to initiate events leading to βNHE activation. Indeed, the Windermere fish included specimens with very low β-AR numbers yet with powerfully expressed βNHEs suggesting that exceptionally low winter temperatures would not necessarily incur greater reductions in βNHE activity.

Experimental elevation of plasma cortisol is able to increase β-AR density, probably by increasing transcription of the β3b-receptor gene. This enlarges the pool of cytosolic, physiologically inactive, β-receptors, which can be mobilised to the plasma membrane on exposure to stress (Reid and Perry, 1991). Whilst we found considerable circannual fluctuations in plasma cortisol with high levels during February-March we failed to observe changes in membrane-bound β-ARs anoxically treated RBCs compared to control RBCs. Cortisol therefore appears to play no role in the seasonal variation of RBC adrenergic responsiveness.

Conclusions

We showed that the number of RBC β-ARs is linearly related both to the level of β-AR transcript and ambient water temperature. Naturally occurring seasonal fluctuation in β-AR numbers were not related to the more inconsistent fluctuations in βNHE activity. On the other hand, βNHE activity was positively related to the βNHE transcript level and depended on the age of the RBC, showing reduced activity in older RBCs. The seasonally

reduced βNHE activity could not be rescued by pharmacological intervention in the transduction cascade suggesting that fluctuations in βNHE activity were a property of the transporter itself rather than any other factor. The inconsistency in timing of the reductions in βNHE activity was matched by corresponding fluctuations in plasma testosterone. Since testosterone stimulates erythropoietic activity in birds and humans, we suggest that the seasonally occurring fluctuations in βNHE activity of trout red cells are caused by changes in the age profile of circulating RBCs. We further hypothesise that this is due to the seasonal influences of testosterone.

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Table 1. β-adrenergically stimulated Na influx in RBCs with normal or seasonally reduced βNHE activity. Values are Na influxes relative to the Na influx in isoproterenol-stimulated RBCs with normal or seasonally reduced βNHE activity. n=3 for each experiment.

Compound	Normal βNHE activity	Reduced βNHE activity
Isoproterenol	100	100
Forskolin	97±15	82±6
Dibutyryl cAMP	52±11	45±19
Calyculin A	61±11	45±15

Table 2. βNHE activity and mean cellular haemoglobin concentration (MCHC) measured in density-separated RBCs and in the original RBC suspension. Statistically significant differences from values for top fraction is indicated by * (P<0.05), **(P<0.01) and ***(P<0.001). n=6 for each experiment.

Cell fraction	Na influx (mmol l^{-1} RBCs h^{-1})	MCHC (mM)
Top (young)	125.6 ± 9.5	3.82 ± 0.16
Middle (older)	90.1 ± 8.5**	4.24 ± 0.14*
Bottom (oldest)	102.0 ± 7.7*	4.57 ± 0.10***
Original RBC suspension	91.0 ± 4.9**	4.10 ± 0.18*

Figure legends

Fig. 1. Seasonal variation in the number of β-adrenergic receptors (bars) and daily ambient water temperature (line) throughout the study. Values are means ± SEM; n=8. The inserted graph shows the linear regression curve for β-AR number vs. mean water temperature (*P*=0.002). Water temperature in the inserted graph as calculated from the temperatures measured over the full period since the previous sampling.

Fig. 2. Seasonality in the β-adrenergically stimulated RBC Na influx (filled circles), daily ambient water temperature (solid line) and plasma cortisol levels (open circles). The dotted line illustrates the mean Na influx at the times of the year when the β-NHE activity is not down-regulated. The crosshatched boxes indicate the spawning periods, defined by high proportions of ovulated females. Values are means ± SEM; n=8.

Fig. 3. The seasonal variations in β-AR numbers (filled circles) and β3b-AR mRNA levels (relative to the August value; open circles). Values are means ± SEM; n=8 for β-AR numbers and n=3-8 for β3b-AR mRNA values. The inserted graph illustrates the linear regression curve for receptor number vs. β3b-AR mRNA levels (*P*=0.005).

Fig. 4. Seasonal changes in the βNHE activity (filled circles) and βNHE mRNA levels (relative to August value; open circles). Note one outlying βNHE mRNA value is plotted separately. Values are means ± SEM; n=8 for βNHE activity values and n=3-8 for βNHE mRNA values. The inserted graph shows the linear regression curve for βNHE activity vs. βNHE mRNA values (*P*=0.038).

Fig. 5. Seasonal fluctuations in plasma testosterone (open circles) and plasma estradiol-17β concentrations (open squares) in relation to fluctuations in RBC βNHE activity (filled circles). The dotted line illustrates the mean Na influx at the times of the year when the β-NHE activity is not down-regulated. Values are means ± SEM; n=8.

Fig. 1

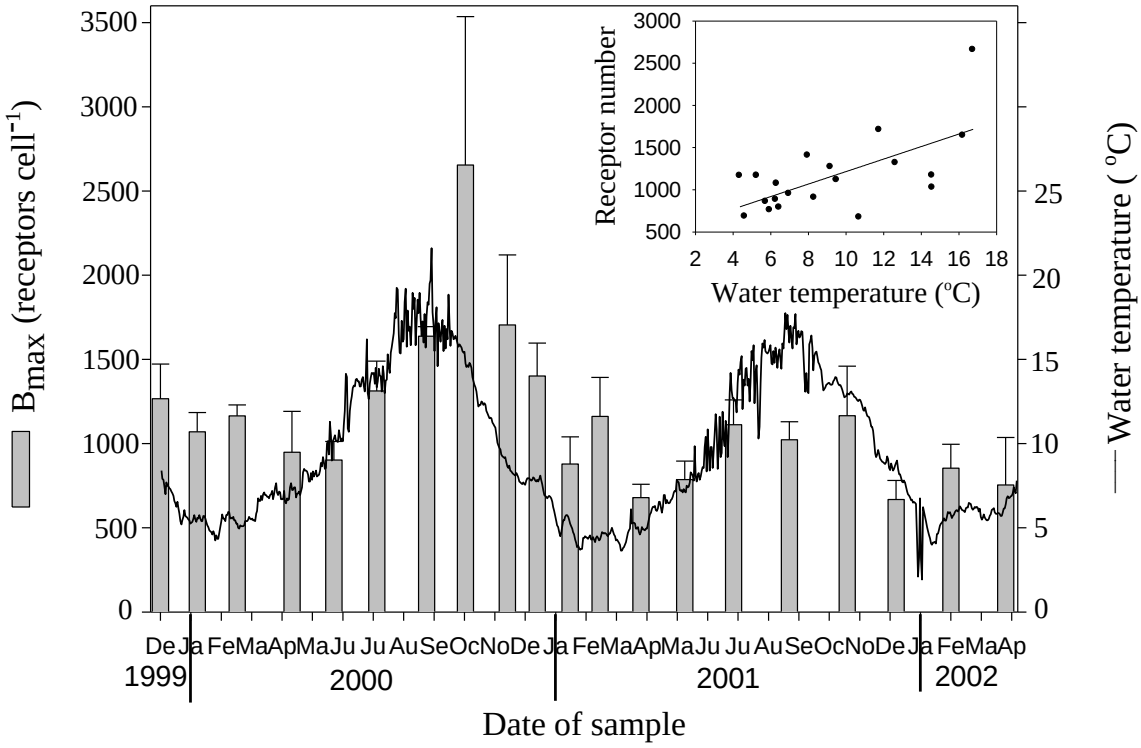

B_max (receptors cell^-1)
Water temperature (°C)
Receptor number
Water temperature (°C)
Date of sample
1999
2000
2001
2002
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap

Fig. 2

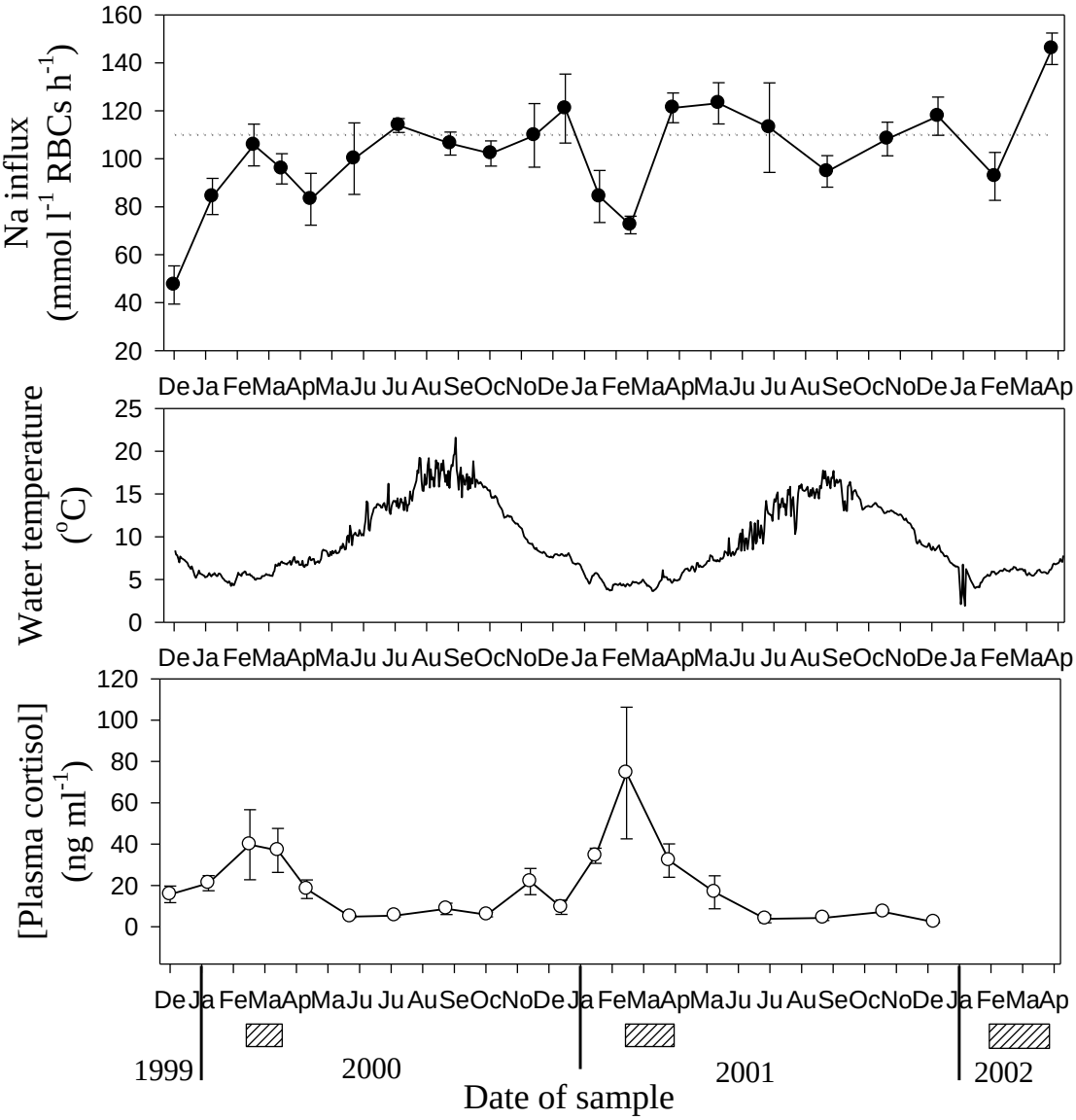

Na influx
(mmol l-1 RBCs h-1)
160
140
120
100
80
60
40
20
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap
Water temperature
(°C)
25
20
15
10
5
0
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap
[Plasma cortisol]
(ng ml-1)
120
100
80
60
40
20
0
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap
1999
2000
2001
2002
Date of sample

Fig. 3

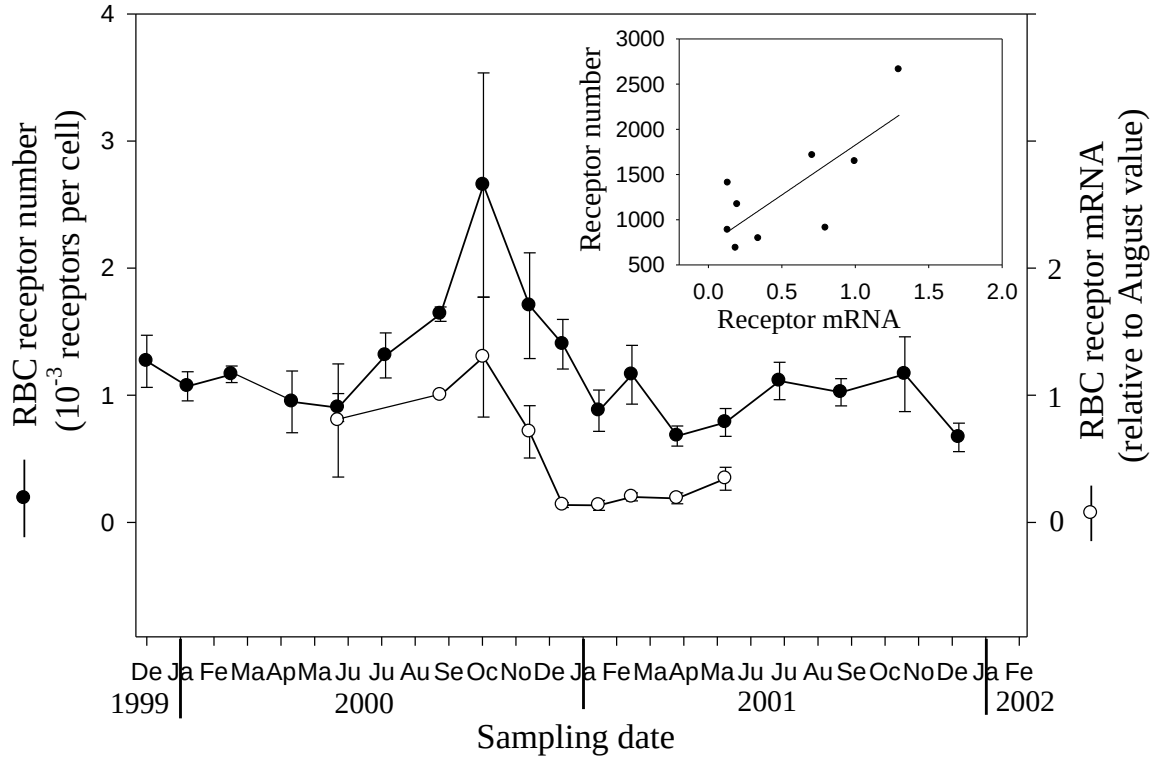

RBC receptor number
(10-3 receptors per cell)
RBC receptor mRNA
(relative to August value)
Receptor number
Receptor mRNA
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe
1999
2000
2001
2002
Sampling date

Fig. 4

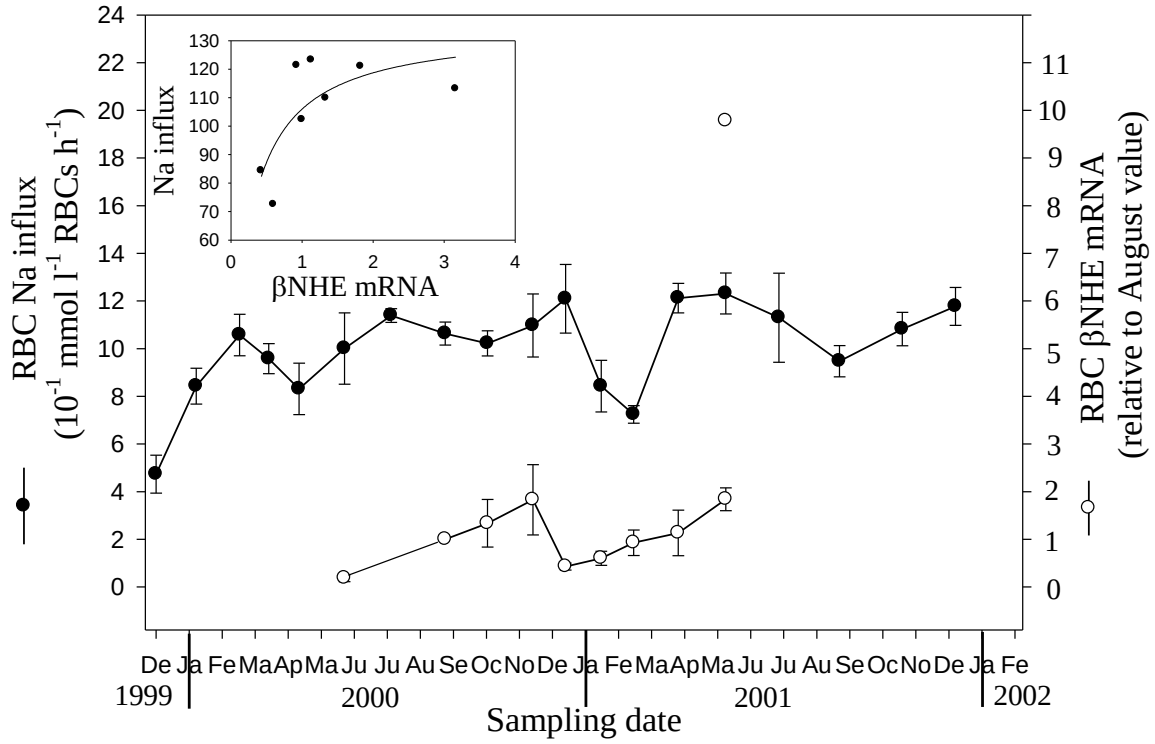
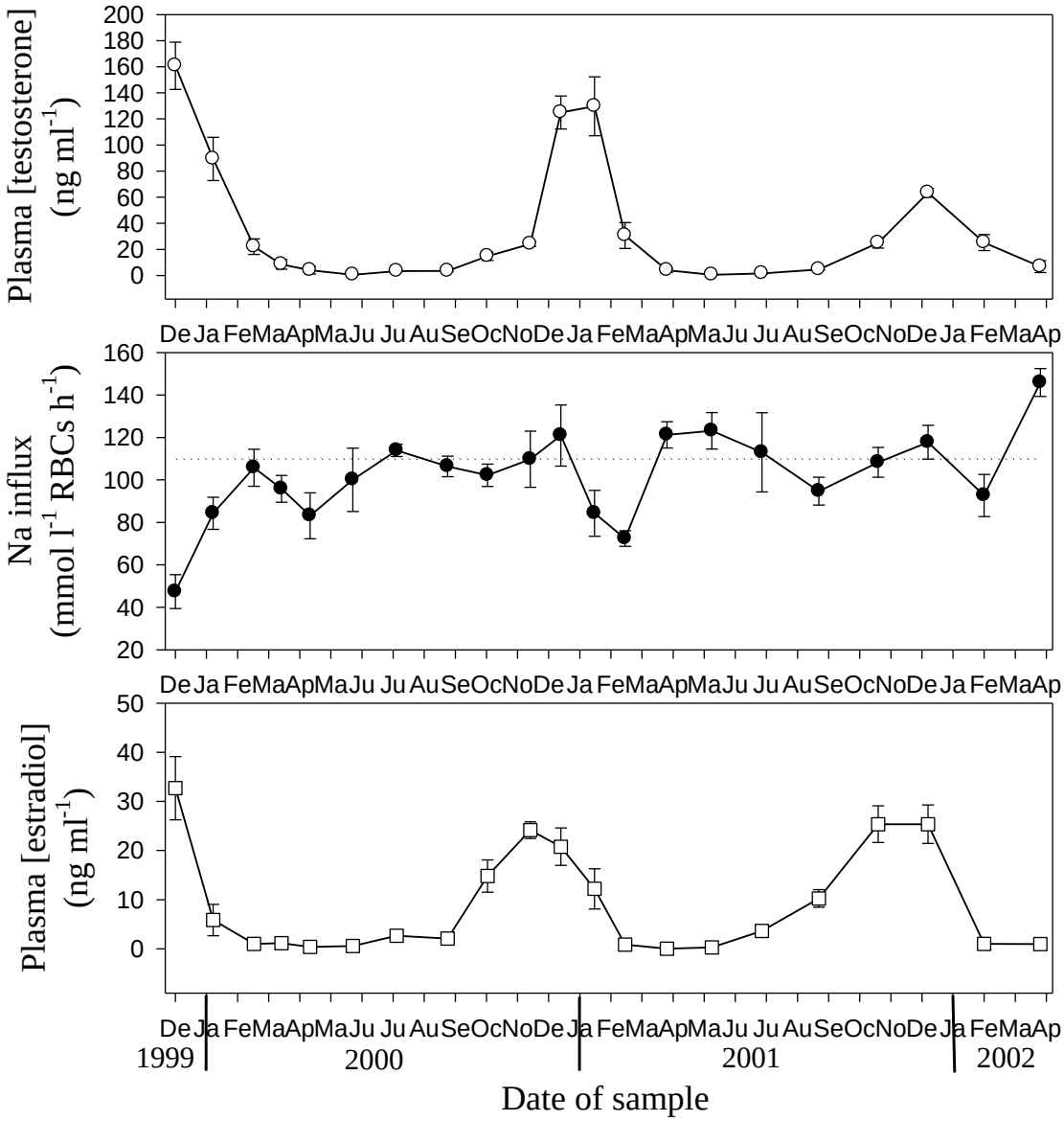

RBC Na influx
(10^{-1} mmol l^{-1} RBCs h^{-1})
RBC βNHE mRNA
(relative to August value)
Na influx
βNHE mRNA
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe
1999
2000
2001
2002
Sampling date

Fig. 5


Plasma [testosterone] (ng ml-1)
Na influx (mmol l-1 RBCs h-1)
Plasma [estradiol] (ng ml-1)
De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap Ma Ju Ju Au Se Oc No De Ja Fe Ma Ap
1999
2000
2001
2002
Date of sample